Immunity, Vol. 15, 71–82, July, 2001, Copyright ©2001 by Cell Press

How Much TCR Does a T Cell Need?

Nathalie Labrecque,[2] Liam Simon Whitfield, Reinhard Obst,[3] Caroline Waltzinger, Christophe Benoist,[1,3] and Diane Mathis[1,3]
Institut de Génétique et de Biologie Moléculaire et Cellulaire
CNRS/INSERM/ULP
1 rue Laurent Fries
67404 Strasbourg-Illkirch
France

Summary

Kinetic features of TCR:MHC/peptide interactions dictate their outcome in vitro, some important parameters of which include the number of molecules engaged and the duration of engagement. We explored the in vivo significance of these findings in transgenic mice expressing TCRs in a quantitatively and temporally controlled manner. As anticipated, reduced TCR levels resulted in attenuated reactivity, but response thresholds were substantially lower than expected—at as low as 1/20th the normal TCR numbers and with no indication of phenotypic skewing at suboptimal levels. We also studied survival of T lymphocytes stripped of their TCRs. Unlike B cells, T cells lacking antigen receptors did not die precipitously; instead, populations decayed gradually, just as previously reported in the absence of MHC molecules.

Introduction

The interaction between TCRs expressed by T cells and MHC/peptide complexes displayed on various cell types is the crux of T lymphocyte differentiation and acquisition of effector functions. Appropriate gauging of the quantity and quality of TCR:MHC/peptide engagements conditions the choices T cells make throughout their life cycle.

In the thymus, the newly assembled TCR of immature thymocytes interacts with MHC/self-peptide complexes on stromal epithelial cells. If strong, this engagement results in clonal deletion of thymocytes by apoptosis. If within an "acceptable" range of avidity, the outcome is positive selection and differentiation into mature, exportable T cells. If engagement is weak or absent, immature thymocytes quickly die of neglect. The affinity/avidity criteria that define acceptable are still unclear, as are the factors that condition commitment to the CD4$^+$ versus CD8$^+$ lineage.

In peripheral lymphoid organs, continued interaction with MHC/self-peptide complexes is required for maintenance of the naive T cell pool. It has been shown, employing a variety of experimental systems, that T cells deprived of peripheral MHC/peptide engagements have a reduced life span (Rooke et al., 1997; Takeda et al., 1996; Kirberg et al., 1997; Brocker, 1997; Witherden et al., 2000; Tanchot et al., 1997; Nesic and Vukmanovic, 1998); engagement of the restricting element that promoted positive selection in the thymus appears to be necessary (Kirberg et al., 1997; Tanchot et al., 1997; Boursalian and Bottomly, 1999; Viret et al., 1999). Yet, a wide range of half-lives in the absence of MHC/peptide interactions has been reported with the different systems, and it is also not clear whether CD4$^+$ and CD8$^+$ T cell populations show the same decay kinetics. Very recently, a few reports even questioned the link between MHC/peptide engagement and T cell survival (Dorfman et al., 2000; Clarke and Rudensky, 2000). TCR:MHC peptide interactions also drive the homeostatic expansion that takes place in response to lymphopenic environments (Ernst et al., 1999, and references therein). Although proliferation in this context appears to be a response to environmental cues signaling that the T cell compartment is not full, TCRs vary in their ability to promote homeostatic proliferation, suggesting that the affinity/avidity constants of MHC/self-peptide recognition may come into play (Ernst et al., 1999; Goldrath and Bevan, 1999; Rocha and von Boehmer, 1991; Clarke and Rudensky, 2000; Kieper and Jameson, 1999; Oehen and Brduscha-Riem, 1999; Ferreira et al., 1999; Surh and Sprent, 2000; Correia-Neves et al., 2001).

Finally, active proliferation and terminal differentiation into effector cells are provoked by antigens presented as short peptide fragments encased within MHC molecules displayed on antigen-presenting cells (APCs). The signal induced by these strong agonist ligands is somehow read as being different from the "tickling" by MHC/self-peptide complexes that promotes the positive selection and survival of T cells, and the resulting proliferative response can be explosive. Again, the strength of the signal imparted through the TCR impinges on the differentiative choice. Cytokine and costimulatory influences on responding T cells certainly play a key role in coaxing them toward a Type 1 (Th1, Tc1) versus Type 2 (Th2, Tc2) phenotype, but there is also evidence that the strength/quality of the TCR signal can exert an important influence (Hosken et al., 1995; Constant et al., 1995; Tao et al., 1997; Rogers and Croft, 1999; Pfeiffer et al., 1995).

Thus, in these many instances, the TCR appears to act as a sensitive rheostat, translating the affinity/avidity of engagement into signals that somehow specify differential cell activity and/or fate. The agonist/antagonist nature of MHC/peptide complexes has generally correlated with differences in the off rate of the complexes they form with TCRs in vitro (Alam et al., 1996; Sykulev et al., 1994; Matsui et al., 1994; Lyons et al., 1996), differences that result in variable duration of productive interactions. The duration of TCR engagement does seem to have an important effect on its outcome, be it in the context of lineage commitment in association with

[1] Correspondence: cb@joslin.harvard.edu (C.B.), dm@joslin.harvard.edu (D.M.)
[2] Present address: Centre de Recherche Guy-Bernier, 5415 Boulevard de l'Assomption, Montréal, Québec H1T 2M4, Canada.
[3] Present address: Section on Immunology and Immunogenetics, Joslin Diabetes Center, and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02215.

positive selection or of effector phenotype choices during the activation of peripheral T cells (Yasutomo et al., 2000; Iezzi et al., 1998; Valitutti et al., 1995a). The number of TCR molecules engaged may be another key parameter of the interaction, having been directly correlated with the outcome (Valitutti et al., 1995b; Matsui et al., 1994; Lanzavecchia et al., 1999). It has been proposed that distinct thresholds, tunable by the concomitant influence of costimulatory molecules, are required for different phenotypic responses (Valitutti et al., 1996; Itoh and Germain, 1997). As a large number of productive interactions (5,000–20,000) seems to be needed, serial engagement of the limited number of MHC molecules loaded by the cognate peptide on each APC (in the hundreds at best) has been hypothesized.

The above conclusions frequently derived from in vitro experiments that entailed selecting cells with diverse TCR levels as the result of a prior exposure to antigen. It seems important to readdress some of the key questions in vivo, in an experimental system where TCR levels can be varied without intentional prior triggering. Thus, exploiting a tetracycline (tet)-based control system (Furth et al., 1994; Kistner et al., 1996), we generated transgenic mice expressing TCRs in a quantitatively and temporally regulated fashion and studied T cell responses to antigen challenges. We also used these animals to reinvestigate survival issues, in a system where $CD4^+$ and $CD8^+$ T cells can be studied in parallel and in the absence of external perturbations.

Results

Tet-Responsive TCR Transgenic Mice

To determine the influence of TCR levels on T cell responsiveness, we generated transgenic mice in which the expression of a transgene-encoded TCR of defined specificity could be modulated after a normal T cell compartment had been established. We chose the OT-1 receptor—specific for the OVA257-264 peptide, K^b-restricted (Kelly et al., 1993), composed of a Vα2/Vβ5 chain combination, and the focus of numerous studies. Mice expressing the OT-1 α chain under tet control were generated using the binary transgenic strategy developed by Bujard and collaborators (Kistner et al., 1996; Furth et al., 1994). The first transgene encodes a tet-sensitive activator, the second a reporter whose expression is controlled by the transactivator. The combination of transgenes employed in the present study is illustrated in Figure 1A. First, the *lck* proximal promoter was used to drive T cell-specific expression of the TetR-VP16 transactivator (tTA). This *lck* promoter-driven vector is active in T-lineage cells, although expression in mature cells varies between different founder lines (Chaffin et al., 1990; Sohn et al., 1998). Second, the cDNA encoding the OT-1 α chain was placed under the control of a minimal promoter augmented with seven TetO sequences. Expression of tTA in T cells should turn on transcription of the TCR α chain, and this transactivation should be sensitive to tet, which inhibits tTA binding to the TetO motif. To complete the OT-1 TCR, we also employed a third transgene encoding the OT-1 TCR β chain and expressed constitutively in all T cells (Correia-Neves et al., 1999).

Transgenic mice carrying each construct were generated by independent microinjection. After analyzing the specificity and levels of transgene expression in offspring from various lck-tTA founders (data not shown), we selected one line, LTH1, whose properties are detailed in Figure 1. According to Northern blot analysis (Figure 1B), the LTH transgene was active only in lymphoid organs (with the exception of a weak signal in the ovaries). Transcripts were localized more precisely in the thymus and lymph nodes by in situ hybridization with a tTA-specific probe (Figure 1C). As might be expected, the thymic cortex labeled more intensely than the medulla, attributable to a lower density of T cells in the medulla compared with the cortex (a pattern characteristic of other probes corresponding to TCR components, such as CD3 [data not shown]). tTA mRNA could be detected only in the T cell areas of the lymph nodes, being largely excluded from the B cell follicles. A more quantitative assessment of tTA transcription in thymocytes was performed by RT-PCR analysis of sorted subpopulations (Figures 1D and 1E). tTA was transcribed in roughly equivalent amounts in $CD4^-CD8^-$ double-negative (DN), $CD4^+CD8^+$ double-positive (DP), and mature $CD4^+CD8^-$ and $CD4^-CD8^+$ single-positive (SP) cells. Among DNs, transcripts were detected in all four subpopulations delineated by the CD25 and CD44 differentiation markers, implying that the LTH-1 transgene was active quite early in T cell differentiation, consistent with a recent analysis of *lck* proximal promoter activity (Buckland et al., 2000).

The LTH1 transgene (hereafter abbreviated as L) was then bred into the TetO-TCRα-OT1 transgenic mice (referred to as TAO, for Tet-controlled Alpha OT-1 TCR) to yield animals with regulatable TCR expression (L/TAO double-transgenic mice). We also introduced into these crosses the TCRα knockout mutation (Philpott et al., 1992; hereafter abbreviated as Cα°), which eliminates endogenously encoded TCR α chain expression and thereby T cell maturation beyond the DP stage; thus, positive selection and the appearance of fully mature T cells in L/TAO/Cα° animals must reflect transgene-encoded TCRα expression. Lymph node T cells from such mice were examined by flow cytometry (Figure 2A). As expected, the reporter transgene alone could not complement the TCRα deficiency: mature T lymphocytes were not found in significant numbers in TAO/Cα° mice. Therefore, this tet-regulatable system is not "leaky." Addition of the LTH-1 transactivator transgene led to full restoration of peripheral $CD4^+$ and $CD8^+$ T cell populations in L/TAO/Cα° mice. Expression of the transgene-encoded TCR was demonstrated by staining with an anti-Vα2 reagent: all T cells stained positively, most of them at levels comparable with those of wild-type mice (68% of WT levels on average). The relatively low proportion of $CD8^+$ T cells in L/TAO/Cα° mice was expected because the OT-1 TCR α chain has been associated with preferential selection into the $CD4^+$ lineage (M. Correia-Neves, personal communication). The OT-1 TCR β chain transgene was then introduced to allow assembly of the complete OT-1 receptor in Vβ5/L/TAO/Cα° mice (third dot plot), yielding a dominant population of $CD8^+$ T cells, as anticipated for a K^b-restricted TCR. Again, all T cells expressed Vα2. The lymphocytes whose maturation was induced by transgene expression

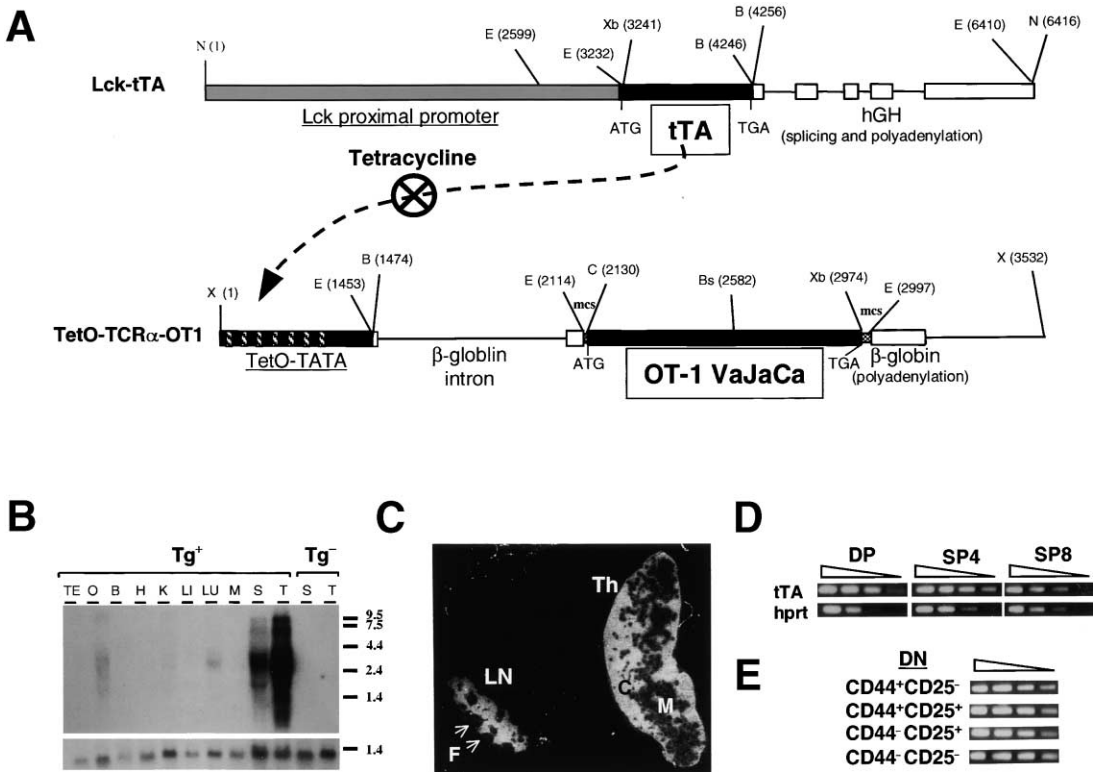

Figure 1. Constructs and Transactivator Expression

(A) Constructs. Top: the lck-tTA transactivator transgene: the microinjected fragment contains the *lck* proximal promoter (Chaffin et al., 1990) (gray bar), the tTA transactivator (solid bar), and several exons of the human growth hormone gene (white bars). Bottom: the TetO-TCRα-OT1 reporter transgene: the fragment contains the TCRα cDNA from the OT-1 hybridoma (K^b-restricted, OVA specific) (solid bar), a minimal cytomegalovirus promoter (gray bar) linked to seven TetO sequences (hatched bars), and the rabbit β-globin intron/exon and polyadenylation sequences (white bar). B, BamHI; Bs, Bst1107i; C, ClaI; E, EcoRI; N, NotI; X, XhoI; Xb, XbaI; hGH, human growth hormone; mcs, multiple cloning site; and polyA, polyadenylation site. The numbers in parentheses indicate the positions of restriction enzyme sites.

(B–E) tTA transgene expression in LTH1 mice. (B) Northern blot analysis of tissue RNA, hybridized with probes specific for tTA (upper panel) and a riboprotein as control (lower panel). Organs were testes (TE), ovary (O), brain (B), heart (H), kidney (K), liver (LI), lung (LU), skeletal muscle (M), spleen (S), and thymus (T). (C) In situ mRNA expression in mesenteric lymph nodes (LN) and thymus (Th). Sections were hybridized with a tTA anti-sense probe. F, B follicles; M, medulla; and C, cortex. (D) Semiquantitative RT-PCR analysis of tTA mRNA expression in $CD4^+CD8^+$ (DP), $CD4^+CD8^-$ (SP4), and $CD4^-CD8^+$ (SP8) thymocytes. (E) $CD4^-CD8^-$ thymocyte subpopulations. Hprt mRNA levels are shown to control for amounts. RT-PCR was performed on serial dilutions (1/10) of cDNA from the different sorted populations with primers specific for the tTA or hGH sequences.

had the phenotypic characteristics of naive T cells—$CD25^-CD69^-CD44^-CD62L^{hi}$ and little cycling (data not shown).

Thus, the combination of transgenes seemed to function as had been hoped for: expression of the OT-1 TCR was transactivator dependent, at levels sufficient for selection and maintenance of a normal complement of T cells. L/TAO/Cα° mice also showed normal thymocyte subsets, including ample populations of mature $CD3^{hi}$ cells absent from Cα° littermates (Figure 2B), with the TCR density expected of thymic subsets. In Vβ5/L/TAO/Cα° mice, the anti-CD4/CD8 staining pattern on gated TCR^{hi} cells (Figure 2C) revealed the existence of a large population of transitional $CD4^+8^{lo}$ cells as is typical for the OT-1 TCR (Hogquist et al., 1994).

It was also important to assess the efficiency of L/TAO expression in a situation where the transgene-encoded product competes with the products of endogenous TCRα loci. The histograms in Figure 2D illustrate that approximately 15%–25% of $CD4^+$ and $CD8^+$ T cells expressed the transgene-encoded TCR α chain, more than double the background of endogenous Vα2 expression. As expected, the presence of a transgene-encoded $V_\beta5$ chain boosted selection into the $CD8^+$ compartment.

We then asked whether TCR levels were responsive to treatment of the mice with tet. The effect was quite graphic, as illustrated in Figure 3A. While sizeable numbers of thymocytes were positively selected into the SP compartments of the untreated Vβ5/L/TAO/Cα° mouse (upper right), a 3 week course of tet treatment completely eliminated SP cells in its littermate (lower right). The absence of mature thymocytes correlated with the shutdown of TCR expression in DP thymocytes (data not shown). It did not represent a toxic effect of the drug itself, since the same treatment had no effect in a wild-type control.

To measure the rate of extinction of TCR expression in peripheral T lymphocytes, L/TAO mice were treated with a high dose (1 mg/ml) of tet, and blood samples were collected at different times and stained with an

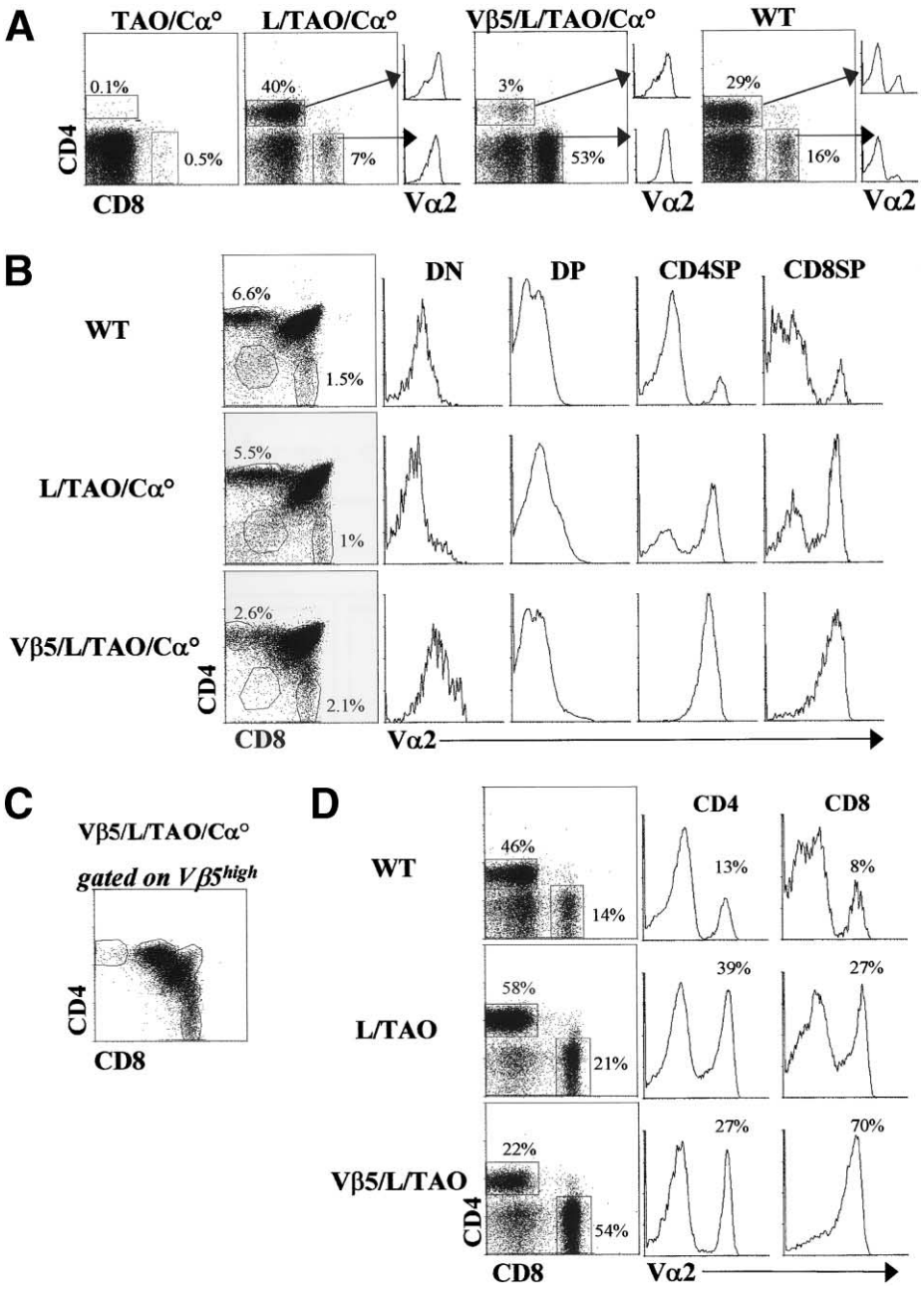

Figure 2. T Cell Compartments

(A) CD4/CD8 profiles of lymph node cells from wild-type, TAO/Cα°, L/TAO/Cα°, and Vβ5/L/TAO/Cα° mice. Juxtaposed small histograms display the expression of the Vα2 chain encoded by the TetO-OT1α transgene in $CD4^+$ and $CD8^+$ T cells.

(B) Thymocyte differentiation in L/TAO/Cα° and Vβ5/L/TAO/Cα° mice: CD4/CD8 profiles are in the left-most panels; Vα2 expression for the different gated populations are to the right of them.

(C) CD4/CD8 profiles on gated Vβ5-high cells from a Vβ5/L/TAO/Cα° thymus.

(D) Expression of the inducible OT-1 TCRα chain in lymph node T cells of Cα-wild-type mice: CD4/CD8 profiles of lymph node cells from wild-type, L/TAO, and Vβ5/L/TAO mice (left panel); Vα2 expression on gated $CD4^+$ or $CD8^+$ cells in the middle and left histograms. The percentage of $CD4^+$ and $CD8^+$ cells in each dot plot is shown and the percentage of $V\alpha2^+$ T cells is indicated on the histograms for Cα-wild-type mice. All stainings were performed on mice that were not tet treated.

anti-Vα2 mAb. A significant decrease in TCR levels was observed already after 4 days of tet treatment, and expression reached baseline values by day 14 (Figure 3B). As expected, cells expressing endogenous Vα2 showed no such drop. The same kinetics were observed for $CD4^+$ and $CD8^+$ T cells. Quantitative flow cytometry with calibrated beads as standards (Davis et al., 1998) (Figure 3C) showed about 40,000 molecules on wild-type $CD4^+$ cells, in good agreement with previous reports (Blichfeldt et al., 1996; Schodin et al., 1996; Valitutti et al.,

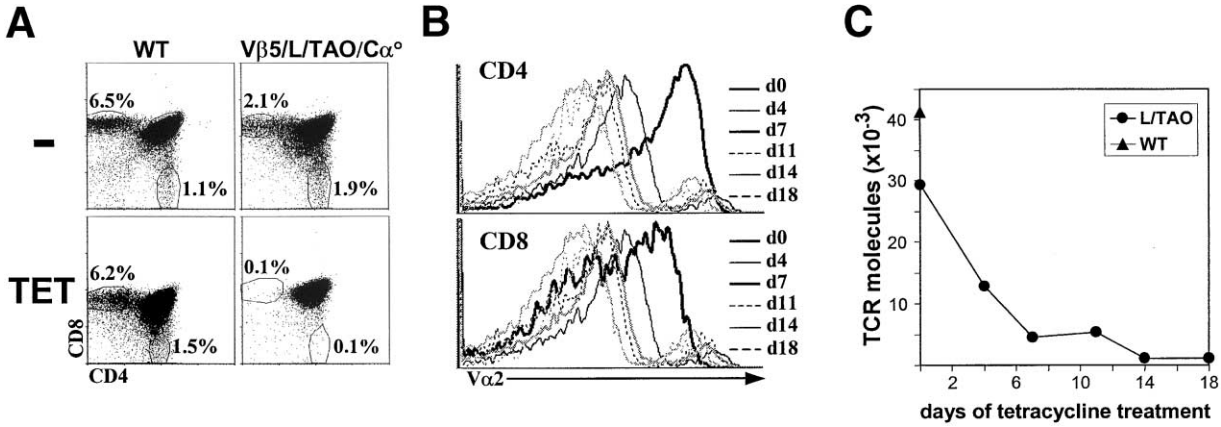

Figure 3. Tet-Controllable TCR Expression

(A) Lack of mature thymocytes in Vβ5/L/TAO/Cα° mice after tet treatment. Wild-type (left panels) or Vβ5/L/TAO/Cα° animals (right panels) were treated (lower panels) with 1 mg/ml of tet for a 3 week period or not (top panels) and their thymi were stained for CD4 and CD8.

(B) Disappearance of TCR expression following tet treatment. Overlay histograms for Vα2 expression in LTAO mice are shown for $CD4^+$ or $CD8^+$ PBL of a single mouse sampled at day 0, 4, 7, 11, 14, and 18 after initiation of tet administration (1 mg/ml in drinking water).

(C) Quantitation of the number of TCR molecules expressed by $CD4^+$ T cells during the course of tet treatment (from [B]).

1995b); 30,000 molecules/cell were found on $CD4^+$ cells from untreated L/TAO mice. Within 2 weeks after initiation of tet treatment, L/TAO cells were essentially denuded of TCRs. A careful analysis of TCR levels over long periods of tet treatment revealed that while the bulk of molecules had disappeared by 14 days, a very low residual expression (equivalent to 200-300 molecules/cell) persisted beyond that time and was stable for months. This low-level persistence, also observed when the cells were stained with anti-CD3 or anti-TCRβ reagents, was distinguishable from the background seen with irrelevant antibody staining and was absent from nontransgenic mice. It is not clear what biochemical form or functional relevance these long-lived TCR molecules might have, although they are clearly incapable of signaling in response to antigenic stimulation (see below) or to promote positive selection in the thymus (Figure 3A).

Graded doses of tet were then used to quantitatively regulate TCR expression levels in Vβ5/L/TAO/Cα° mice. The protocol, chosen for its reliability, was to leave the animals with no tet (permitting full TCR expression) for their first 3 weeks of life and then to switch them to 0, 5, 10, or 400 μg/ml of tet for 2 weeks or more. TCR expression was maintained stably at intermediate levels in animals treated with these low tet doses and levels correlated with tet concentration (Figure 4). TCR levels could thus be "adjusted" to 20- to 40-fold below normal (using cells expressing endogenous Vα2 as an internal standard).

TCR Density and T Cell Responsiveness

The possibility of regulating TCR levels, both quantitatively and temporally, in these transgenic mice permitted us to analyze the influence of TCR density on T cell responses in vivo. To quantitate TCR levels in individual experimental mice, a single inguinal lymph node was surgically removed from Vβ5/L/TAO animals fed different tet doses. Two days after the biopsy, the animals were challenged by intravenous injection of the antigenic peptide SIINFEKL. Two doses were used: 0.1 μg, which is near saturating for the induction of a proliferative response in mice expressing the normal complement of OT-1 TCRs; and 20 μg, a 100-fold excess. This

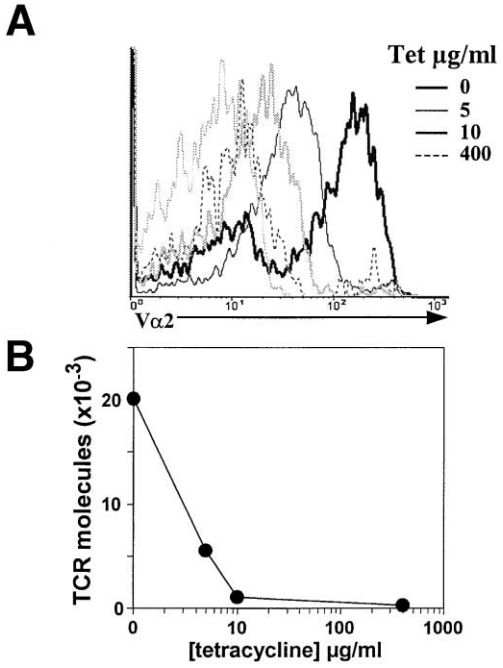

Figure 4. Tet Dose-Dependent Expression of the TCR in Vβ5/L/TAO Mice

(A) The overlay histogram represents the expression of the transgene-encoded α chain by lymph node $CD8^+$ T cells from Vβ5/L/TAO mice treated for 2 weeks with different tet doses (0, 5, 10, and 400 μg/ml) in a representative experiment.

(B) Quantitation of the number of TCR molecules using standardization microbeads containing a known number of fluorescent molecules.

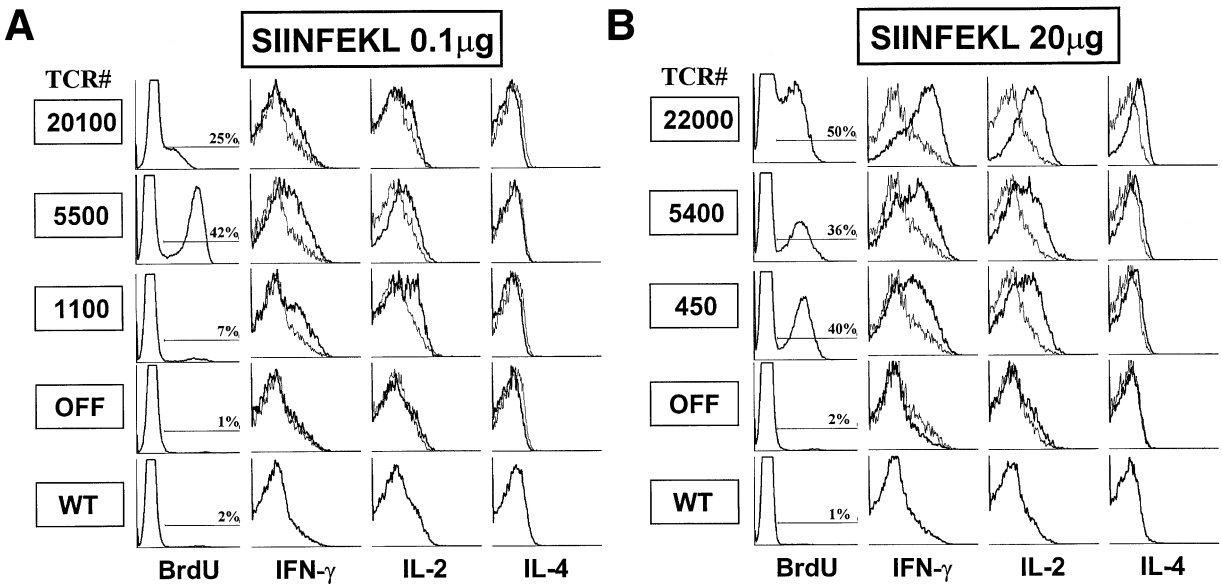

Figure 5. T Cell Responses in Vβ5/L/TAO Mice Expressing Various TCR Levels

Vβ5/L/TAO animals treated with different doses of tet for 16 days were immunized with 0.1 μg (A) or 20 μg (B) SIINFEKL peptide intravenously, and T cell responses were analyzed 48 hr later. The histograms, from left to right, show BrdU incorporation, IFN-γ, IL-2, and IL-4 production by $CD8^+$ mesenteric lymph node T cells from control nontransgenic (thin line), and Vβ5/L/TAO (thick line) mice. TCR densities are indicated at the left (determined as above). The tet doses were 0, 5, 10, and 400 μg/ml (from top to bottom). The fifth line of histograms shows results for an immunized nontransgenic control mouse (WT).

response is dependent on both TCR transgenes (there is no response in mice or cells expressing the Vβ5 transgene together with endogenous Vα chains [data not shown]). The antigen induces a downmodulation of the transgene-encoded TCR (4-fold at the lowest dose, complete at the highest). To test for proliferation, we injected the animals with bromodeoxyuridine (BrdU) 34 and 42 hr after immunization and sacrificed them at 48 hr. Spleen and lymph node T cells were stained for BrdU incorporation. As illustrated in Figure 5A, the lowest doses of immunizing peptide elicited significant proliferation only in mice exhibiting the two highest TCR densities. Perhaps surprisingly, mice with as few as 500–1000 TCR molecules per $CD8^+$ T cell responded, although substantially only at the high immunogen dose. In no case did we see any proliferation, beyond that observed with unimmunized or nontransgenic mice, in animals treated with the maximum tet dose, indicating that the residual TCR molecules displayed by these cells did not suffice to induce a response to even a high density of agonist ligand.

Cytokine production was also examined by intracytoplasmic staining of the same cells 48 hr after immunization. Substantial IL-2 and γ-IFN induction was seen only at the high dose of immunizing peptide. The proportion of positive cells and cytokine levels decreased with diminishing TCR density, but cytokines were still clearly detectable in cells displaying <1000 surface TCR molecules. Importantly, the decreased γ-IFN expression in mice showing a low TCR density did not correlate with an induction of IL-4, absent in all mice examined except for untreated animals receiving the highest peptide dose.

These points are highlighted in the compiled indices of Figure 6. At the high antigen dose, the cycling of T cells with even very low TCR density resulted in a marked expansion of the $CD8^+$ compartment. At the low antigen dose, there was more of a correlation between the degree of proliferation and TCR levels. In addition, cytokine responses seemed to correlate more with antigen dose than with TCR numbers. This was particularly clear for IL-2.

In short, not only was the threshold of TCR density for responsiveness to antigen surprisingly low, but the intensity of the response seemed more cued on the density of antigen ligands than on the number of TCRs available to engage them.

Turnover of $CD4^+$ and $CD8^+$ T Cells in the Absence of TCR Expression

Given the profound and fairly rapid shutdown of TCR expression that can be induced in L/TAO mice and the loss of thymocyte positive selection and peripheral T cell responsiveness that ensues, it seemed worthwhile to exploit this system to investigate the in vivo fate of mature T cells denuded of TCRs subsequent to undergoing a normal selection process. As discussed above, a number of investigators have claimed that persistent engagement of TCRs by MHC/self-peptide complexes is necessary for the long-term survival of naive peripheral T cells, although population decay kinetics have varied for $CD4^+$ and $CD8^+$ cells, and of late there has been some debate on the whole issue. Furthermore, a recent study (Lam et al., 1997) reported that B cells show a strikingly rapid demise after elimination of their surface Igs via inducible gene targeting, as if the very presence of an assembled antigen receptor is required for B cell survival. We wondered whether a parallel shutdown of

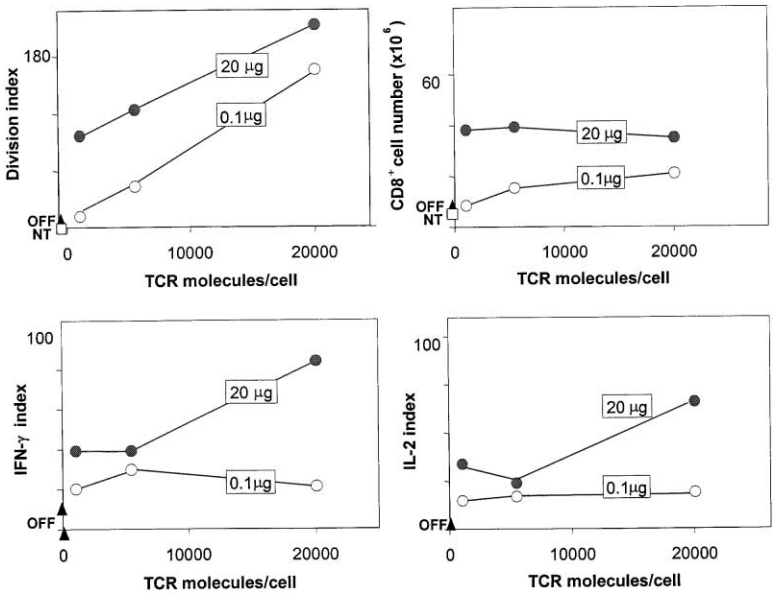

Figure 6. TCR Density and T Cell Responsiveness

$CD8^+$ T cell divison factor (upper left), $CD8^+$ T cell number (upper right), IFN-γ production index (bottom left), and IL-2 production index (bottom right) following intravenous immunization with SIINFEKL peptide are plotted as a function of the number of TCR molecules on the surface of T cells. The division factor was determined using the percentage of $BrdU^+$ cells and correcting for cells that had divided more than once (see Experimental Procedures); $CD8^+$ T cell numbers were quantitated for mesenteric lymph nodes; cytokine production indices were calculated from the percentage of cytokine-positive cells and their level of expression (see Experimental Procedures). The number of surface molecules/cell was determined as in Figure 4. The control values obtained with totally shutoff expression ("off") or in nontransgenic mice (NT) are shown.

TCR expression on T cells (though by another genetic trick) would rapidly precipitate their death.

These experiments were performed on L/TAO or Vβ5/L/TAO mice harboring a wild-type endogenous TCRα locus, thereby allowing for sizeable populations of mature T cells that do or do not express the inducible TCRα transgene (Figure 2D). The behavior of transgene-positive cells "in a crowd" of cells expressing other clonotypes should more closely mimic the normal situation and avoid complications due to the "homeostatic" proliferation in a lymphopenic setting. T cells that have lost TCR expression can easily be monitored by staining with anti-CD3 and anti-CD4 or -CD8 mAbs, registering as CD3-negative, CD4-, or CD8-positive cells (Figure 7A). Both L/TAO and Vβ5/L/TAO animals were used because $CD4^+$ and $CD8^+$ T cells predominate in the former and latter, respectively (Figure 2). The animals were allowed to generate normal lymphocyte compartments until 6 weeks of age, when TCR expression was shut off. T cell numbers and phenotypes were then followed over time in individual mice by sequential surgical removal of single lymph nodes (inguinal and axillary nodes, which we find to have essentially identical cell numbers in individual control mice over time), followed by cytofluorimetry. Since tet administration also halts thymic positive selection (Figure 3), these experiments tracked the survival of peripheral T cells without the complicating influences of thymocyte export.

A reproducible decline in both the $CD4^+$ and $CD8^+$ populations of individual mice was observed (Figure 7B). This was not due just to the tet treatment, because no loss of T cells was observed in control animals that drank the same antibiotic-laced water (open triangles). The decay was also not due to toxicity of the residual, unpaired TCRβ chain, as there are numerous examples of fully viable cells expressing unpaired TCRβ chains (Mertsching et al., 1997; Saint-Ruf et al., 1994). The data were replotted after normalization on the starting T cell number for each mouse and exponential curve fitting, taking day 8 as the starting point to account for the time needed to substantially shut down surface TCR levels, visible as a lag in the upper right panel, for example. Calculated half-lives were 26.8 and 19.0 days for $CD4^+$ and $CD8^+$ populations, respectively (bottom panels). These values are quite consistent with those obtained by most investigators for T cells placed in an MHC-deficient environment. Thus, the life expectancy of T cells without TCRs is remarkably similar to that in the absence of MHC/self-peptide ligands.

Discussion

The combination of transgenes engineered for these studies performed as we had hoped. The OT-1 TCR α chain was expressed at near-normal levels in the absence of tet and effectively promoted the selection and maintenance of $CD4^+$ and $CD8^+$ T cells. The partitioning of T cells into thymic and peripheral subsets mimicked the peculiarities of the original OT-I TCR transgenic line (Hogquist et al., 1994). Upon high-dose tet treatment, TCR expression was extinguished over a matter of days; it could also be maintained at intermediate levels with limited tet doses. While it has not been possible to reproduce in mice the 1000-fold dynamic range of reporter gene expression reported for tissue culture transfectants, the 20-fold range in TCR display does allow very useful analyses. This system offers the possibility of modulating or eliminating TCR:MHC/peptide contacts in a noninvasive manner, in an otherwise normal environment, in particular without the perturbations implicit in cell labeling and transfer protocols.

Number of TCRs Needed for T Cell Responsiveness

Very few surface TCR molecules (1000 or less) were needed for T cells to respond to immunization in vivo, and the density/response relationship was surprisingly nonexponential. This was true when suboptimal tet treatments resulted in a low level of TCR expression (Figures 4 and 5) and also when mice were immunized 8 days after tet administration, when incomplete shutoff

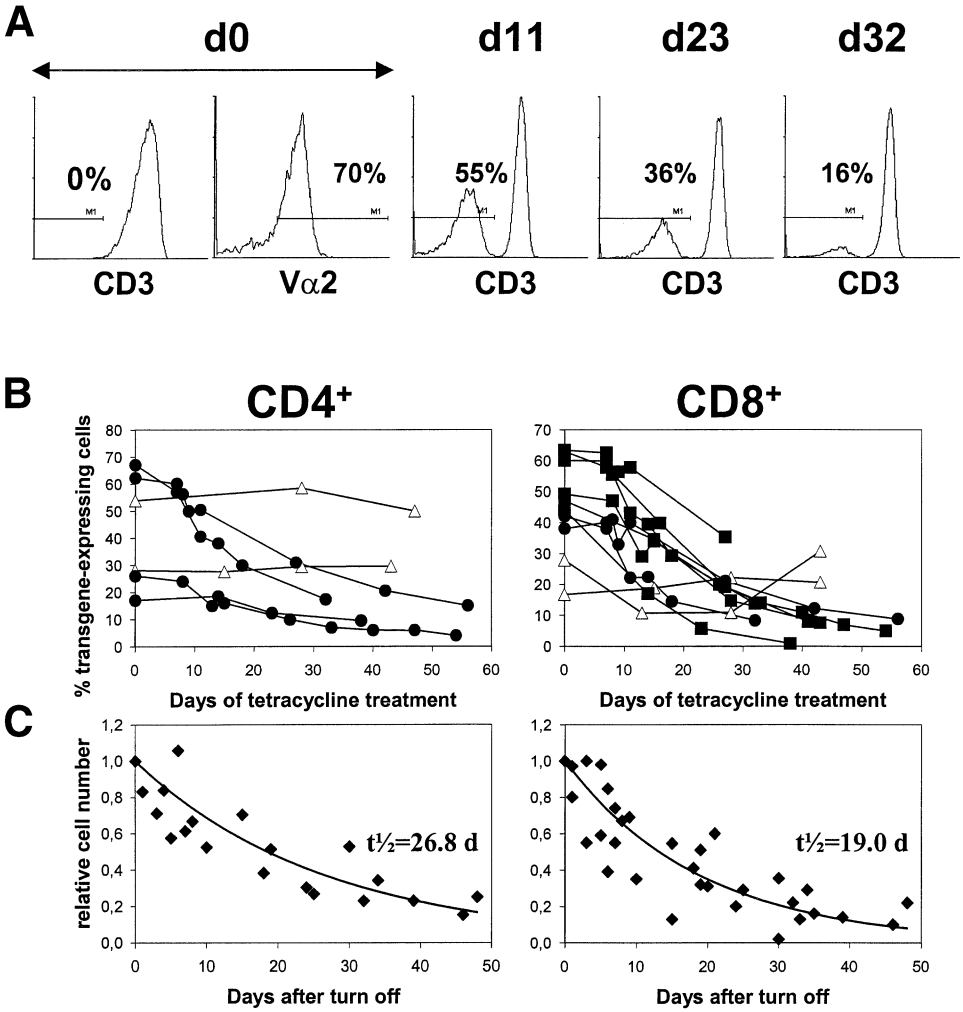

Figure 7. Decay of CD4$^+$ and CD8$^+$ T Cells Stripped of TCR Molecules

(A) Representative profiles of gated CD8-positive cells of a single Vβ5/L/TAO mouse, from individual lymph nodes surgically removed at different times of high-dose tet treatment.

(B) The proportion of transgene-expressing CD4$^+$ or CD8$^+$ T cells from tet-treated L/TAO (black circles), Vβ5/L/TAO (black squares), or control (open triangles) mice was determined as in (A); transgene-expressing T cells were identified as CD3$^-$ cells after the shutoff.

(C) Regression analysis of the data from (B), normalized on the pretreatment value for each mouse. To account for the 7–10 day delay before the surface TCR is effectively cleared, the regression assumes that the decline initiates on day 8. The half-life of the T cell population is indicated.

still left a thousand or so molecules at the cell surface (data not shown). One might argue that a minor fraction of the cells was still displaying intermediate to high TCR levels in treated mice and accounted for the responses observed. However, this is very unlikely. For example, in the mouse with a mean level of 450 molecules per CD8$^+$ cell, the 40% of cells that incorporate BrdU or produce γ-IFN 48 hr after immunization cannot have been generated from the gaussian trail (<5%) of cells that express more than 5000 molecules in this mouse (particularly as only 50% of cells respond even at full TCR levels). These results are consistent with those from studies that employed laser-mediated micromanipulation in vitro to show that the engagement of 200–400 TCR molecules by anti-CD3 was sufficient to induce a robust Ca flux (Wei et al., 1999). Schodin et al. (1996) had also shown that a lower number of active TCR was sufficient for target killing by a CTL clone in vitro. There is also an interesting parallel with patients carrying mutations in the CD3ε gene, some of which have T lymphocytes with low TCR levels but comparatively robust T cell function (Soudais et al., 1993). On the other hand, they contrast with reports that T cell reactivity showed a marked dependence on TCR density, in particular one describing up to a 10^4-fold drop in antigen sensitivity when the number of TCR molecules/cell was lowered from 25,000 to 8,000, reflected in a highly exponential density/response profile (Viola and Lanzavecchia, 1996; Blichfeldt et al., 1996). In most of these experiments, however, cells of varying TCR densities were obtained by 3 day preculture with antigen to induce TCR downmodulation; this manipulation may have conferred a refractory state on the T cells. Another explanation for the differences may be a strong influence of costimulatory

and adhesion molecules active in the natural in vivo setting, amplifying the response and lowering the numbers of TCRs that need to be engaged. Indeed, the clones used by Viola and Lanzavecchia were CD28 negative, and costimulatory molecules tuned the T cells to lower thresholds for activation in these assays. Finally, it is also conceivable that T cells "adapt" to progressively lower TCR levels by an increased sensitivity of downstream signal transduction pathways, an adaptation perhaps not possible in the time frame of the in vitro experiments.

T cells face a daunting challenge in maintaining specificity: the TCR recognizes its MHC/peptide ligand with very low affinity, there is often only a limited number of the specific ligand molecules available on APCs in vivo, and TCR:MHC/peptide engagement must persist over tens of hours. How can TCR:MHC/peptide interactions with half-lives on the order of 10 s account for such prolonged cellular interactions? Some element of TCR and MHC/peptide clustering has been invoked (reviewed in Germain, 1997; Dustin and Chan, 2000). Two different lines of reasoning have been advanced. First, TCRs and MHC molecules may have a natural propensity to dimerize, there being some structural data in support of this notion (Brown et al., 1993; Fernandez-Miguel et al., 1999; Reich et al., 1997); productive TCR triggering would entail crosslinking via multimerization of the TCR and MHC molecules into stable two-dimensional "pseudo-crystals" (reviewed in Germain, 1997). Here, the TCR:MHC/peptide complexes would drive the clustering, possibly through preformed TCR dimers or multimers, as recently suggested for antigen receptors on B cells (Schamel and Reth, 2000). The second proposal is the "immunological synapse" concept: shortly after initial TCR:MHC/peptide contact, a major rearrangement of the cytoskeleton draws TCR molecules into the center of a "bull's eye" formation. The cytoskeletal reorganization induced by the very first contacts would be the driving force that clusters TCR molecules, whether or not they have already been engaged (reviewed in Dustin and Chan, 2000). Our results are clearly more consistent with the latter proposal: a model invoking a higher order assembly driven by the crosslinking of TCR molecules themselves would be more highly dependent on TCR density; on the contrary, cytoskeletal herding of TCR molecules into the synapse could be far less dependent, drawing on whatever molecules the cell displays.

A few years ago, the serial engagement model was advanced to explain the seeming paradox of thousands of TCR molecules being engaged by only a few hundred MHC/peptide ligands (Valitutti et al., 1995b; Viola and Lanzavecchia, 1996). The key assumption underlying this model, that each molecule downmodulated from the cell surface upon activation, was one that had been productively engaged and resultingly internalized. This plausible interpretation was supported by a close correlation between the degree of downmodulation and the extent of cell activation. Recent results make one question this interpretation, however, as TCR downmodulation is now shown to result from an absence of reexpression instead of an induced internalization, with no evidence that individual TCR molecules that fail to be reexpressed are those that have been engaged (Liu et al., 2000). If anything, the opposite is true (Niedergang et al., 1997). Our results render serial engagement unnecessary, as it is not 10,000 to 20,000 TCR molecules that must be engaged to elicit activation, but only several hundred. Thus, these results are compatible with models in which TCR:MHC/peptide complexes form on a one to one basis and persist monogamously for a long time thereafter, as in an immunological synapse.

Can T Cells Survive without TCRs?

Initially, yes. Our results on the decay of TCR-negative T cell populations differ significantly from those on Ig-negative B cells (Lam et al., 1997). The latter died precipitously, such that it was essentially impossible to visualize B cells devoid of surface Igs in cytometric profiles. In contrast, denuded $CD4^+$ and $CD8^+$ T cells were readily observed (Figures 3B and 7A). These divergent outcomes may well stem from differences in the physiology of B and T cells. On the other hand, it is conceivable that the downmodulation of MHC class I molecules induced on B cells when their Igs were lost (Lam et al., 1997) rendered them efficient NK cell targets soon after the gene excision event, accelerating their death.

The $CD4^+$ and $CD8^+$ T cell populations decayed progressively after extinction of TCR expression. This behavior is consistent with results from diverse experimental setups indicating that $CD4^+$ T cells have only limited survival potential in the absence of MHC molecules, with a half-life very similar to that observed here (Takeda et al., 1996; Rooke et al., 1997; Witherden et al., 2000). It contrasts with the long-term persistence of naive $CD4^+$ T cell pools in thymectomized mice. Recent experiments, however, did not detect any differences in population decay when $CD4^+$ T cells were directly transferred into MHC-proficient and -deficient hosts, and invoked possible artifacts due to homeostatic proliferation to explain the discrepancy with prior results (Clarke and Rudensky, 2000; Dorfman et al., 2000). Our data serve to reaffirm the need for TCR:MHC/peptide engagement for the maintenance of naive T cell pools. Proliferation did not play a role in our experiments, as significant cycling was not observed, whether or not the cells had been exposed to tet (Figure 5). It is probably more than a concidence that the two dissenting results came after simple transfer of CFSE-labeled cells, rather than the more elaborate experimental protocols that generated peripheral T cell populations via natural export of thymocytes (Takeda et al., 1996; Rooke et al., 1997; Witherden et al., 2000).

Comparison of the results from different experimental systems has given the impression that populations of $CD8^+$ T cells decay more rapidly than $CD4^+$ populations when deprived of their MHC/peptide ligands (discussed in Witherden et al., 2000). However, in all of these systems, only one or the other of the two compartments could be studied. The results reported here, exploiting an experimental setup that permits simultaneous analysis of the $CD4^+$ and $CD8^+$ populations, argue for decay kinetics of the same order, only slightly faster for $CD8^+$ cells. This conclusion bolsters prior proposals (Murali-Krishna et al., 1999; Dorfman et al., 2000) that the near-instantaneous disappearance of $CD8^+$ T cells described in certain cases (e.g., Tanchot et al., 1997) may have been complicated by rejection phenomena.

Our findings show that quantitative relationships between TCR density and T cell responsiveness that were clear in in vitro settings did not hold in an in vivo environment, likely due to the influence of elaborately structured microenvironments and the opportunity for the full panoply of costimulatory and adhesion molecules to impinge on the process. Likewise, a striking characteristic of B cells denuded of their antigen receptors was not a feature of TCR-less T cells. No doubt more examples of such divergences will arise in the future as regulatable, cell-type-specific transgenesis systems permit us to explore in vivo questions that before could only be addressed in vitro.

Experimental Procedures

Construction and Analysis of Transgenic Mice

Details of the constructs, mouse genotyping, and RNA analysis are presented as supplemental data (see http://www.immunity.com/cgi/content/full/15/1/71/DC1). Flow cytometry was performed as previously described (Rooke et al., 1997). The quantification of the number of TCR molecules on T cells was done by cytofluorometry, comparing T cells stained with a saturating concentration of anti-Vα2 mAb with standardization microbeads containing a known number of fluorescent molecules (QuantibritePE beads, Becton Dickinson). T cells were stained with saturating concentrations of anti-Vα2 mAb and their staining intensity (minus background autofluorescence) was compared to a standard curve of microbeads carrying known numbers of phycoerythrin molecules. The Vβ5 transgenic and C$\alpha^{\circ/\circ}$ lines have been described previously (Correia-Neves et al., 1999; Philpott et al., 1992). Tet treatment was performed by supplementing the drinking water with the indicated concentration of tet plus 2% sucrose. Bottles were changed twice a week during the course of the treatment.

In Vivo T Cell Responses

Vβ5/L/TAO mice treated for 2 weeks with 0, 5, 10, or 400 μg/ml of tet in the drinking water (supplemented with 2% sucrose) were immunized intravenously with either 0.1 μg or 20 μg of SIINFEKL peptide diluted in sterile PBS, and T cell responses were analyzed 48 hr afterward. To measure T cell proliferation, mice were injected with 1 mg of BrdU intra peritoneally 36 and 42 hr after immunization, sacrificed 6 hr after the last injection and their lymph nodes removed. Cells were stained for BrdU incorporation as described (Gilfillan et al., 1994). In brief, cells were stained with anti-CD4-PE and anti-CD8-tricolor and then fixed in 70% ethanol for 30 min. After washing, cells were permeabilized overnight in 1% paraformaldehyde, 0.1% Tween-20 in PBS, treated with Dnase, and stained with anti-BrdU-FITC (Becton Dickinson). At high proliferation rates, the intensity of BrdU staining decreases (Figure 5), likely through secondary divisions or stimulation of de novo pyrimidine synthesis. To correct for this effect, a "proliferation index" was calculated by multiplying the percent labeled cells by the shift in the mean fluorescence intensity (MFI) relative to the MFI of unstimulated cells. Cytokine production was analyzed by flow cytometry as described (Openshaw et al., 1995). Lymph node cells were stimulated for 4 hr in vitro with PMA (50 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma); brefeldin A (Sigma) was added to a final concentration of 10 μg/ml during the last 2 hr of incubation. Cells were then fixed in 1% formaldehyde for 30 min and washed in PBS. The fixed cells were stained for intracellular cytokines in the presence of 0.5% saponin with anti-IL-2-FITC, anti-IL-4-FITC, and anti-γIFN-FITC mAbs (Pharmingen), counterstained with anti-CD4-PE and anti-CD8-tricolor, and analyzed on a flow cytometer. A cytokine production index was determined by summing the fraction of positive cells with the shift in mean fluorescence intensity.

T Cell Survival Assays

To follow in individual mice the fate of T cells lacking TCR expression, we surgically removed lymph nodes at different time points, as described previously (Rooke et al., 1997). The right axillary lymph node was taken before the beginning of tet treatment, and three other data points were collected at various times by removal of the left inguinal, and right and left axillary lymph nodes. The mesenteric lymph nodes were taken at the time of sacrifice. When more than six points are shown, the other data derive from blood sampling, taken in between the removal of lymph nodes. Cells were stained with anti-CD4-PE, anti-CD8-Tricolor, and anti-CD3 (KT3) followed by goat anti-rat-FITC.

Acknowledgments

We thank H. Bujard and F. Carbone for DNA constructs, A. Gonzalez, H.M. Van Santen, and M. Maccioni for helpful discussions, and P. Gerber, C. Ebel, and J. Hergueux for assistance. N.L. received a Centenial Fellowship from the Medical Research Council of Canada, L.S.W. was supported by the European Molecular Biology Organization and the Human Frontier Science Program, and R.O. by the European Molecular Biology Organization and the German Research Council (DFG). This work was supported by institute funds from the INSERM, the CNRS, the Hopital Universitaire de Strasbourg, and by a grant from the Association pour la Recherche contre le Cancer.

Received January 29, 2001; revised May 11, 2001.

References

Alam, S.M., Travers, P.J., Wung, J.L., Nasholds, W., Redpath, S., Jameson, S.C., and Gascoigne, N.R. (1996). T-cell-receptor affinity and thymocyte positive selection. Nature *381*, 616–620.

Blichfeldt, E., Munthe, L.A., Rotnes, J.S., and Bogen, B. (1996). Dual T cell receptor T cells have a decreased sensitivity to physiological ligands due to reduced density of each T cell receptor. Eur. J. Immunol. *26*, 2876–2884.

Boursalian, T.E., and Bottomly, K. (1999). Survival of naive CD4 T cells: roles of restricting versus selecting MHC class II and cytokine milieu. J. Immunol. *162*, 3795–3801.

Brocker, T. (1997). Survival of mature CD4 T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells. J. Exp. Med. *186*, 1223–1232.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature *364*, 33–39.

Buckland, J., Pennington, D.J., Bruno, L., and Owen, M.J. (2000). Co-ordination of the expression of the protein tyrosine kinase p56lck with the pre-T cell receptor during thymocyte development. Eur. J. Immunol. *30*, 8–18.

Chaffin, K.E., Beals, C.R., Wiklie, T.M., Forbush, K.A., Simon, M.L., and Perlmutter, R.M. (1990). Dissection of thymocyte signaling pathways by in vivo expression of pertussis toxin ADP-ribosyltransferase. EMBO J. *9*, 3821–3829.

Clarke, S.R., and Rudensky, A.Y. (2000). Survival and homeostatic proliferation of naive peripheral CD4+T cells in the absence of self peptide: MHC complexes. J. Immunol. *165*, 2458–2464.

Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T., and Bottomly, K. (1995). Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. J. Exp. Med. *182*, 1591–1596.

Correia-Neves, M., Waltzinger, C., Wurtz, J.M., Benoist, C., and Mathis, D. (1999). Amino acids specifying MHC class preference in TCR V alpha 2 regions. J. Immunol. *163*, 5471–5477.

Correia-Neves, M., Waltzinger, C., Mathis, D., and Benoist, C. (2001). The shaping of the T cell repertoire. Immunity *14*, 21–32.

Davis, K.A., Abrams, B., Iyer, S.B., Hoffman, R.A., and Bishop, J.E. (1998). Determination of CD4 antigen density on cells: role of antibody valency, avidity, clones, and conjugation. Cytometry *33*, 197–205.

Dorfman, J.R., Stefanova, I., Yasutomo, K., and Germain, R.N. (2000). $CD4^+$ T cell survival is not directly linked to self-MHC-induced TCR signaling. Nat. Immunol. *1*, 329–335.

Dustin, M.L., and Chan, A.C. (2000). Signaling takes shape in the immune system. Cell *103*, 283–294.

Ernst, B., Lee, D.S., Chang, J.M., Sprent, J., and Surh, C.D. (1999). The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. Immunity *11*, 173–181.

Fernandez-Miguel, G., Alarcon, B., Iglesias, A., Bluethmann, H., Alvarez-Mon, M., Sanz, E., and de la Hera, A. (1999). Multivalent structure of an alphabeta T cell receptor. Proc. Natl. Acad. Sci. *16*, 1547–1452.

Ferreira, C., Barthlott, T., Garcia, S., Zamoyska, R., and Stockinger, B. (1999). Differential survival of naive CD4 and CD8 T cells. J. Immunol. *165*, 3689–3694.

Furth, P.A., St. Onge, L., Böger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H., and Hennighausen, L. (1994). Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. Proc. Natl. Acad. Sci. *91*, 9302–9306.

Germain, R.N. (1997). T-cell signaling: the importance of receptor clustering. Curr. Biol. *7*, R640–R644.

Gilfillan, S., Waltzinger, C., Benoist, C., and Mathis, D. (1994). More efficient positive selection of thymocytes in mice lacking terminal deoxynucleotidyl transferase. Int. Immunol. *6*, 1681–1686.

Goldrath, A.W., and Bevan, M.J. (1999). Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. Immunity *11*, 183–190.

Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. (1994). T cell receptor antagonist peptides induce positive selection. Cell *76*, 17–27.

Hosken, N.A., Shibuya, K., Heath, A.W., Murphy, K.M., and O'Garra, A. (1995). The effect of antigen dose on CD4+T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. J. Exp. Med. *182*, 1579–1584.

Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity *8*, 89–95.

Itoh, Y., and Germain, R.N. (1997). Single cell analysis reveals regulated hierarchical T cell antigen receptor signaling thresholds and intraclonal heterogeneity for individual cytokine responses of CD4+ T cells. J. Exp. Med. *186*, 757–766.

Kelly, J.M., Sterry, S.J., Cose, S., Turner, S.J., Fecondo, J., Rodda, S., Fink, P.J., and Carbone, F.R. (1993). Identification of conserved T cell receptor CDR3 residues contacting known exposed peptide side chains from a major histocompatibility complex class I-bound determinant. Eur. J. Immunol. *23*, 3318–3326.

Kieper, W.C., and Jameson, S.C. (1999). Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. Proc. Natl. Acad. Sci. *96*, 13306–13311.

Kirberg, J., Berns, A., and von Boehmer, H. (1997). Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. J. Exp. Med. *186*, 1269–1275.

Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J., Ullmer, C., Lübbert, H., and Bujard, H. (1996). Doxycycline-mediated, quantitative and tissue-specific control of gene expression in transgenic mice. Proc. Natl. Acad. Sci. *93*, 10933–10938.

Lam, K.P., Kuhn, R., and Rajewsky, K. (1997). In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell *90*, 1073–1083.

Lanzavecchia, A., Lezzi, G., and Viola, A. (1999). From TCR engagement to T cell activation: a kinetic view of T cell behavior. Cell *96*, 1–4.

Liu, H., Rhodes, M., Wiest, D.L., and Vignali, D.A. (2000). On the dynamics of TCR:CD3 complex cell surface expression and downmodulation. Immunity *13*, 665–675.

Lyons, D.S., Lieberman, S.A., Hampl, J., Boniface, J.J., Chien, Y., Berg, L.J., and Davis, M.M. (1996). A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. Immunity. *5*, 53–61.

Matsui, K., Boniface, J.J., Steffner, P., Reay, P.A., and Davis, M.M. (1994). Kinetics of T-cell receptor binding to peptide/I-Ek complexes:correlation of the dissociation rate with T-cell responsiveness. Proc. Natl. Acad. Sci. *91*, 12862–12866.

Mertsching, E., Wilson, A., MacDonald, H.R., and Ceredig, R. (1997). T cell receptor alpha gene rearrangement and transcription in adult thymic gamma delta cells. Eur. J. Immunol. *27*, 389–396.

Murali-Krishna, K., Lau, L.L., Sambhara, S., Lemonnier, F., Altman, J., and Ahmed, R. (1999). Persistence of memory CD8 T cells in MHC class I-deficient mice. Science *286*, 1377–1381.

Nesic, D., and Vukmanovic, S. (1998). MHC class I is required for peripheral accumulation of CD8+ thymic emigrants. J. Immunol. *160*, 3705–3712.

Niedergang, F., Dautry-Varsat, A., and Alcover, A. (1997). Peptide antigen or superantigen-induced down-regulation of TCRs involves both stimulated and unstimulated receptors. J. Immunol. *159*, 1703–1710.

Oehen, S., and Brduscha-Reim, K. (1999). Naive cytotoxic T lymphocytes spontaneously asquire effector function in lymphocytopenic recipients: a pitfall for T cell memory studies. Eur. J. Immunol. *29*, 608–614.

Openshaw, P., Murphy, E.E., Hoskin, N.A., Maino, V., Davis, K., Murphy, K., and O'Garra, A. (1995). Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. J. Exp. Med. *182*, 1357–1367.

Pfeiffer, C., Stein, J., Southwood, S., Ketelaar, H., Sette, A., and Bottomly, K. (1995). Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. J. Exp. Med. *181*, 1569–1574.

Philpott, K.L., Viney, J.L., Kay, G., Rastan, S., Gardiner, E.M., Chae, S., Hayday, A.C., and Owen, M.J. (1992). Lymphoid development in mice congenitally lacking T cell receptor $\alpha\beta$-expressing cells. Science *256*, 1448–1452.

Reich, Z., Boniface, J.J., Lyons, D.S., Borochov, N., Wachtel, E.J., and Davis, M.M. (1997). Ligand-specific oligomerization of T-cell receptor molecules. Nature *387*, 617–620.

Rocha, B., and von Boehmer, H. (1991). Peripheral selection of the T cell repertoire. Science *251*, 1225–1228.

Rogers, P.R., and Croft, M. (1999). Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. J. Immunol. *163*, 1205–1213.

Rooke, R., Waltzinger, C., Benoist, C., and Mathis, D. (1997). Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenoviruses. Immunity *7*, 123–134.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H.J., and von Boehmer, H. (1994). Analysis and expression of a cloned pre-T cell receptor gene. Science *266*, 1208–1212.

Schamel, W.W., and Reth, M. (2000). Monomeric and oligomeric complexes of the B cell antigen receptor. Immunity *13*, 5–14.

Schodin, B.A., Tsomides, T.J., and Kranz, D.M. (1996). Correlation between the number of T cell receptors required for t cell activation and TCR-ligand affinity. Immunity *5*, 137–146.

Sohn, S.J., Forbush, K.A., Nguyen, N., Witthuhn, B., Nosaka, T., Ihle, J.N., and Perlmutter, R.M. (1998). Requirement for Jak3 in mature T cells: its role in regulation of T cell homeostasis. J. Immunol. *160*, 2130–2138.

Soudais, C., de Villartay, J.P., Le Deist, F., Fischer, A., and Lisowska-Grospierre, B. (1993). Independent mutation of the human CD3-epsilon gene resulting in a T cell receptor/CD3 complex immunodeficiency. Nat. Genet. *3*, 77–81.

Surh, C.D., and Sprent, J. (2000). Homeostatic T cell proliferation. How far can T cells be activated to self-ligands? J. Exp. Med. *192*, F9–F14.

Sykulev, Y., Brunmark, A., Jackson, M., Cohen, R.J., Peterson, P.A., and Eisen, H.N. (1994). Kinetics and affinity of reaction between an antigen-specific T cell receptor and peptide-MHC complexes. Immunity *1*, 15–22.

Takeda, S., Rodewald, H.R., Arakawa, H., Bluethmann, H., and Shimizu, T. (1996). MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. Immunity *5*, 217–228.

Tanchot, C., Lemonnier, F.A., Pérarnau, B., Freitas, A.A., and Rocha, B. (1997). Differential requirements for survival and proliferation of CD8 naïve or memory T cells. Science *276*, 2057–2058.

Tao, W., Constant, S., Jorritsman, P., and Bottomly, K. (1997). Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. J. Immunol. *159*, 5956–5963.

Valitutti, S., Dessing, M., Aktories, K., Gallati, H., and Lanzavecchia, A. (1995a). Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T action cytoskeleton. J. Exp. Med. *181*, 577–584.

Valitutti, S., Muller, S., Cella, M., Padovan, E., and Lanzavecchia, A. (1995b). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature *375*, 148–151.

Valitutti, S., Müller, S., Dessing, M., and Lanzavecchia, A. (1996). Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. J. Exp. Med. *183*, 1917–1921.

Viola, A., and Lanzavecchia, A. (1996). T cell activation determined by T cell receptor number and tunable thresholds. Science *273*, 104–106.

Viret, C., Wong, F.S., and Janeway, C.A., Jr. (1999). Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. Immunity *10*, 559–568.

Wei, X., Tromberg, B.J., and Cahalan, M.D. (1999). Mapping the sensitivity of T cells with an optical trap: polarity and minimal number of receptors for Ca(2+) signaling. Proc. Natl. Acad. Sci. *96*, 8471–8476.

Witherden, D., van Oers, N., Waltzinger, C., Weiss, A., Benoist, C., and Mathis, D. (2000). Tetracycline-controllable selection of $CD4^+$ T cells: half-life and survival signals in the absence of major histocompatibility complex class II molecules. J. Exp. Med. *191*, 355–364.

Yasutomo, K., Doyle, C., Miele, L., and Germain, R.N. (2000). The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate. Nature *404*, 506–510.