Homeostatic Competition Among T Cells Revealed by Conditional Inactivation of the Mouse *Cd4* Gene

Qi Wang, Julie Strong, and Nigel Killeen

Department of Microbiology and Immunology, University of California at San Francisco, San Francisco CA 94143

Abstract

Absence of CD4 impairs the efficiency of T cell receptor (TCR) signaling in response to major histocompatibility complex (MHC) class II–presented peptides. Here we use mice carrying a conditional *Cd4* allele to study the consequences of impaired TCR signaling after the completion of thymocyte development. We show that loss of CD4 decreases the steady-state proliferation of T cells as monitored by in vivo labeling with bromo-deoxyuridine. Moreover, T cells lacking CD4 compete poorly with CD4-expressing T cells during proliferative expansion after transfer into lymphopenic recipients. The data suggest that T cells compete with one another during homeostatic proliferation, and indicate that the basis of this competition is TCR signaling.

Key words: CD4 • T lymphocytes • helper • gene targeting • CD4-positive T lymphocytes

Introduction

Ligand-dependent signaling by TCRs determines multiple aspects of T cell development and differentiation. In the absence of encounter with peptide–MHC ligands, T cells become apoptotic in the thymus and do not undergo clonal expansion during immune responses in the periphery. They also demonstrate survival defects and proliferative problems when placed into MHC-deficient environments, suggesting that peptide–MHC engagement is a necessary component of general T cell homeostasis (1–5).

Whereas the significance of TCR signaling in the thymus and during immune responses is clear, the nature of its role in T cell homeostasis and postthymic selection of naive T cells is unresolved. It is unclear, for instance, whether TCR signaling in response to endogenous peptide–MHC ligands constitutes a potent or minor selection for naive T cells. Moreover, it remains to be determined whether selection of this sort confers any obvious benefit on the immune system in terms of its ability to mount pathogen-specific responses and/or to avoid autoimmune responses.

The CD4 coreceptor is an important part of the TCR signaling machinery used by helper lineage T cells (6–8). Its presence during antigen recognition enhances the recognition of low affinity ligands by TCRs and reduces the threshold concentration required for T cell activation by high affinity ligands (9–11). Nonetheless, CD4 can be dispensable for T cell development in the thymus and activation in the periphery (12–14), reflecting the modest nature of its contribution to TCR signaling. An induced CD4 deficiency is therefore one means for selectively impairing the efficiency of the TCR signal without completely eliminating it (9).

In this paper, we have used conditional gene inactivation systems to study the consequences of impairing the TCR signal after the completion of thymic-positive selection. The data provide evidence of competition between T cells based on the strength of the TCR signal they receive and are consistent with the existence of extra-thymic MHC–peptide-dependent T cell selection.

Materials and Methods

Gene Targeting and Generation of Mutant Mice. To generate the *Cd4* targeting construct, a 2.2-Kb *Bgl* II fragment containing exons 2 and 3 of the *Cd4* gene was cloned into a modified form of pBluescript-KS$^+$. A double-stranded oligonucleotide linker containing a *loxP* and a *Bam*H I site was then inserted into a *Sac* I site upstream of the initiator methionine in exon 2. A downstream 6.5-Kb *Bgl* II-*Asp*718 I fragment containing exons 4 and 5 of the *Cd4* gene was added to the construct, followed by the blunt-ended insertion of a human *CD52* cDNA fused to a *loxP*-flanked *MC1-neo* gene (gift of Hua Gu, National Institutes of Health, Bethseda, MD) into the *Bgl* II site in the third intron. The construct was linearized and electroporated into JM-1 ES cells (129/SvJ) (15). ES cell colonies were screened using a probe

Address correspondence to Nigel Killeen, Dept. of Microbiology and Immunology, University of California at San Francisco, San Francisco, CA 94143-0414. Phone: 415-502-5495; Fax: 415-502-8424; E-mail: nigel@itsa.ucsf.edu

from the first intron of the *Cd4* gene hybridized to Southern blots of *Sac* I–digested genomic DNA according to standard procedures (16). Cells carrying the desired mutation were transiently transfected with a Cre expression vector and replated at low density on irradiated primary embryonic fibroblasts. Colonies that had lost the *loxP*-flanked *MC1-neo* gene by Cre recombination were identified by Southern blot analysis (as above), and cells from them were subsequently microinjected into C57BL/6 blastocysts. Chimeric male mice were crossed to C57BL/6 females to establish lines of mice carrying the *Cd4* mutation.

The generation of *dLck-hcre* transgenic mice expressing a codon optimized form of the *cre* open reading frame under the control of the distal *Lck* promoter (17) will be described elsewhere (unpublished data). These mice were bred to animals carrying the conditional CD4 allele and then backcrossed to $Cd4^{-/-}$ mice to generate $Cd4^{lox/-}$ *dLck-hcre* mice. All mice were housed in the Parnassus Heights pathogen-free barrier facility at the University of California at San Francisco.

Antibodies and Flow Cytometry. FITC-, PE-, or TC-conjugated antibodies and Annexin V were purchased from Caltag or BD PharMingen. 0.3×10^6 lymphocytes or thymocytes were incubated for 30 min on ice in a volume of 25 μl of FACS® buffer (PBS with 0.3% BSA and 0.01% NaN_3) containing antibodies at saturating concentrations. The cells were washed twice in FACS® buffer, stained with secondary reagents as necessary, washed again, and then analyzed using a FACScan™ flow cytometer (Becton Dickinson) and CELLQuest™ or Flowjo software. Annexin V staining was performed following the manufacturer's protocol (BD PharMingen).

BrdU Labeling and Detection. 8-wk-old mice were provided bromo-deoxyuridine (BrdUrd)* (0.8 mg/ml) in their drinking water for a period of 20 d. On days 5, 10, 15, and 20, mesenteric and inguinal lymph nodes were harvested from selected mice and stained for BrdUrd incorporation using a modification of published methods (18). Briefly, 10^6 cells were stained in FACS® buffer with antibodies, washed twice, and then resuspended in 40 μl of ice-cold 0.15 M NaCl. While mixing gently, 96 μl of ice-cold 95% ethanol were added drop-wise to the cells; they were then incubated on ice in the dark for 30 min before washing with 160 μl of cold PBS. The cells were fixed for 30 min at room temperature by adding 150 μl of PBS containing 1% paraformaldehyde and 0.03% Tween-20. After this step, the cells were spun down and incubated with 15,000 U of DNase I (Sigma-Aldrich) in 100 μl of 4.2 mM $MgCl_2$, 0.15 M NaCl, pH 5.0, for 10 min at room temperature. After washing with 200 μl of PBS, the cells were incubated in the dark at room temperature for 30 min with 50 μl of a 1:5 dilution of FITC-anti-BrdUrd (BD PharMingen). Finally, the cells were washed twice with PBS, resuspended in FACS® buffer and analyzed using the FACScan™. Electronic gating was used to quantify cells that stained brightly with FITC anti-BrdUrd and were therefore likely to have undergone postthymic proliferation (49).

In Vivo Depletion of $CD4^+$ T Cells, KLH Immunization, and In Vitro Antigen Restimulation Assays. Mice were injected with 0.5 mg of purified anti-CD4 mAb (GK1.5) intraperitoneally on 3 d consecutively. A fourth intraperitoneal injection of 1 mg of antibody was given on day 6 before immunization on day 7. CD4-depleted or control mice were immunized intraperitoneally with 100 μg of KLH in Alum (Pierce Chemical Co.), and their spleens were harvested 7 d after immunization. 10^6 CD8-depleted splenocytes (containing equivalent numbers of CD4 lineage cells) were stimulated in vitro with 50 μg/ml KLH in 96-well flat-bottomed plates. Supernatants were removed from the stimulated cultures 48 h after initiation, and these were assayed for cytokine content by ELISA using antibodies and standards purchased from BD PharMingen.

Cell Transfers and CFSE Labeling. Bone marrow preparations harvested from $Cd4^{lox/-}$ *dlck-hcre*^{3778} and *dlck-hcre*^{3779} mice were depleted of T cells using anti-Thy1.2 MACS® beads and Miltenyi LS^+ columns. 5×10^6 T cell–depleted bone marrow cells were injected into the tail veins of irradiated (1,100 rads) 6-wk-old C57BL/6 female mice. The recipients received water containing neomycin (1.1%; Sigma-Aldrich) and polymixin B sulfate (850 U/ml; Paddock Laboratories) for 6–8 wk.

Single cell suspensions of pooled lymph nodes and spleen were prepared from C57BL/6, $Cd4^{-/-}$, and *dlck-hcre* $Cd4^{lox/-}$ bone marrow chimeras. $CD4^+$ T cells were removed from selected samples using anti-CD4 MACS® beads and LS^+ columns. The cells were washed in PBS and incubated in 2.5 μM CFSE in PBS for 8 min at room temperature. FCS was added to quench the CFSE and the cells were washed twice in IMDM/10%FCS medium. 3×10^7 labeled cells were injected into the tail veins of recombination activating gene (RAG)-$1^{-/-}$ mice.

Results

Generation and Analysis of Mouse Lines that Allow for Stage-specific Inactivation of the CD4 Gene. A conditional allele of the mouse *Cd4* gene was created in ES cells by targeted mutagenesis. The targeting construct used for this purpose featured *loxP* sites both upstream of the initiator methionine in exon 2 and also downstream of exon 3 within the third intron (Fig. 1 A). Cre-mediated recombination between these two *loxP* sites deletes a critical part of the gene which encodes both the CD4 signal peptide and a part of the molecule that is necessary for MHC class II binding (19). ES cells carrying the desired mutation were identified by standard procedures (Fig. 1 B) and were subsequently transiently transfected with a Cre recombinase expression vector to delete the linked *loxP*-flanked *MC1-neo* gene. The conditional *Cd4* allele (hereafter referred to as $Cd4^{lox}$) was established in the germline of mice by blastocyst microinjection of the mutant ES cells.

To inactivate the *Cd4* gene at different stages in T cell development, we used three recently developed lines of mice that express the Cre recombinase under the control of the distal promoter of the *Lck* gene. The developmental onset of Cre recombination in these mice is variable and has been studied using a Cre-dependent reporter line and other approaches (unpublished data). Cre recombination in line 3785 is highly penetrant in T cells, with its onset being largely after the preTCR signal has been delivered. In contrast, Cre recombination occurs later in lines 3778 and 3779, primarily after cells have rearranged the locus encoding TCR-α and progressed through the TCR-αβ checkpoint to the TCR^{hi} stage of development. Cre recombination in T cells of line 3778 is less efficient than in 3779, which itself shows less recombination than 3785.

Abbreviations used in this paper: BrdUrd, bromo-deoxyuridine; LACK, *Leishmania major* homologue of mammalian RACK1; RAG, recombination activating gene.

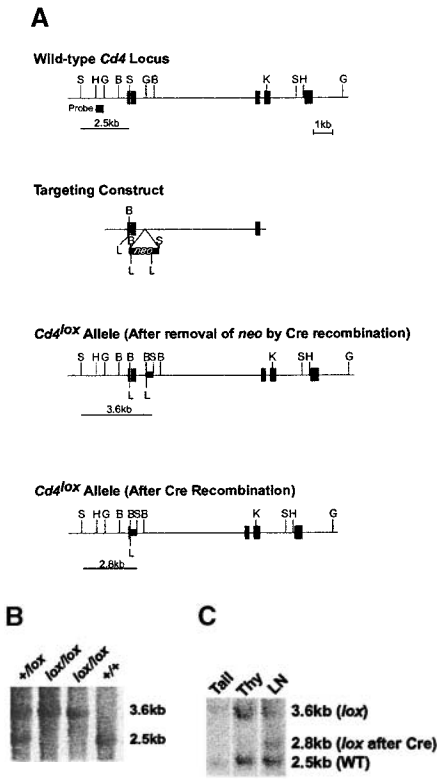

Figure 1. Gene targeting to create a conditional *Cd4* allele. (A) Design of the targeting construct used to insert *loxP* sites into the *Cd4* gene and structure of the mutant *Cd4* allele before and after Cre recombination. The location of the intron 1 probe used on Southern blot analyses is indicated, as are the sizes of the diagnostic *Sac* I fragments it detects. Enzyme sites are labeled as follows: *Bam*H I – B; *Bgl* II – G; *Hind* III – H; *Sac* I – S; *Kpn* I – K; and *loxP* – L. (B) Southern blot analysis of *Sac* I–digested tail DNA from mice of the indicated genotypes probed with the intron 1 probe. (C) Southern blot analysis of *Sac* I–digested tail, thymus, and lymph node DNA from *dLCk-hcre* transgenic $Cd4^{lox/+}$ mice hybridized with the intron 1 probe. The sizes of the wild-type and recombined or unrecombined $Cd4^{lox}$ bands are indicated.

Cre transgenic mice were generated that had both a null and conditional allele of *Cd4* (i.e., $Cd4^{lox/-}$ *dLck-hcre* mice) so that recombination on only one chromosome would be required to cause loss of CD4. Thymocytes and T cells from the mice were then analyzed by flow cytometry as shown in Fig. 2. Inactivation of the *Cd4* gene in line 3785 occurred early during thymocyte development so that roughly half of the TCR^{lo} small cells failed to express CD4. These mice were similar to $Cd4^{-/-}$ mice in the overabundance of $CD8^+$ T cells found in their lymph nodes, and their paucity of peripheral CD4 lineage T cells (identifiable by expression of the αβ TCR and absence of expression of CD8). Such a phenotype indicates that most double-positive stage thymocytes in the mice had inactivated the *Cd4* gene before undergoing positive selection where the function of CD4 first becomes significant. This interpretation was substantiated by analysis of cells that had upregulated the TCR (Fig. 2) or CD69 (data not shown), most of which expressed very little or no CD4 in line 3785. The similar representation of CD4 lineage cells in $Cd4^{lox/-}$ *dLck-hcre*^{3785} and $Cd4^{-/-}$ mice also confirmed that the $Cd4^{lox}$ allele performed as expected, causing loss of CD4 expression and function after Cre recombination.

Lines 3778 and 3779 showed near normal distributions of CD4 expression at the TCR^{lo} stage of development, with loss of CD4 becoming prevalent primarily at the more mature TCR^{hi} stage. $Cd4^{lox/-}$ *dLck-hcre*^{3778} and $Cd4^{lox/-}$ *dLck-hcre*^{3779} mice had substantial numbers of mature CD4 lineage cells in their lymph nodes (85 ± 10 [n = 15] and 73 ± 12 [n = 18] percent of the nontransgenic controls, respectively). This was true even though 29 ± 3% and 81 ± 4% of the CD4 lineage cells lacked CD4 expression in these mice. Steady-state analysis of this sort indicated that in contrast to mice showing early loss of CD4 ($Cd4^{lox/-}$ *dLck-hcre*^{3785} or $Cd4^{-/-}$ mice), CD4-deficient cells could accumulate in substantial numbers when CD4 loss occurred late in development.

Immune Responses Mediated by CD4-deficient T Cells. We have found previously that Th cells in $Cd4^{-/-}$ mice use TCRs that have similar ligand-binding properties to those in CD4-expressing mice (9) consistent with a modest role for CD4 in regulating the selection of the TCR repertoire. Nevertheless, it was of interest to examine the antigen-responsiveness of CD4 lineage cells that had lost CD4 as a late-thymic or postthymic event, as in $Cd4^{lox/-}$ *dLck-hcre*^{3778} mice. Most, if not all of the CD4-deficient T helper cells in these mice would have undergone thymic-positive selection in the presence of CD4, allowing for a direct assessment of how postthymic loss of CD4 might impair antigen responsiveness.

In one series of experiments, we immunized cohorts of mice with KLH and then used an in vitro restimulation assay to monitor the outcome of in vivo clonal expansion. The $Cd4^{lox/-}$ *dLck-hcre*^{3778} mice were pretreated with cytotoxic anti-CD4 mAb, to deplete CD4-expressing cells and thus ensure that the responsive T cells lacked CD4 throughout the immunization procedure. As shown in Fig. 3 A, the CD4-deficient T cells were capable of vigorous responses to this antigen. Interestingly, as observed previously for $Cd4^{-/-}$ Th cells (20–22) the late loss of CD4 significantly compromised T_H2-mediated IL-4 secretion, but did not impair T_H1 responses (Fig. 3 A). Thus, these data reveal the capacity of the CD4-deficient T cells to proliferate in vivo in response to antigenic stimulation, while also providing further support for the importance of CD4 in T_H2 differentiation.

In related experiments, we immunized $Cd4^{lox/-}$ *dLck-hcre*^{3778} and $Cd4^{lox/-}$ *dLck-hcre*^{3779} mice with the *Leishmania major* homologue of mammalian RACK1 (LACK) antigen (23, 24). To increase the precursor frequency of antigen-reactive cells in the mice we used animals expressing a transgene-derived LACK-reactive TCR-β chain (9), and detected cells carrying LACK-reactive TCR heterodimers using a fluorescent MHC–peptide multimer (25). These TCR-β transgenic mice have a diverse TCR repertoire because of the rearrangement of endogenous TCR-α chain genes, but they mount vigorous responses to LACK that are characterized by the rapid accumulation of large num-

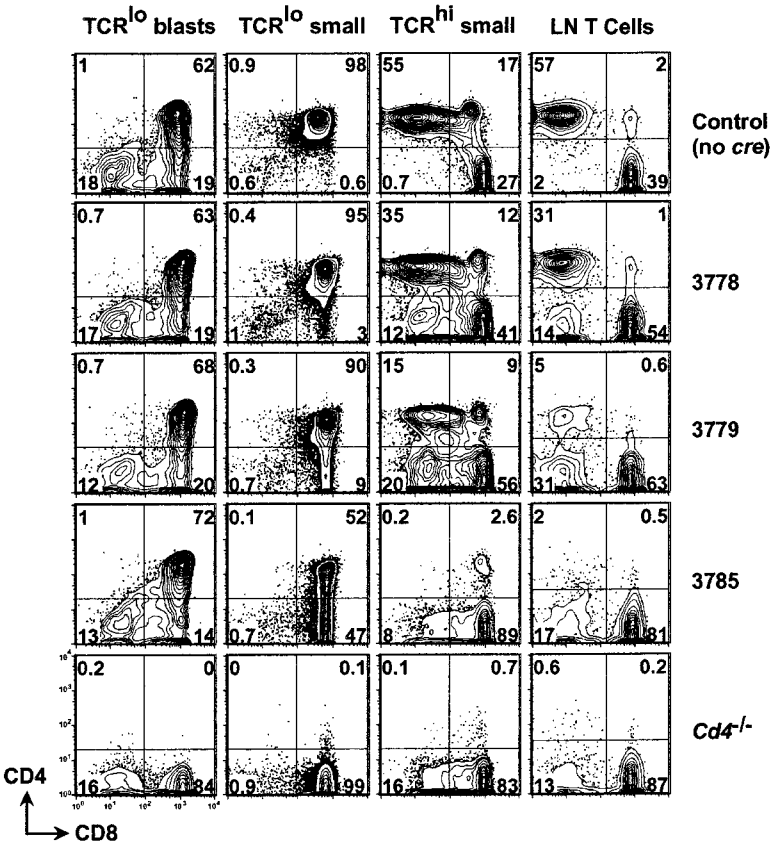

Figure 2. T cell populations in *Cd4*$^{lox/-}$ *dLck-hcre* transgenic mice. Thymocytes at progressively increasing stages of maturity (first three columns) and T cells were analyzed by three-color flow cytometry using directly conjugated fluorescent anti-CD4, CD8, and TCR-β mAbs. All but the last row of contour plots (from *Cd4*$^{-/-}$ mice) show cells from mice that carried the conditional *Cd4* allele balanced by a null *Cd4* allele, and that were also transgenic for the indicated *dLck-hcre* insertions. The contour plots show the relative representation of populations defined by CD4 and CD8 expression.

bers of LACK-reactive T cells in the draining lymph nodes (9). In both *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} and *Cd4*$^{lox/-}$ *dLck-hcre*^{3779} mice, LACK-reactive cells were equivalently represented in both the CD4-expressing and CD4-deficient populations of T helper cells by flow cytometry (Fig. 3 B and C). These results again indicate that despite the expected deficiency in TCR function, cells that lack CD4 retain the capacity to recognize and mount effective proliferative responses to antigens.

Differences in Th Cell Turnover Dependent on the Timing of CD4 Loss. Increased apoptosis of CD4 lineage cells has been observed previously in *Cd4*$^{-/-}$ mice (26) and in mice with an MHC class II mutation that blocks CD4 binding (27). In *Cd4*$^{-/-}$ mice, this apoptosis is partially counteracted by a marked increase in homeostatic T helper cell proliferation, with the net result of both effects being a reduced but nonetheless stable complement of CD4 lineage cells (26). To test whether late loss of CD4 (in lines 3778 and 3779) was also associated with increased apoptosis and homeostatic proliferation of CD4$^-$CD8$^-$ T cells, the *Cd4*$^{lox/-}$ *dLck-hcre* transgenic mice were fed BrdUrd continuously in their drinking water. We then monitored the mice by flow cytometry to track the accumulation of T cells that had undergone cell division and thereby incorporated BrdUrd into their DNA. The results of this analysis are shown in Fig. 4 A.

Early loss of CD4 (in *Cd4*$^{-/-}$ or in *Cd4*$^{lox/-}$ *dLck-hcre*^{3785} mice) led to exaggerated proliferation in peripheral CD4 lineage cells. These cells were also enriched for the display of markers typical of memory cells (28) or cells expanding in a T cell void (29, 30) such as low expression of CD62L or high expression of CD44 (Fig. 4 B and data not shown). There was also an increase in apoptosis among fresh ex vivo CD4 lineage T cells from these mice (Fig. 4 C). Together, these findings are entirely consistent with previously published results showing decreased survival and enhanced turnover when Th cells undergo positive selection without CD4 (26).

In contrast to the consequences of early loss of CD4, its later loss in *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} or *dLck-hcre*^{3779} mice was not associated with increased proliferation (Figs. 4 A, 6 B, and data not shown). Th cells in these mice incorporated BrdUrd at a rate that was consistently lower than CD4$^+$ T cells in control mice that did not express Cre. Indeed, CD4$^+$ T cells in the *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} or *dLck-hcre*^{3779} mice reproducibly showed enhanced proliferation relative to the CD4$^-$CD8$^-$ T cells in the same mice. The CD4$^-$CD8$^-$ population was not enriched for memory phenotype cells nor was it prone to apoptosis, as detected by annexin V staining (Fig. 4 B and C). Thus, late loss of CD4 in these mice did not cause the enhanced turnover and increased apoptosis found in the CD4 lineage cells of *Cd4*$^{-/-}$ or *Cd4*$^{lox/-}$ *dLck-hcre*^{3785} mice. Instead, if anything, late loss of CD4 caused reduced turnover of the CD4-deficient Th cells, perhaps indicating that they were less capable of making a homeostatic proliferative response than the

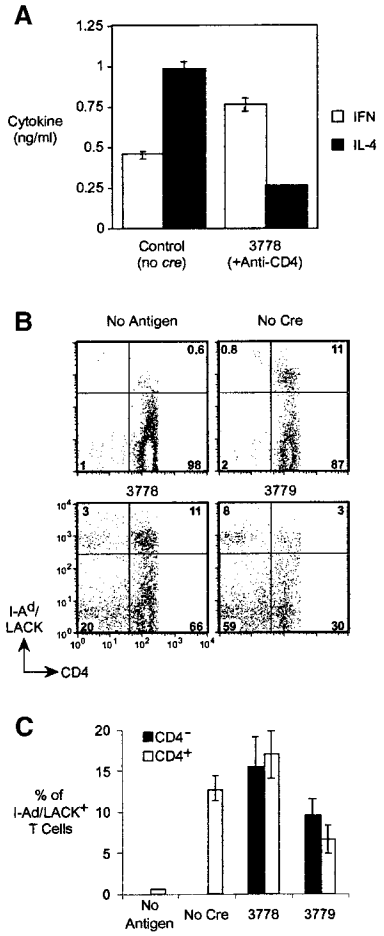

Figure 3. Immune responses mediated by CD4-deficient T cells. (A) Cytokine production by T cells from immunized mice after antigen restimulation in vitro. CD8-depleted spleen cells (containing equivalent numbers of CD4 lineage cells) from immunized mice (100 μg KLH given intraperitoneally in alum 7 d beforehand) were restimulated in vitro with 50 μg/ml KLH for 48 h before harvesting supernatants for IL-4 or IFN-γ-specific ELISAs. *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} mice were pretreated with cytotoxic anti-CD4 mAb (as described in Materials and Methods) to eliminate CD4-expressing cells (see Fig. 6 A). (B) Antigen-driven clonal expansion of LACK-reactive T cells in the absence of CD4. *H-2*^{db} *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} and *dLck-hcre*^{3779} mice carrying the WT15 LACK-reactive TCR-β transgene (reference 9) were immunized with *Escherichia coli*–derived recombinant LACK in the footpads using Freund's Complete Adjuvant. Popliteal lymph nodes were removed 7 d after immunization and the LACK-reactive T cells they contained were identified by multi-color flow cytometry using a LACK/I-A^d fluorescent multimer (reference 25). The percentages of CD4-expressing and CD4-deficient T helper cells from the immunized mice that stained with the multimer are shown in C.

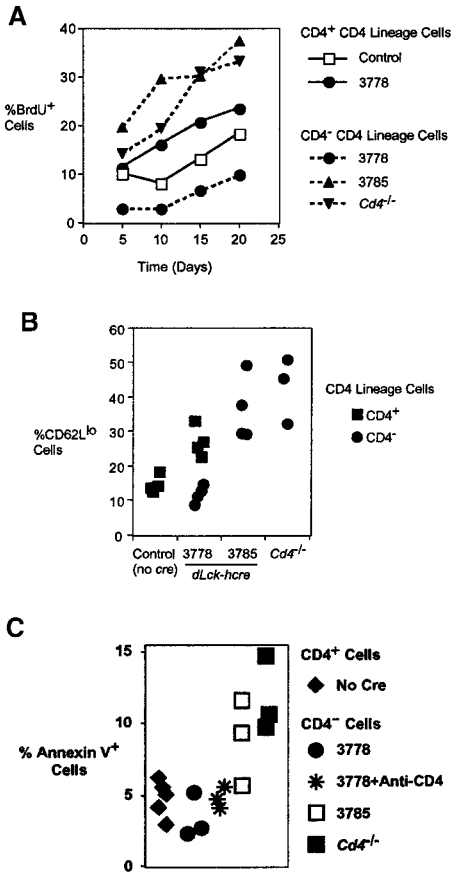

Figure 4. Proliferation and phenotype of CD4-deficient T cells. (A) The in vivo rates of cell division for CD4-expressing and CD4-deficient T cells were examined by continuous labeling with BrdUrd. Cells that had undergone DNA synthesis in vivo were identified by multi-color flow cytometry using an anti-BrdUrd mAb. The graph shows the accumulation of cells that stained brightly (reference 49) with the anti-BrdUrd antibody in individual mice analyzed at different time points. The data are representative of those obtained in two independent experiments. (B) Representation of CD62L-expressing CD4$^+$ and CD4$^-$ T cells in early (3785) and late (3778) -acting *dLck-hcre* transgenic *Cd4*$^{lox/-}$ mice. (C) Frequency of Annexin V-stained CD4$^+$ and CD4$^-$ cells in *Cd4*$^{lox/-}$ *dLck-hcre* transgenic and control mice. Three of the *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} mice were treated with cytotoxic anti-CD4 mAb as shown in Fig. 6.

cells that retained CD4 in the same mice. To examine this last possibility, we performed two types of experiments that tested the capacity of CD4-deficient T cells to proliferate under circumstances of induced T cell deficiency. The first of these experiments involved labeling T cells from the mutant mice with CFSE and then transferring them into T cell–deficient recipients by intravenous injection. The other type of experiment examined the proliferative response of CD4$^-$ T cells in *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} mice that had been depleted of their CD4-expressing cells by injection of cytotoxic anti-CD4 mAb.

To facilitate adoptive transfer experiments between mice that were not on the same genetic background, we first generated bone marrow chimeras using cells from *dLck-hcre*^{3778} *Cd4*$^{lox/-}$ mice as donors and irradiated C57BL/6 mice as recipients. T cells developing in these chimeras would be tolerized to the C57BL/6 environment and would therefore not mediate graft-versus-host responses when subsequently transferred into C57BL/6 RAG-1$^{-/-}$ congenic recipients. Donor cells from the bone marrow chimeras were labeled with CFSE before the secondary adoptive transfers, so that early rounds of proliferation could be monitored. FACS® data from a representative experiment are shown in Fig. 5 A. CD4-deficient T cells from *Cd4*$^{-/-}$ mice decreased in representation immediately after transfer (Fig. 5 B), consistent

with their high apoptotic index and previously published data (26). At later times, the numbers of these cells increased substantially (Fig. 5 B) and the CFSE profiles showed that many of the transferred cells had proliferated extensively (data not shown). As observed previously (4, 31), two rates of proliferation were evident in all of the transferred populations we followed: some cells underwent >7 rounds of division by 4–6 d while the majority appeared to require >30 d to go through only 2–3 rounds.

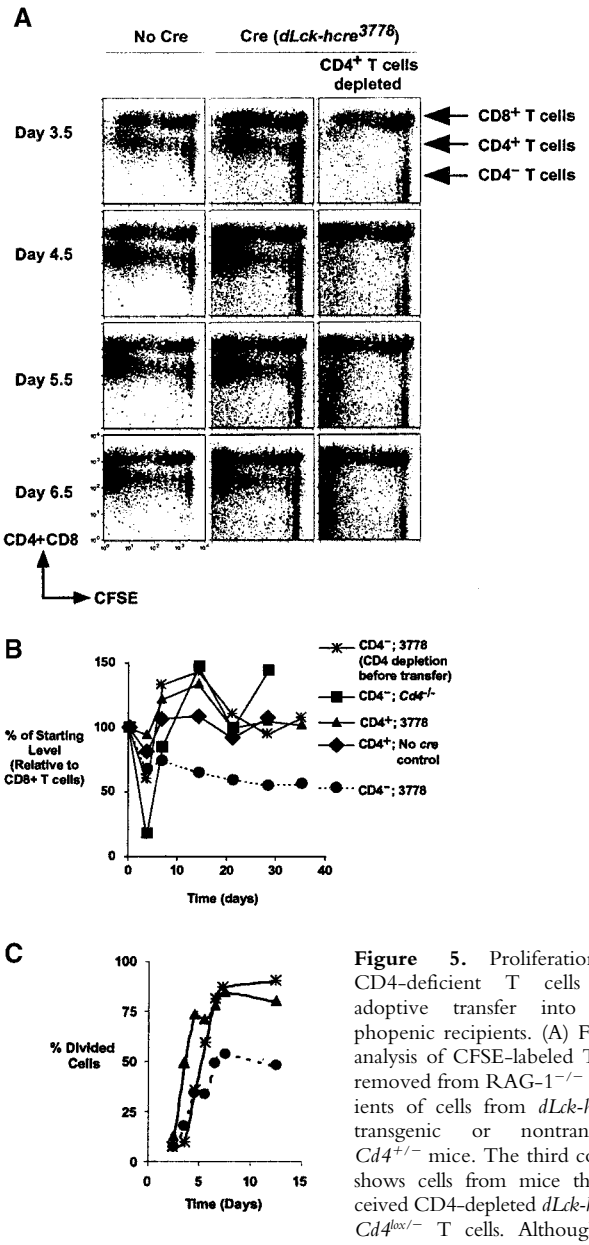

Figure 5. Proliferation of CD4-deficient T cells after adoptive transfer into lymphopenic recipients. (A) FACS® analysis of CFSE-labeled T cells removed from RAG-$1^{-/-}$ recipients of cells from *dLck-hcre*^{3778} transgenic or nontransgenic *Cd4*$^{+/-}$ mice. The third column shows cells from mice that received CD4-depleted *dLck-hcre*^{3778} *Cd4*$^{lox/-}$ T cells. Although tricolor-conjugated anti-CD4 and anti-CD8 antibodies were used together, CD4- and CD8-expressing cells could still be distinguished from one another by their different levels of fluorescence, as indicated. (B) Changes in the relative representation of $CD4^-$ and $CD4^+$ T cells in RAG-$1^{-/-}$ mice that received the indicated cell populations. The results of this experiment are representative of two others. Changes in the fraction of cells that had undergone cell division (assessed by CFSE fluorescence) are shown in C.

Like cells that had lost CD4 as an early event, CD4-deficient cells from the late-acting Cre transgenic mice (line 3778) also responded to the T cell–deficient environment by proliferating (Fig. 5 A and B). Interestingly, these cells appeared to suffer a substantial competitive disadvantage in this response when they were cotransferred with $CD4^+$ T cells. Thus, the magnitude of their proliferative response was considerably increased when CD4-expressing cells were depleted from the inoculum immediately before adoptive transfer. In the presence of competitor $CD4^+$ T cells, the transferred CD4-expressing T cells predominated and there was a progressive decrease in the relative frequency of CD4-deficient cells. Therefore, the data are consistent with the BrdUrd studies presented in Fig. 4, and suggest that competition during normal homeostasis may discriminate between T cells on the basis of TCR/coreceptor signaling.

Injection of cytotoxic anti-CD4 mAb into *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} mice eliminated CD4-expressing cells (Fig. 6 A) and caused marked proliferation of the remaining CD4-deficient T helper cells, as monitored by BrdUrd incorporation (Fig. 6 B). In contrast to the phenotype of CD4-deficient cells in *Cd4*$^{lox/-}$ *dLck-hcre*^{3785} and *Cd4*$^{-/-}$ mice, this proliferation was not associated with increased apoptosis

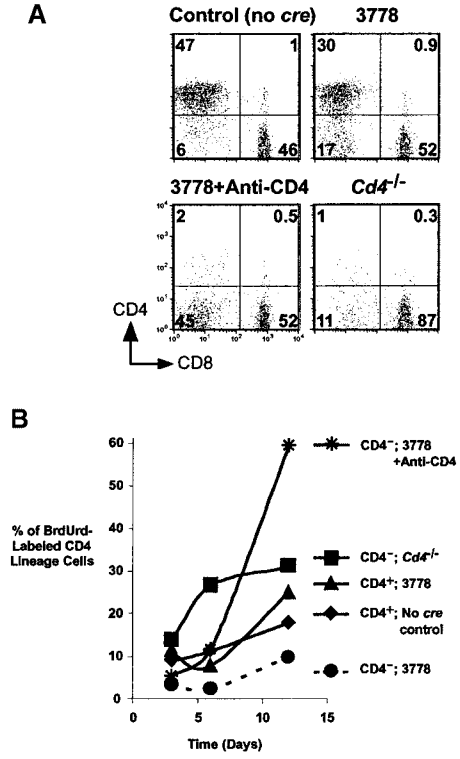

Figure 6. Proliferation of CD4-deficient T cells after in vivo depletion of CD4-expressing cells. (A) *dLck-hcre*^{3778} *Cd4*$^{lox/-}$ mice were injected four times with 500 μg each of cytotoxic anti-CD4 antibody on days 0, 1, 2, and 6, while being fed BrdUrd continuously in their drinking water. The FACS® plots show the extent of in vivo CD4 depletion after 7 d. (B) Accumulation of BrdUrd-labeled $CD4^+$ and CD4-deficient cells in anti-CD4–treated and untreated *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} transgenic mice and nontransgenic controls.

(Fig. 4 C). Together with the results of immunization experiments (Fig. 3) these data show that cells that have lost CD4 expression late in their development are not obviously defective in their capacity to proliferate. Their decreased incorporation of BrdUrd (Fig. 4 A) and restrained expansion in RAG-$1^{-/-}$ mice (Fig. 5) can, however, be explained by competition with CD4-expressing cells, since the removal of these cells reveals their proliferative potential in both cases (Figs. 5 and 6).

In a final series of experiments, we examined the expression of the CD5 molecule on CD4-expressing and CD4-deficient T cells in *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} and *Cd4*$^{lox/-}$ *dLck-hcre*^{3779} mice. CD5 is a negative regulator of TCR signaling (32, 33) whose level of expression on the T cell surface changes in response to the antigenic environment in which the T cell moves (34). For example, $CD4^+CD8^+$ thymocytes from MHC-deficient mice show decreased cell surface display of CD5 relative to thymocytes from wild-type mice, whereas autoreactive $CD4^+$ T cells show enhanced levels of cell surface CD5 compared with normal $CD4^+$ T cells (34). If the absence of CD4 impairs the response of T cells to endogenous peptide–MHC ligands, then this might be expected to correlate with decreased cell surface expression of CD5 (35, 36). Consistent with this expectation, the CD4-deficient population of cells was relatively more enriched for low expression of CD5 compared with CD4-expressing T cells in the same mice (Fig. 7 A and B). Thus, impaired homeostatic TCR signaling, as shown here with loss of CD4, induces decreased expression of the CD5 negative regulator, presumably as a means of augmenting TCR sensitivity and consequently homeostatic competitiveness.

Discussion

Using conditional CD4 deficiency as a means to interfere with the function of the TCR, we have shown that impaired TCR/coreceptor signaling decreases the competitiveness of Th cells during homeostatic proliferation. Cells that had lost CD4 because of Cre recombination showed a competitive disadvantage compared with CD4-expressing cells when they were transferred into RAG-$1^{-/-}$ recipients. Eliminating competitor $CD4^+$ T cells relieved the block to proliferation in these adoptive transfer experiments. CD4-deficient T cells proliferated rapidly in response to an induced T cell void caused by antibody-mediated depletion of $CD4^+$ T cells, and they also could undergo efficient clonal expansion in response to intentional antigenic stimulation. Thus, T cells lacking CD4 retained the capacity to proliferate, but failed to realize it fully during homeostatic expansion in the presence of CD4-expressing cells. Taken together, the results reveal evidence of competition among T cells for homeostatic proliferation cues, and suggest that the basis of this competition is TCR/coreceptor signaling.

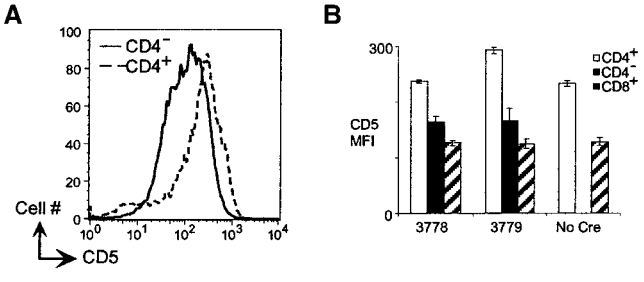

Figure 7. Decreased surface expression of CD5 on CD4-deficient T cells in *dLck-hcre Cd4*$^{lox/-}$ mice. (A) Cell surface CD5 on $CD4^+$ and $CD4^-CD8^-$ T cells from *dLck-hcre*^{3778} *Cd4*$^{lox/-}$ mice. (B) Mean CD5 fluorescence intensity (MFI) of $CD4^+$, $CD4^-$, and $CD8^+$ T cells in *dLck-hcre*^{3778} and *dLck-hcre*^{3779} *Cd4*$^{lox/-}$ mice.

The significance of TCR/coreceptor signaling in regulating the survival of peripheral T cells and selection of the T cell repertoire after thymic development has been somewhat controversial. Although T cells have shown impaired survival after transfer to MHC-deficient hosts in some settings (37), the magnitude of this effect has been variable or undetectable in others (2, 4, 38). Using alternative approaches to restrict expression of MHC molecules solely to the thymus (1, 5, 39), other groups have reported reduced survival of $CD4^+$ T cells in the periphery, consistent with a necessary role for TCR signaling for T cell persistence. Direct support for this position has recently come from reports showing that postthymic loss of TCR expression impairs T cell survival (40, 41), in a less dramatic, but related fashion to the way in which loss of BCR expression interferes with B cell survival (42). Here, we have described diminished T cell competitiveness and reduced in vivo proliferation caused by impairing the TCR signal through absence of CD4. These data are consistent with the emerging view that TCR–ligand interactions are important for postthymic T cell survival and indicate that, for $CD4^+$ T cells, the contribution of CD4 coreceptor is required for survival signaling.

During immune responses, T cells will compete with one another for access to antigen (43, 44). This competition appears to place an upper limit on the number of cell divisions experienced by an antigen-reactive clone. Thus, when precursor frequencies are high, T cells undergo fewer cell divisions than when these frequencies are low (43, 44). Increasing the display of antigen to T cells, by injecting mice with antigen-pulsed dendritic cells, can increase the number of cell divisions a clone experiences (44) presumably by alleviating competition for access to antigen. Competition of this sort has the potential to explain selection for T cells bearing high affinity TCRs as immune responses mature (45–48), because such TCRs may enhance competitiveness at the surface of antigen presenting dendritic cells allowing for increased access to proliferative cues. The data presented in this paper suggest that TCR-dependent competition may also be a normal part of naive T cell homeostasis, again favoring cells that make high affinity engagements with peptide–MHC ligands at the expense of those making weaker engagements.

Competition between $CD4^+$ and $CD4^-$ T cells could be most readily visualized in the adoptive transfer experi-

ments, but we also observed that CD4-deficient T cells incorporated BrdUrd at a significantly reduced rate under steady-state conditions compared with CD4-expressing cells in the same mice. In principal, at least some of this steady-state difference could be attributed to enrichment for memory T cells within the $CD4^+$ population in the context of previous work showing that memory T cells can have a shorter cell cycle than naive T cells (49). Quantifying the frequencies of memory versus naive cells in the two populations is unfortunately made difficult by the fact that actively proliferating naive T cells can adopt a cell surface phenotype that is typical of memory cells, despite the absence of antigen encounter (4, 29, 30). Even with this in mind, however, we found only a two- to threefold enrichment for memory T cells within the $CD4^+$ compartment of *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} mice (data not shown), which seems unlikely to account for the observed differences in BrdUrd incorporation. Using LACK or KLH immunization, we have also shown that CD4-deficient T cells are capable of engaging in vigorous immune responses, even in the context of competition with $CD4^+$ T cells. Thus, the differences in steady-state proliferation of $CD4^+$ and $CD4^-$ T cells in *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} mice and the differences in expansion of these cells after adoptive transfer both seem likely to reflect the same type of TCR-dependent competitive effect.

TCR-based competition between naive T cells during expansion under lymphopenic conditions and during steady-state homeostasis implies that the naive TCR repertoire is under continual selection beyond the thymus. Specifically, the data suggest that there is a growth advantage conferred on T cells that bind endogenous peptide–MHC ligands with high affinity, and that such cells would tend to dominate over time at the expense of cells whose TCRs bind endogenous ligands with lower affinity. There are no direct experiments that address whether such refinement of the TCR repertoire is in fact beneficial for the organism. It seems possible, for instance, that the refined repertoire may be relatively more enriched for T cells that will be useful during pathogen-specific immune responses, than a repertoire that has not yet undergone refinement. Alternatively, in the absence of significant thymic output, this type of repertoire refinement could focus the repertoire over a long period of time in a fashion that might not be beneficial to the organism. Although we have shown here that impaired homeostatic competitiveness did not detectably impair the highly focused immune response to the *Leishmania major* LACK antigen, additional experiments will be required to test more adequately the fitness of CD4-deficient T helper cells, or other populations of cells that also have suboptimal TCR-dependent signaling responses to endogenous peptide–MHC ligands.

The mechanism by which TCR signaling would enhance competitiveness remains to be addressed. Whereas it is possible that TCR signaling itself is directly responsible for regulating the cell cycle of naive T cells, it is also possible that the TCR signal induces a state in the cell that makes it more responsive to other proliferative cues, such as might be provided by cytokines. For instance, IL-7 has recently been shown to regulate the proliferation and survival of both naive and memory T cells (2, 50–53), and there are increased levels of circulating IL-7 under conditions of lymphopenia (54). Similarly, memory $CD8^+$ T cells rely on a balance of IL-15 and IL-2 receptor signals for normal homeostasis (50). Thus, it is possible that TCR signaling might induce or downregulate sensitivity to a cytokine either at the level of the receptor itself or the signaling molecules downstream of it. Polyclonal $CD4^+$ and $CD4^-$ populations that differ in their homeostatic competitiveness should be useful tools for examining these possibilities.

Early loss of CD4 in the thymus, as in *Cd4*$^{-/-}$ mice or *Cd4*$^{lox/-}$ *dLck-hcre*^{3785} mice, creates Th lineage cells that are both prone to apoptosis and have a high mitotic index (26). In contrast, cells that lose CD4 later in the developmental pathway (e.g., as found in *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} mice) are not abnormally apoptotic and, as just mentioned they also demonstrate a low rate of proliferation. Thus, the loss of CD4 incurs two very different phenotypes on cells, dependent on whether it occurs before or primarily after positive selection in the thymus. The low rate of proliferation in *Cd4*$^{lox/-}$ *dLck-hcre*^{3778} mice is at least partly explained by competition as it can be significantly if not completely eliminated by deletion of CD4-expressing cells from the mice (Figs. 5 and 6). Importantly, however, proliferation of $CD4^-$ cells after in vivo depletion of $CD4^+$ T cells, does not increase the frequency of apoptotic cells over the time course examined (Fig. 4). This finding suggests that the two phenotypes observed in *Cd4*$^{-/-}$ mice (26) can be separable, *i.e.*, proliferation of Th cells that lack CD4 can occur in the absence of an associated increase in cell death. The cause of the apoptotic phenotype exhibited by *Cd4*$^{-/-}$ T cells is therefore not necessarily a consequence of the proliferative stress incurred by reduced thymic output of helper lineage cells. Instead, it may well reflect a more direct consequence of CD4 deficiency, such as aberrant or incomplete differentiation of thymocytes when they lack efficient TCR/coreceptor signaling. Such an interpretation makes clear the benefit of the conditional mutagenesis approach, and the types of mouse strains used here, because they permit a dissociation of pre- and post-developmental effects of genes that have multiple roles in a linear differentiation pathway.

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