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The Role of CD4 in Peripheral T Cell Homeostasis

by

Julie Strong

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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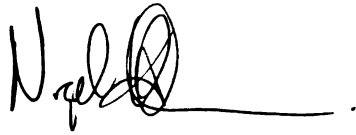# THE ROLE OF CD4 IN PERIPHERAL T CELL HOMEOSTASIS

Julie Strong

Abstract

Signal transduction in response to ligand recognition by T cell receptors (TCRs) regulates T cell fate within and beyond the thymus. The participation of the coreceptor CD4 can significantly augment the sensitivity of TCR-mediated signaling. Here we have examined the functions of the CD4 molecule in peripheral T cells, particularly in the regulation of T helper cell survival.

Using animals defective in CD4 expression, we find that despite adequate export of naïve T cells from the thymus, the loss of CD4 results in a marked deficiency in T helper cell representation. T helper cells that lack CD4 expression are prone to apoptosis and decline sharply in numbers after adoptive transfer to irradiated recipients. The helper lineage in CD4$^{-/-}$ animals is also enriched for cells exhibiting a memory cell phenotype, and such cells are less prone to apoptose, indicating that transition of naïve cells to a memory state diminishes their dependence on survival signals that involve CD4. Our findings suggest a critical role for CD4 in the homeostasis of T helper cells, particularly naïve cells.

In a separate set of experiments, we have used gene targeting in embryonic stem cells to generate mice carrying a conditional allele of CD4. Using new and previously described strains of Cre-expressing mice, we have studied the consequences of inducible or stage-specific loss of CD4 expression. We show that naïve cells which lose surface CD4 extrathymically fail to persist

after loss of CD4 expression, whereas cells which have been previously activated do not. These data complement the findings in CD4-deficient animals and demonstrate a key role for CD4 in the regulation of T helper cell survival and extrathymic selection of the T cell repertoire.

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INTRODUCTION

Thymic Development and Selection

Early Thymic Development

The T cell component of the immune system expresses a vast diversity of antigen receptors (Arstila et al., 1999) that can specifically recognize peptides in the context of self Major Histocompatibility molecules (MHC) (Zinkernagel and Doherty, 1974). The process of shaping the T cell receptor (TCR) repertoire to the particular range of MHC molecules present in a given individual begins in the thymus. T cell development is initiated by multipotent stem cells that migrate from the bone marrow to seed the thymus (Ardavin et al., 1993; Shortman and Wu, 1996). In the thymus stem cells become committed to the T lineage and to the $\gamma\delta$ or $\alpha\beta$ fate (Dudley et al., 1995; Godfrey et al., 1993). To continue maturation, nascent $\alpha\beta$ T cells must rearrange the germline gene segments of the TCR to make a functional protein that can interact with the MHC on the thymic stroma (Fehling and von Boehmer, 1997; Haks et al., 1999). Rearrangement of the TCRβ locus occurs prior to α rearrangement (Raulet et al., 1985; Snodgrass et al., 1985).

Productive rearrangement of a TCRβ gene allows for expression of a TCRβ chain, which forms the pre-TCR when paired with the pre-T cell receptor α chain (pre-Tα) (Groettrup et al., 1993). The successful rearrangement of a TCRβ chain is critical for progression from the CD4$^-$8$^-$ stage of thymocyte development to the CD4$^+$8$^+$ stage (Dudley et al., 1994; Fehling and von Boehmer, 1997; Mallick et al., 1993). Signaling through the pre-TCR

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triggers cell division and upregulation of CD4 and CD8 (Falk et al., 1996; Penit et al., 1995) and allelic exclusion of the β locus (Uematsu et al., 1988). Additionally, germline transcripts of the mature TCRα locus appear following this signal (Hozumi et al., 1998; Villey et al., 1996) and the cells then begin to rearrange the TCRα locus (Raulet et al., 1985).

Thymic Selection

At the CD4$^+$CD8$^+$ stage of development, cells are subject to a process of selection that favors the maturation of cells having an appropriate level of responsiveness to the MHC-peptide complexes displayed on the thymic stroma. Cells that do not successfully express a mature αβ receptor or do not have a minimum level of reactivity to self-MHC molecules do not receive a positive selection signal, which if received rescues the cells from death by neglect (reviewed in Goldrath and Bevan, 1999b; von Boehmer, 1994). During this period several changes occur in expression of cell-surface molecules, including upregulation of the TCR (Borgulya et al., 1992; Ohashi et al., 1990) as well as expression of activation markers such as CD69 (Bendelac et al., 1992). Additionally, successful positive selection results in the downregulation of the recombinase-activating genes *RAG-1* and *RAG-2*, preventing the further rearrangement of the α locus (Borgulya et al., 1992; Brandle et al., 1992). At this stage cells also cease expression of one of the coreceptors to become CD4$^+$8$^-$ or CD4$^-$8$^+$ (Lucas et al., 1994).

In order to prevent autoreactive cells from exiting the thymus, nascent T cells are also subject to negative selection (reviewed in Nossal, 1994; Sebzda

et al., 1999). This process is highly sensitive (Adelstein et al., 1991) and results in the deletion of cells specific for self-antigen (Kappler et al., 1987; Kisielow et al., 1988a; MacDonald et al., 1988). Interestingly, the unselected TCR repertoire appears to have some intrinsic affinity for MHC (Merkenschlager et al., 1997; Zerrahn et al., 1997), consistent with the possibility that negative selection is the dominant component of the selection process.

Several models have been proposed for the factors that may tip the balance between positive and negative selection. One such model is the differential-avidity model, in which high avidity interactions result in negative selection, whereas lower avidity interactions promote positive selection (Ashton-Rickardt et al., 1994; Ashton-Rickardt and Tonegawa, 1994; Jameson et al., 1995). In this model, signals from multiple TCR-MHC interactions are integrated, so that the outcome is based on a summation of the strength and duration of signals generated by individual TCR-MHC complexes. This model is supported by many studies, including those which demonstrate that the same peptide can mediate both positive and negative selection in a concentration-dependent manner (Ashton-Rickardt et al., 1994; Fukui et al., 1997; Sebzda et al., 1994). Some conclusions from these studies have been challenged, though, on the grounds that the cells produced may not be fully functional (Cook et al., 1997; Girao et al., 1997).

More recently data has emerged that suggests that a different parameter may be critical. A study by Allen and colleagues provided support for the hypothesis that the kinetics of the interaction between an individual TCR and its ligand determine the outcome, not simply the sheer number of

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TCR-MHC/peptide complexes (Williams et al., 1999). The authors were able to correlate the half-life of a given TCR-ligand complex *in vitro* with its activity in thymic selection to demonstrate that longer half-lives resulted in negative selection, while shorter ones allowed for positive selection. In the model supported by these data, a high affinity ligand would induce negative selection even at very low doses. Although some controversy remains, the preponderance of the data clearly argue for a key role of the strength and duration of the TCR-mediated signal in determining the fate of the developing T cell.

Coreceptor Function in Selection

The CD4 and CD8 coreceptors act to improve the efficiency of TCR-derived signaling by two distinct but interacting mechanisms (Killeen and Littman, 1995). They foster interaction of the TCR with MHC by binding directly to nonpolymorphic regions of the MHC (Doyle and Strominger, 1987; Gao et al., 1997; Konig et al., 1992; Norment et al., 1988; Potter et al., 1989; Salter et al., 1990). In transfection systems (Doyle and Strominger, 1987; Norment et al., 1988) and in experiments with primary T cells *ex vivo* (Metz et al., 1997), both CD8 and CD4 can improve cell adhesion. In addition to stabilizing the T cell-APC interaction, both CD4 and CD8 are associated with the intracellular protein tyrosine kinase p56[lck] (Barber et al., 1989; Rudd et al., 1988; Veillette et al., 1988), which becomes activated upon coreceptor stimulation (Veillette et al., 1989a; Veillette et al., 1989b). Recruitment of p56[lck] to the TCR-MHC interface by the coreceptor dramatically improves the reactivity of T cells to ligand (Collins et al., 1992; Glaichenhaus et al., 1991;

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Hampl et al., 1997; Killeen and Littman, 1993; Madrenas et al., 1997; Miceli et al., 1991).

The consequences of the participation of CD4 and CD8 in the T cell-APC interaction during selection are made clear by experiments in which coreceptor function is abrogated. Mutation of the CD8 binding site on Class I MHC showed that interference with the CD8-MHC interaction prevents development of the CD8 lineage (Aldrich et al., 1991; Killeen et al., 1992; Potter et al., 1989). Gene disruption experiments clearly demonstrated the critical role for CD4 and CD8 in positive selection of their respective lineages. In the absence of CD8, no development of the cytotoxic lineage was observed (Fung-Leung et al., 1991), much like the situation in mice that lack Class I expression due to a disruption in the beta-2 microglobulin (β_2m) gene (Koller et al., 1990). Similarly, mice lacking CD4 exhibit a significant reduction in the helper lineage (Killeen et al., 1993; Rahemtulla et al., 1991). Interestingly, however, the block is not absolute: a small number of helper lineage cells are found in the peripheral T cell pool of CD4-deficient mice (Locksley et al., 1993; Rahemtulla et al., 1994), unlike in the case of the CD8-deficient mouse (Bachmann et al., 1995; Fung-Leung et al., 1991). Furthermore, studies with Class I molecules mutant in the CD8 binding site illuminated a role for coreceptor in negative selection as well (Ingold et al., 1991; Killeen et al., 1992).

CD4 Versus CD8 Lineage Commitment

The correlation between the MHC restriction of a given TCR and the coreceptor found on the cell is extremely tight (Kaye et al., 1989; Kisielow et

al., 1988b; Sha et al., 1988; Teh et al., 1988). Despite much research, though, the details of the process of CD4 versus CD8 lineage commitment remain controversial. Two major models have been put forth. On the one hand, the instructive model (von Boehmer, 1986) postulates that the cell would retain expression of both coreceptors until MHC engagement had occurred and would downregulate the non-interacting receptor. A necessary component of this model is that the signals transmitted via CD4 and CD8 are distinct from each other, allowing the cell to distinguish which coreceptor has engaged MHC. On the other hand, the stochastic/selective model (Robey et al., 1991) suggests that prior to selection, cells would downregulate one of the coreceptors at random. Only those cells in which the coreceptor and TCR could engage the same MHC would receive a survival signal.

Initial attempts to distinguish the models yielded contradictory results. For instance, the stochastic model would predict that cells in which the remaining coreceptor and the TCR did not recognize the same MHC could be rescued from death by constitutive expression of the appropriate coreceptor. However, in many cases transgenic expression of coreceptors failed to allow for maturation of cells that inappropriately retained expression of the other receptor (Borgulya et al., 1991; Robey et al., 1991). These results appeared to support the instructive model. However, data from Mathis and colleagues were consistent with a stochastic model (Chan et al., 1993), based on characterization of thymic subsets in animals lacking either Class I or Class II MHC molecules. This study found thymocytes that appeared to have been selected on an MHC that did not match the coreceptor specificity. The

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assumptions underlying the analysis by Mathis and colleagues, as well as other studies, were challenged by the elegant analysis of patterns of coreceptor expression performed by Germain and coworkers (Lucas and Germain, 1996). Their study illuminated the complexity of such expression patterns, particularly in the period immediately following a positive selection signal. The data demonstrate that cells that had previously been thought to be committed to one lineage may in fact be progressing towards the other. These findings call into question the interpretation of earlier experiments supporting the stochastic model. Recent data are also consistent with the instructive model (Itano and Robey, 2000). Additionally, the involvement of accessory molecules such as Notch (Robey et al., 1996) has also been suggested, although these data are controversial. Data from Bevan and coworkers support the idea that Notch is primarily involved in thymocyte survival and not lineage commitment per se (Deftos et al., 1998).

Final Steps in Thymic Maturation

The final stage of thymic maturation is the CD4$^+$ 8$^-$ or CD4$^-$8$^+$ stage, at which the cell has successfully survived both positive and negative selection. Such cells appear to be resident in the thymus for up to two weeks (Egerton et al., 1990). Multiple changes occur in cell-surface molecules during this period, including upregulation of Qa-2 and CD45RB as well as downregulation of HSA (Lucas et al., 1994). However, the purpose of this period and the signals that allow the mature thymocyte to migrate to the periphery remain poorly understood. Regardless, the cells eventually exit the thymus to become part of the peripheral repertoire.

Peripheral T Cell Homeostasis

Homeostasis of Lymphocyte Subpopulations

Many levels of homeostatic control determine the contribution of each of the various mature lymphocyte subsets to the peripheral pool (Freitas and Rocha, 2000; Goldrath and Bevan, 1999b). At the broadest level, the numbers of T and B cells appear to be independently regulated (Tanchot et al., 1997b). For instance, in mice lacking T cells, the B cell population is approximately normal size (Mombaerts et al., 1992) and the converse is true in mice lacking B cells (Kitamura et al., 1991). Within the T cell population, the CD4 and CD8 subsets seem to occupy the same niche, in that each subset expands to result in a normal total T cell compartment in the absence of the other (Fung-Leung et al., 1991; Killeen et al., 1993; Koller et al., 1990; Rahemtulla et al., 1991). However, when cells of either subset are transferred into wild-type mice, the ratio of CD4 to CD8 lineages rapidly returns to that found prior to the transfer, suggesting tight control over the lineages under conditions in which the T cell compartment is normal size (Rocha et al., 1989).

Additionally, the numbers of naïve and memory cells are highly regulated (Freitas and Rocha, 1999; Goldrath and Bevan, 1999b). The cellularity of the memory compartment has also been shown to be under significant homeostatic control. The size of the pool appears fixed, expanding only to the size of a normal memory compartment even in the absence of other T cells and even when large numbers of memory cells are added to the system (Tanchot and Rocha, 1995).

Mechanisms of Peripheral T Cell Homeostasis

The size of the various T cell subsets can be regulated at many steps, including input of newly generated cells as well as expansion and survival of resident cells. Although some studies have suggested that thymic output is independent of the peripheral pool size (Berzins et al., 1998), other findings support the possibility that human thymic function may increase in the case of severe T cell depletion (McCune, 1998; Poulin et al., 1999; Smith et al., 2000). However, the size of the naïve cell pool can be independent of thymic output, since animals lacking thymic output were able to maintain normal numbers of naïve cells (Bell et al., 1987; Tanchot and Rocha, 1997). The mechanisms by which recent thymic emigrants are incorporated into the T cell pool remain somewhat unclear, although newly generated cells may contribute a key part of the TCR repertoire. Rocha and coworkers demonstrated that newly produced T cells replace naïve cells but not memory cells, preserving the response to recall antigens while simultaneously maintaining the diversity of the TCR repertoire (Tanchot and Rocha, 1997).

In addition to regulation at the level of incorporation of new cells, division of resident cells is a significant point of homeostatic regulation. Cytokines may have significant effect on the numbers of naïve versus memory cells. For example, animals deficient in the common cytokine receptor gamma chain exhibit an accumulation of activated/memory phenotype CD4$^+$ cells in an antigen-dependent fashion (Nakajima et al., 1997), suggesting a role for signals transduced through the common gamma chain in maintaining the balance between naïve and memory cells. Interleukin-15 (IL-15) has been

shown to promote division (Ku et al., 2000; Lodolce et al., 1998; Zhang et al., 1998a) as well as appropriate homing (Lodolce et al., 1998) of memory T cells; interleukin-2 appears to antagonize this function of IL-15 (Ku et al., 2000). Furthermore, Type I interferons have been shown to induce turnover of memory cells in an antigen-nonspecific fashion (Tough et al., 1996), probably through induction of IL-15 (Zhang et al., 1998a).

An important emerging mechanism of control is at the level of peripheral T cell survival as determined by interaction with MHC (Brocker, 1997; Takeda et al., 1996; Tanchot et al., 1997a). An early demonstration of this reliance on the presence of MHC molecules for T cell persistence came in an investigation of survival requirements for CD8$^+$ T cells (Tanchot et al., 1997a). Cells bearing a transgenic TCR required the restricting Class I molecule in order to persist after transfer to lymphopenic hosts. In the absence of the appropriate MHC, the transferred cell population decayed rapidly. Monoclonal CD4$^+$ populations also fail to persist without the selecting Class II allele, declining with relatively similar kinetics (Freitas and Rocha, 1999; Kirberg et al., 1997). The evidence concerning polyclonal populations was initially somewhat confusing, and appeared to suggest that although long-term engraftment did require the selecting MHC, the kinetics of decay were significantly different than had been found in the transgenic situations (Rooke et al., 1997; Takeda et al., 1996). In the latter studies, naïve T cells were generated in Class II-deficient hosts by thymic transplant (Takeda et al., 1996) or by transient induction of MHC expression using a retroviral system (Rooke et al., 1997), whereas previous data had been generated using transfers of

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mature cells. The details of the dependence on MHC are still emerging, and will require further study.

The survival requirements for memory cells appear somewhat less stringent than for naïve cells (Freitas and Rocha, 1999). The selecting MHC allele does not seem to be necessary for long-term survival of CD4$^+$ memory cells (Swain et al., 1999). Data from Rocha and coworkers as well as others suggested that some interaction with the correct class of MHC molecule was important for CD8$^+$ memory cells, as they declined in hosts devoid of any Class I MHC (Markiewicz et al., 1998; Tanchot et al., 1997a). However, these data were contradicted by an elegant study by Ahmed and colleagues in which *in vivo*-generated antigen-specific memory cells were transferred to Class I-deficient or normal hosts (Murali-Krishna et al., 1999). In this case, no MHC was necessary for long-term memory cell survival. The effects seen in earlier studies may be due to specific properties of the transgenic systems used (Tanchot et al., 1997a) or the methods of generation of memory cells (Markiewicz et al., 1998).

Peptide Recognition in T Cell Homeostasis

The role of specific peptide recognition in the delivery of survival signals is somewhat more controversial. Here we must make a distinction between mere persistence and the homeostatic proliferation that occurs as a result of lymphopenia. In a system using T cells bearing a transgenic TCR, the presence of the antigenic peptide was critical for expansion of cells transferred

into lymphopenic hosts, but not for naïve cell survival per se (Tanchot et al., 1997a).

Whether the peptides that can promote cell division in cases of lymphopenia are distinct from the selecting ligands remains in dispute. Through the use of irradiated and genetically T-deficient hosts, Surh and colleagues suggested that in the absence of a full T cell compartment, naïve T cells become overtly reactive to self-peptides, acquiring an activated phenotype and proliferating to fill the compartment (Ernst et al., 1999). These data are consistent with the possibility that the selecting ligand (but presumably not a conventionally antigenic peptide) is critical for the generation of homeostatic signals, at least in this context. Furthermore, data from Goldrath and Bevan (Goldrath and Bevan, 1999a) demonstrated a difference between antagonist ligands (those able to promote thymic selection but not peripheral activation of transgenic T cells) and agonist or activating ligands. Only the former could support proliferation in lymphopenic hosts. In contrast, others have suggested that the selecting peptide cannot promote division of T cells in similar settings (Bender et al., 1999). The exact nature of this process will require finer dissection. Antigenic peptide does not appear to be necessary for survival of memory cells (Markiewicz et al., 1998; Murali-Krishna et al., 1999; Tanchot et al., 1997a).

A Role for Coreceptor in Peripheral T Cell Homeostasis?

The critical role of MHC and, in some cases, peptide for survival and homeostatic proliferation of T cells implies that the relevant signal is

almost certainly delivered via the TCR. The importance of TCR-derived signals raises the possibility that optimal signaling might also depend on the participation of the coreceptor. Through its capacity to bind MHC and recruit $p56^{lck}$ to the TCR signaling complex, CD4 can substantially improve the antigen responsiveness of T cells (Collins et al., 1992; Glaichenhaus et al., 1991; Hampl et al., 1997; Killeen and Littman, 1993; Madrenas et al., 1997; Miceli et al., 1991). Since the survival effect of MHC/peptide is likely to be mediated via the TCR, it is reasonable to hypothesize that CD4 might participate in the transmission of such a survival signal. Although CD4 appears to function independently of TCR in some cases (Cruikshank et al., 1996; Newell et al., 1990), due to the weak affinity of CD4 for MHC (Margulies et al., 1996; Ward and Qadri, 1997) any role for the coreceptor in homeostasis would likely be through improving the sensitivity of the TCR-derived signal. Unlike the CD8$^{-/-}$ mouse, in which no CD8 lineage cells emerge from the thymus, the CD4-deficient mouse has provided an opportunity to study the functions of CD4 in the periphery due to the residual CD4 lineage population found in such mice (Killeen et al., 1993; Rahemtulla et al., 1991).

The existence of a population of CD4 lineage cells without surface CD4 provides an opportunity to address the roles for CD4 in mature peripheral T cells. Specifically, we have tested the hypothesis that CD4 contributes substantially to the delivery of survival signals to helper T cells. Here we use a constitutively CD4-deficient system to demonstrate that cells lacking CD4 are markedly compromised in their ability to survive. Such cells are more apoptotic than their wild-type counterparts and decline rapidly when

transferred to wild-type or lymphopenic hosts. We further show that naïve cells are significantly more impaired in their survival than are memory cells. Additionally we create a system for conditional expression of CD4 in which the functions of CD4 can be examined without the complication of coreceptor-independent thymic selection. Using this conditional allele we show that naive cells that lose CD4 extrathymically become apoptotic, whereas previously-activated cells do not. The data demonstrate that the propensity to apoptose in the absence of CD4 is in fact indicative of a specific role for CD4 in the generation of survival signals in naïve peripheral T helper cells. This conditional genetic system is particularly versatile in that control of CD4 expression can be exerted in a cell-type or stage-specific manner, allowing the examination of the role of CD4 in many aspects of peripheral T cell function.

MATERIALS AND METHODS

Mice. Adult C57Bl/6, B6.Ly5.1, B6.RAG-1$^{-/-}$, B6.CD4$^{-/-}$, B6.β_2m$^{-/-}$, MRL, MRL/*lpr*, B10.D2 and Eμ-bcl-2-25 mice were obtained from the Jackson Laboratory, Charles River Laboratories or were bred in house in the Parnassus Heights Barrier Facility at UCSF. MRL/*lpr* CD4$^{-/-}$ mice were a kind gift of David Wofsy, UCSF. CD4$^{-/-}$CD5$^{-/-}$ mice were bred in-house and have been described elsewhere (Pena-Rossi et al., 1999). Mice expressing a Tva transgene controlled by CD4 regulatory elements (*cd4-TVA*) have been described elsewhere (Wang et al., in preparation). In CD4$^{+/+}$ *cd4-TVA* transgenic mice, Tva, the receptor for Subgroup A Avian Sarcoma and Leukosis Viruses (ASLV-A) (Bates et al., 1998), is expressed on all thymocytes and T cells in a

fashion that follows endogenous CD4 expression. Tva is therefore a marker

for the CD4 lineage, akin to similar previously used markers (Bendelac et al.,

1994; Chan et al., 1998; Sawada et al., 1994). Mx-cre mice were a kind gift of

Ralf Kuhn, Institute for Genetics, University of Cologne, Cologne, Germany

via Steven Young, Gladstone Institute of Cardiovascular Disease/UCSF.

**Gene Targeting and Generation of Mice Expressing a Conditional Allele of

CD4.** CD4^{off} mice were generated by gene targeting. The targeting construct

spanned the 8.7 kb *SacI-KpnI* region of the CD4 locus containing exons II, II, IV

and a portion of exon V and was generated in a modified form of pBS-KS$^+$ in

which the multiple cloning site had been replaced with a linker containing a

more limited number of restriction sites. A *loxP* site was inserted into the *SacI*

site in exon II of CD4 by addition of a pair of linkers having the sequences

5'GGCCGCTCTAGAAAGCTTATAACTTCGTATAGCATACATTATACGAA

G-TTATAG3' and

5'AATTCTATAACTTCGTATAATGTATGCTATACGAAGTTA-

TAAGCTTTCTAGAGC3'. Into the *BglII* site in intron III we inserted

sequences containing a neomycin resistance cassette (*neo^r*) under the control of

the MC1 (HSV-thymidine kinase) promoter and flanked by a pair of *loxP*

recognition sites in the same orientation as that inserted in exon II. This *neo^r*

cassette was derived from pL$_2$neo, which was a kind gift of Dr. Hua Gu,

National Institutes of Health, Bethesda, MD. Immediately downstream of the

loxP-neo^r and oriented parallel to the CD4 coding sequences we placed the

coding region for human CD52, which was generated by polymerase chain

reaction and sequenced to confirm accuracy.

The construct was linearized by digestion with *KpnI* and 20μg of DNA were transfected into 2.0×10^7 JM-1 embryonic stem (ES) cells by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA) set at 500μF and 0.25kV. Cells were plated on layer of primary embryonic feeder cells that had received 2000 Rads of γ-irradiation. G418 selection was applied at 200 μg/ml 48 hours after transfection, and colonies were picked to 96-well plates containing feeder cells after ten days. Clones were expanded and split for freezing and genomic analysis. A *SacI* digest was used with the 5' probe to identify potential targeted colonies, and the mutation was confirmed using a 3' probe and a *BglII* digest. Correctly targeted clones were transiently transfected with pMC-Cre to induce partial recombination to remove the *neo*[r] sequences. Clones derived from this procedure were then screened using the above Southern blot strategy to identify those that had lost only the *neo*[r] sequences. Clones were injected into 3.5-day old blastocysts using standard procedures. Chimeric mice were bred to C57Bl/6 females to obtain germline transmission, and the resulting mice were bred to appropriate strains for use in the experiments described below.

Antibodies and Reagents. ASLV-A envelope-Ig fusion protein (SUA-rIgG), used to detect Tva, was provided by Kurt Zingler (Zingler and Young, 1996). The following antibodies were purchased from Pharmingen (San Diego, CA): anti-CD3ε (145-2C11), anti-T cell receptor β (TCRβ) (H57-597), anti-CD69 (H1.2F3), anti-CD2 (RM2-5) anti-Ly5.1 (CD45.1) (A20), anti-CD44 (IM7), anti-CD62L (MEL-14) and anti-IFNγ-FITC (XMG1.2). Anti-B220 (RA3-6B2), anti-CD8α (CT CD8a), anti-CD4 (S3.5), streptavidin-FITC and streptavidin-

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TriColor were purchased from Caltag Laboratories (Burlingame, CA). Anti-BrdU, Streptavidin-SpectralRed and Annexin V-FITC were purchased from Becton-Dickinson (San Jose, CA), Southern Biotechnology Associates, Inc. (Birmingham, AL) and R&D Systems (Cambridge, MA) respectively. Donkey-anti-rabbit conjugates were obtained from Jackson ImmunoResearch (West Grove, PA) except for the donkey anti-rabbit horseradish peroxidase conjugate, which was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Anti-Cre monoclonal antibody (7.23) was obtained from Berkeley Antibody Co. (Berkeley, CA). Anti-Cre polyclonal antiserum (B4197) was a kind gift of Louis Reichardt, UCSF.

5'-Bromo-2'-Deoxyuridine (BrdU), DNase I, flourescein isothiocyanate (FITC), 2-2-2 tribromoethanol, 2-amyl alcohol, neomycin, phorbol 12-myristate 13-acetate (PMA), ionomycin and anti-murine IgG-agarose were purchased from Sigma (St. Louis, MO). Polymyxin was obtained from Paddock Laboratories (Minneapolis MN) and Carboxyflourescein, succimidyl ester (CFSE) from Molecular Probes (Eugene, OR). Pefabloc and leupeptin protease inhibitors were obtained from Boehringer Mannheim (Indianapolis, IN). Immobilon-P PVDF membrane was purchased from Millipore (Burlington, MA) and Hybond-N$^+$, Rediprime labeling kits, S-200 Microspin columns and ECL detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Flow Cytometry. Single cell suspensions were prepared from lymph node, spleen and thymus as noted and incubated with appropriate antibodies for 30 minutes at 4° C in PBS/0.3% BSA/0.01% NaN$_3$. Cells were washed once in the

above solution between staining steps and three times before analysis. 2-30x10^5 scatter-gated cells were analyzed using a FACScan® and CellQuest® software from Becton-Dickinson. Anti-BrdU staining was performed as previously described (Carayon and Bord, 1992; Tough and Sprent, 1994). Briefly, cells were stained for surface markers, fixed in 95% EtOH, washed and then incubated in 1% paraformaldehyde/0.01% Tween 20 and pelleted. The cells were then incubated with DNase I prior to washing and staining with FITC-conjugated anti-BrdU.

IFNγ staining was performed on cells sorted for Tva and either high or low CD62L expression using a MoFlo® (Cytomation Inc., Fort Collins, Colorado) cell sorter. After sorting, the cells were activated with PMA (50 ng/ml) and ionomycin (2 μg/ml) for four hours, with Brefeldin A (10 μg/ml) present during the last two hours. The cells were then fixed in 4% paraformaldehyde, washed, permeabilized in 0.5% saponin in PBS/1%FCS and stained with anti-IFNγ-FITC in the permeabilization buffer. The cells were washed in the same buffer twice before analysis using the FACScan®.

Apoptotic cells were detected by flow cytometry with Annexin V-FITC according to the manufacturer's instructions.

Cell culture and *in vitro* activation. Cells were cultured at 1x10^6/ml in RPMI-1640 supplemented with 10% FCS, penicillin (0.05 U/ml), glutamine (0.29 mg/ml), MEM non-essential amino acids (0.1mM) and β-mercaptoethanol (0.12μM). For activation, 5 ng/ml PMA and 200 ng/ml ionomycin were added to the culture for the indicated length of time.

Intrathymic Injections. Injections were performed as previously described (Donovan and Brown, 1994). In brief, 4-5 week old mice were anaesthetized with avertin (tribromoethanol). An incision was made in the sternum to reveal the thymus, and approximately 20 μl of a 1 mg/ml FITC solution in PBS were injected into each thymic lobe. Pooled cells from the spleen, mesenteric, inguinal, brachial and cervical lymph nodes were analyzed by FACS® 24 hours after injection for the presence of FITC-labeled recent thymic emigrants.

Fetal Liver Transplants. CD4$^{-/-}$ males that were homozygous for the *cd4-TVA* transgene were mated to either B6.Ly5.1$^+$ or CD4$^{-/-}$Ly5.1$^-$ females and fetal liver tissue was obtained by dissection of embryos on day 14 of gestation. Cell suspensions were prepared in IMDM supplemented with 20% FCS by passing tissue through a 22-gauge needle followed by a 25-gauge needle. Fetal liver preparations from each genotype were pooled and combined in the indicated mixtures. 4x10^6 cells were then injected into the tail veins of irradiated (1000 Rads) 6-week old C57BL/6 mice. Transplanted animals received water containing neomycin (1.1% w/v) and polymixin B sulfate (850 U/ml) until analysis 6-8 weeks after transplantation.

Cell Transfers and CFSE Labeling. Single cell suspensions of pooled lymph nodes and/or spleen as indicated were prepared from the indicated donors, washed in PBS and incubated in 0.1μM (for transfers to C57Bl/6 recipients) or 3.3μM (for transfers to RAG-1$^{-/-}$ recipients) CFSE in PBS for 8 minutes at room temperature (Lyons and Parish, 1994). At the end of the incubation FCS was added to quench the CFSE and the cells were washed twice in RPMI

containing 15% FCS. The labeled cells were then injected into the tail vein of sex-matched 8-12 week-old RAG-1$^{-/-}$ mice (1x10^7 or 5x10^7 cells per recipient). For transfers of purified T cells, the single cell suspensions described above were sorted by negative selection using anti-B220, anti-MHC Class II and anti-CD8 MACS® microbeads and passing over LS$^+$ magnetic columns (Miltenyi Biotec, Auburn, CA). Wild-type and CD4-deficient cells were then mixed at a 1:1 ratio and 5x10^6 total CD4 lineage cells were transferred into RAG-1$^{-/-}$ recipients as above.

Immunoprecipitation and Western blotting of Cre. Whole cell lysates were prepared in a solution of 10mM Tris pH 8.0, 150mM NaCL and 2mM EDTA pH 8.0 containing 1% NP-40 and supplemented with 1mM Pefabloc and 50 µg/ml leupeptin. A volume corresponding to 19x10^6 cells was then incubated for 30 minutes at 4° C with 25 µl of packed anti-mouse IgG beads which had been preblocked for 1 hour in a solution of the above buffer supplemented with 0.1% bovine serum albumin. After this preclearing step, the lysate was transferred to a fresh tube and 25 µl of fresh beads were added, along with 5 µl (5 µg) of 7.23 anti-Cre antibody. The precipitation was allowed to proceed for 2 hours at 4° C, after which the lysate was removed, the beads were boiled in 25 µl of SDS-PAGE sample buffer and the supernatant loaded on a 10% SDS-polyacrylamide gel. Protein was transferred to PVDF membrane by standard procedures (Burnette, 1981; Towbin et al., 1979). The membrane was then blocked in 100mM Tris pH 8.0, 150mM NaCl and 0.5% NP-40 supplemented with 5% dry milk and incubated at 4° C overnight with B4197 anti-Cre polyclonal antiserum diluted 1:3000 in the above solution. The

membrane was then washed three times in the above solution, incubated for 45 minutes at room temperature with donkey-anti-rabbit horseradish peroxidase diluted 1:4000 in the above solution, washed twice and detected using ECL reagents according to manufacturer instructions.

Southern Blotting. Southern blotting of genomic DNA was according to standard procedures (Botchan et al., 1976; Jeffreys and Flavell, 1977). Briefly, DNA was digested with the indicated enzyme overnight, resolved on a 1% agarose gel and transferred to Hybond N$^+$ in 0.4N NaOH. The membrane was then probed with an appropriate segment of DNA labeled with ^{32}P-dCTP using the Rediprime random labeling kit according to manufacturer's instructions.

CHAPTER 1:

THE EFFECTS OF CD4 DEFICIENCY ON THYMIC SELECTION AND PERIPHERAL SURVIVAL OF CD4 LINEAGE CELLS

Summary

The participation of the CD4 coreceptor in T cell-APC interactions has important functional consequences both in the thymus and in the periphery. Here we demonstrate that CD4 is centrally involved in the provision of survival signals to helper T cells, such that in the absence of CD4 expression they fail to persist effectively *in vivo* and are prone to apoptosis. CD4 regulates naïve cell survival more potently than that of memory cells, which are represented at higher frequencies and are less apoptotic than naïve counterparts in animals lacking CD4. The data reveal a critical role for CD4 in the provision of effective survival signals to helper T cells.

Introduction

Through binding to MHC and recruiting the tyrosine kinase p56[lck] to the TCR signaling complex, CD4 can significantly improve the antigen responsiveness of both developing and mature T cells (Collins et al., 1992; Glaichenhaus et al., 1991; Hampl et al., 1997; Killeen and Littman, 1993; Madrenas et al., 1997; Miceli et al., 1991). A pivotal role for CD4 in the development of the helper T lineage was revealed by studies of animals with a disrupted CD4 gene (Killeen and Littman, 1993; Killeen et al., 1993; Rahemtulla et al., 1991). Strikingly, these animals exhibited a population of mature MHC Class II-restricted CD4⁻CD8⁻ αβ T cells in the periphery (Locksley et al., 1993; Rahemtulla et al., 1994). Such cells can respond to Class II-restricted antigens and exhibit allo-reactivity as well as provide stimulation to B cells for antibody production (Killeen et al., 1993; Rahemtulla et al., 1991; Rahemtulla et al., 1994). Additionally, these helper lineage cells can assist in the defense against pathogens such as *Leishmania major* (Locksley et al., 1993).

Despite retaining significant helper function in the absence of CD4 expression, the CD4-deficient cells do exhibit a distinct impairment in development to the Th2 subset both *in vitro* and *in vivo* (Fowell et al., 1997). Interestingly, a tailless CD4 molecule did not restore the differentiation to a Th2 phenotype, suggesting that recruitment of p56[lck] to the TCR complex by CD4 may be critical (Brown et al., 1997). These data add to previous studies in cell lines and hybridomas showing that the participation of CD4, and in particular association of p56[lck] with CD4, in the interaction between the T cell

and the APC can promote T cell activation and interleukin-2 production (Collins et al., 1992; Glaichenhaus et al., 1991; Miceli et al., 1991). The data from the CD4$^{-/-}$ mouse in particular suggest that although cells manage to emerge from the thymus without the contribution of CD4, such cells are not functionally equivalent to wild-type cells.

In parallel with the conditional genetic system to be described later, we have used the CD4-deficient mouse to reveal essential functions of CD4 in the periphery. Here we show that, in addition to its crucial role during thymocyte development and immune responses, CD4 is also critically involved in promoting the survival of T helper cells. We find that the lack of CD4 expression significantly increases the likelihood of T helper cell apoptosis and leads to a dramatic depletion of naïve cells from the T helper cell population. The data make clear a critical role for CD4 in peripheral selection of the T helper cell repertoire.

Results

Reduced Numbers of T Cells in CD4$^{-/-}\beta_2$m$^{-/-}$ Mice.

To facilitate the analysis of CD4-deficient cells, we had previously generated CD4$^{-/-}\beta_2$m$^{-/-}$ doubly-deficient animals (Locksley et al., 1993). Mice lacking CD4 alone as well as the double-nulls contained mature CD4 lineage cells that were capable of helper cell responses despite the absence of CD4 (Brown et al., 1997; Fowell et al., 1997; Locksley et al., 1993; Rahemtulla et al., 1994). These cells normally make up approximately 10% of the peripheral T cell pool in CD4$^{-/-}$ mice. Similar to mice that lack either MHC class II or CD8 expression (Fung-Leung et al., 1991; Grusby et al., 1991; Viville et al., 1993) mice lacking CD4 or β_2m alone had close to normal total numbers of T cells in their peripheral lymphoid tissues (Killeen et al., 1993; Koller et al., 1990; Rahemtulla et al., 1991; Zijlstra et al., 1990). In contrast, the CD4$^{-/-}\beta_2$m$^{-/-}$ double-null mice had significantly reduced numbers of mature T cells relative to wild-type mice (Figure 1 and Table 1).

Whereas lymph nodes from C57Bl/6 control animals had an average of 62.4% T cells, those of CD4$^{-/-}\beta_2$m$^{-/-}$ animals contained only 12.8% T cells. The same relative proportions of T cells were observed in both young and old (>6 months) mice. Although the absence of CD4 impairs positive selection of the CD4 lineage in the thymus (Killeen et al., 1993; Rahemtulla et al., 1991, see also below), wild type T helper cells are capable of extensive proliferation and can readily expand from small numbers to reconstitute peripheral compartments (Rocha et al., 1989). We therefore sought a better understanding of why the
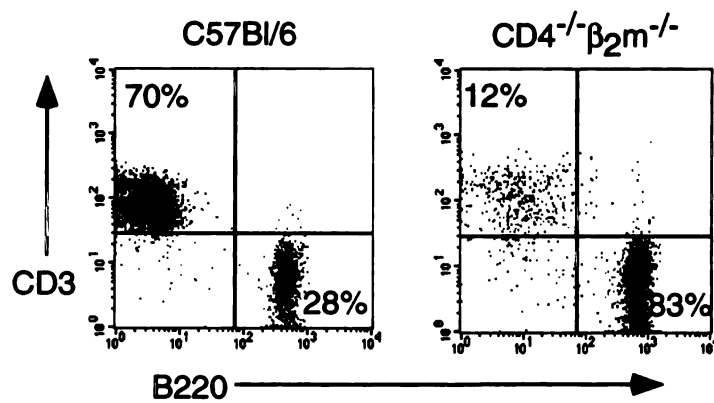
FIG. 1. $CD4^{-/-}\beta_2m^{-/-}$ mice show reduced frequency of peripheral T cells. Lymph node cells from C57Bl/6 and $CD4^{-/-}\beta_2m^{-/-}$ mice were analyzed by flow cytometry using anti-B220 and anti-CD3ε antibodies. The data shown are representative of ten animals of each genotype examined in three separate experiments.

Table 1. T cell numbers in CD4-deficient mice. Spleen cells from mice of the indicated genotypes were counted and analyzed by FACS®.

T Cell Numbers ($\times 10^6$)

	Total	CD4 Lineage	CD8 Lineage
C57Bl/6	29.8±2.3	18.3±2.1	11.0±0.8
CD4$^{-/-}$	20.4±4.0	5.7±2.4	14.8±5.4
CD4$^{-/-}$β$_2$m$^{-/-}$	8.4±3.1	6.8±2.4	1.6±0.7

absence of CD4 impaired the representation of peripheral T helper cells in CD4$^{-/-}$ and CD4$^{-/-}$β$_2$m$^{-/-}$ animals.

Reduced Positive Selection but Efficient Thymic Egress in the Absence of CD4.

To determine the magnitude of the effect of CD4 on the efficiency of positive selection, CD4$^{-/-}$β$_2$m$^{-/-}$ and CD4-expressing β$_2$m$^{-/-}$ animals were injected with BrdU to pulse-label a cohort of dividing cortical thymocytes. The development of these cells into the CD4 lineage was then followed by flow cytometry over the next five days, using upregulation of CD3 and CD69 as markers of positive selection (Figure 2). β$_2$m-deficient animals were used so that the selection of cells on MHC class II molecules could be examined in the absence of MHC class I-dependent selection (Chan et al., 1994). As shown in Figure 2, the percentage of labeled positively selected thymocytes (CD3^{hi}CD69$^+$ cells) was typically five-fold lower as measured on day 4 in the absence of CD4 (5.9% labeled cells) than in the presence of CD4 (30.6% labeled cells). Similar results were obtained when mature thymocytes were identified by upregulation of CD2 (Teh et al., 1997) rather than CD69 (not shown). These kinetic data indicate that positive selection of CD4 lineage thymocytes is about five-fold less efficient in the absence of CD4 – a figure that is consistent with the steady state representation of mature T helper lineage cells in CD4-expressing vs. CD4$^{-/-}$ thymuses (Killeen et al., 1993; Rahemtulla et al., 1991).

To examine the possibility that loss of CD4 might impair the efficiency of emigration from the thymus, we used an intrathymic FITC injection
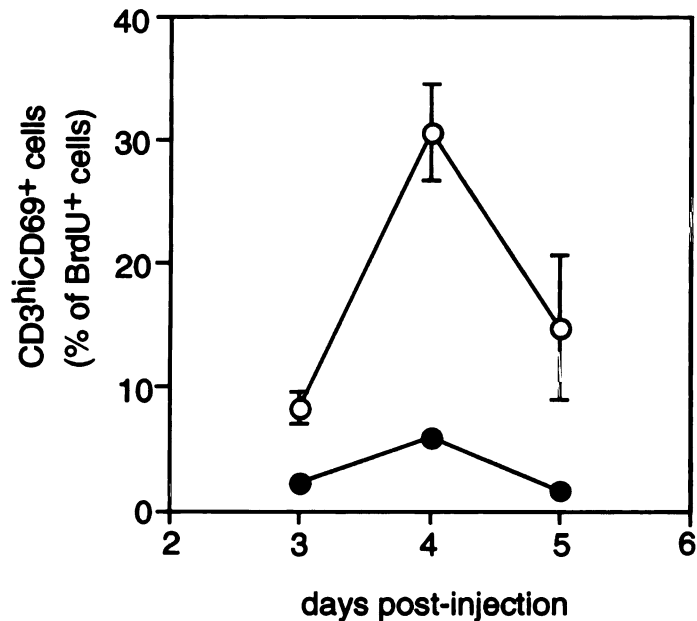
FIG. 2. Decreased positive selection of CD4 lineage cells in the absence of CD4. A cohort of dividing thymocytes was labeled by injecting 5-7 week old CD4-expressing $\beta_2m^{-/-}$ (open circles) or CD4$^{-/-}\beta_2m^{-/-}$ (filled circles) mice intraperitoneally with two doses of 1 mg BrdU each, four hours apart. At the indicated times, thymocytes were stained using anti-CD3ε, anti-CD69 and anti-BrdU. The figure shows the percentage of BrdU$^+$ cells that express high levels of CD3 as well as CD69. Each point represents three animals of each strain. The data shown are representative of four independent experiments.

procedure (Donovan and Brown, 1994) to compare the export of CD4 lineage cells from the thymuses of CD4$^{-/-}$ and wild-type mice. Unlike BrdU, the FITC label is taken up by virtually all thymocytes, both resting and cycling. As shown in Figure 3, FITC-labeled CD8$^-$ T cells (i.e., those that include primarily the CD4 lineage) could be detected in the peripheral lymphoid organs of both genotypes of mice 24 hours after FITC injection. In the CD4$^{-/-}$ animals, the representation of these recent thymic emigrant cells was, if anything, increased relative to the representation of the precursor (CD3^{hi}CD8$^-$) population in the thymus. Similar results were found when animals were fed on water containing BrdU and then monitored for the accumulation of weakly BrdU-labeled cells in their peripheral lymphoid tissues (not shown), a method which has been previously used to track recent thymic emigrants (Tough and Sprent, 1994). From these data, we conclude that the loss of CD4 does not obviously impair the rate of export of CD4 lineage cells from the thymus to the periphery.

Cumulatively, the data show that in the absence of CD4, the output of CD4 lineage cells from the thymus is about 20% of normal due to inefficient positive selection, a reduction that seems insufficient to account for the marked decrease in T helper cells in the periphery. We therefore hypothesized that an additional, peripheral defect must account for the reduced numbers of CD4 lineage cells found in CD4$^{-/-}$ or CD4$^{-/-}\beta_2$m$^{-/-}$ mice. As one approach to confirm this, we established radiation chimeras in which recipient mice received a roughly equal mixture of fetal liver from CD4-expressing and CD4-deficient embryos. In these chimeras, wild-type donor
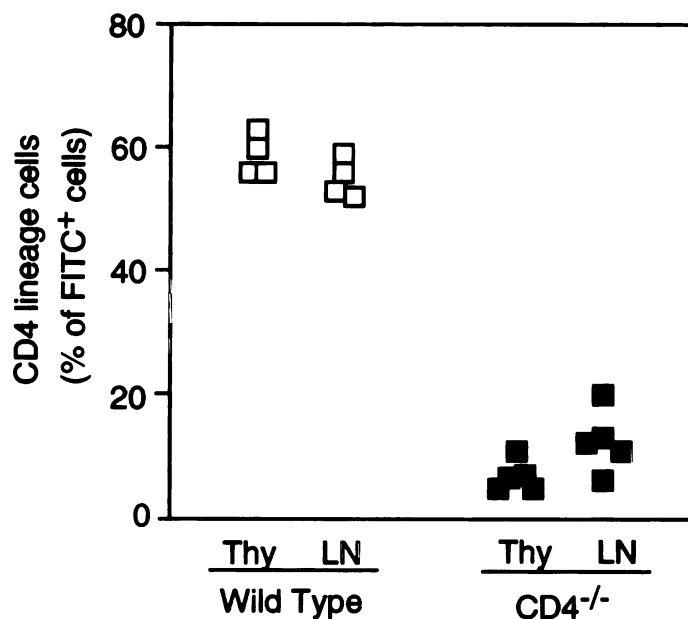
FIG. 3. Thymic emigration in the absence of CD4. 4-5 week-old β_2m-expressing mice were injected intrathymically with FITC in PBS, labeling virtually all thymocytes. 24 hours after injection, cells from the mesenteric, brachial, inguinal and cervical lymph nodes were pooled and examined by flow cytometry for $FITC^+$ T cells. The graph shows the percentage of total $CD3^{hi}$ thymocytes or $FITC^+CD3^+$ lymph node cells that were $CD8^-$ in either wild-type (open symbols) or $CD4^{-/-}$ (filled symbols) animals. Each point represents one animal and the data shown are representative of three independent experiments.

cells were distinguished by expression of Ly5.1, and CD4 lineage cells from wild-type and mutant donors were marked by expression of a transgenic CD4 lineage-specific reporter (*cd4-TVA*, a chicken Tva cDNA controlled by CD4 regulatory elements) (Figure 4A). Using this combination of surface molecules, the relative representation of CD4$^{-/-}$ vs. CD4-expressing T helper lineage cells could be determined in both the thymuses and periphery of chimeric animals.

Two conclusions could be drawn from the analysis of the chimeric mice. First, in agreement with the observations above, CD4$^{-/-}$ cells were about three-to-four-fold less frequent than CD4-expressing cells at the 'single-positive' stage of development than at the 'double-positive' stage (Figure 4B). The magnitude of this reduction in positive selection is close to that expected from the kinetic data described above. Second, the chimeras revealed a marked reduction in the frequency of CD4 lineage cells between the single-positive thymocyte compartment and the periphery. This last reduction was unaffected by the input ratio of CD4$^{-/-}$ to CD4-expressing cells and typically accounted for a two-three-fold decrease in the cellularity of the CD4 lineage (Figure 4B). Since we could find no evidence for a defect in exit of CD4$^{-/-}$ cells from the thymus, the data suggest that the absence of CD4 impairs some aspect of peripheral T cell homeostasis, with a defect in either T cell proliferation or survival being likely candidates.
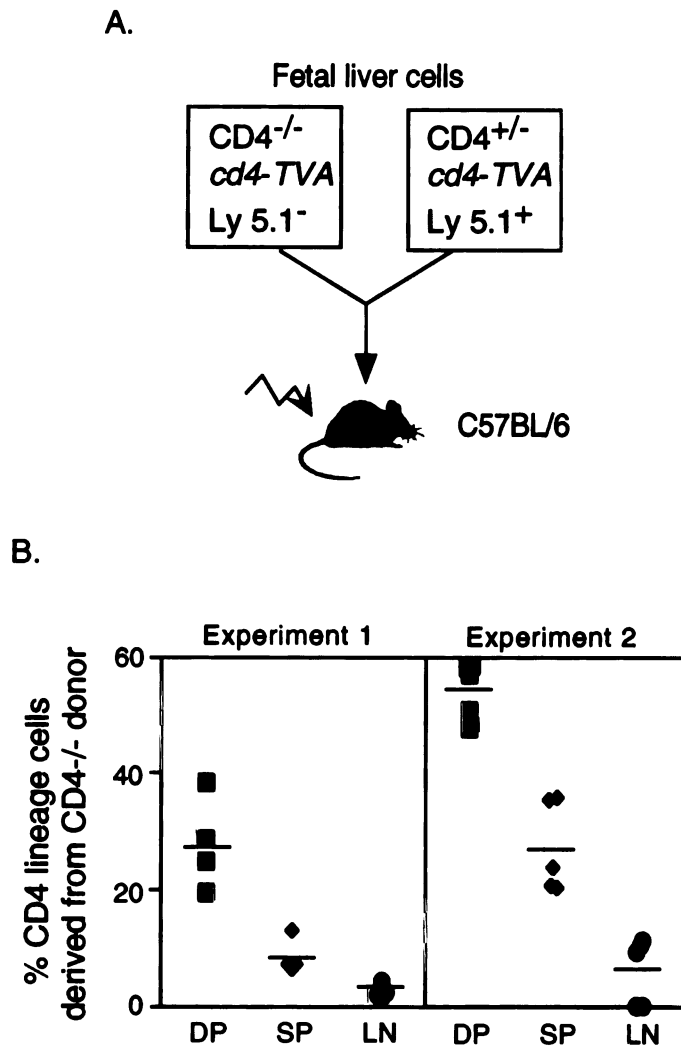
FIG. 4. Representation of CD4-deficient T helper cells in mixed fetal-liver chimeras. (A) Fetal liver (e14) cells from CD4$^{+/-}$Tva$^+$ Ly5.1$^+$ and CD4$^{-/-}$ Tva$^+$ Ly5.1$^-$ animals were mixed at a 70:30 (Experiment 1) or a 40:60 (Experiment 2) ratio and injected into lethally-irradiated C57BL/6 hosts. Recipients were analyzed 6-8 weeks later by flow cytometry. Donor-derived CD4 lineage thymocytes and lymphocytes were identified by expression of Tva; CD4 genotype was inferred by expression of Ly5.1. (B) The figure shows the percentage of thymocytes that were Tva$^+$ CD8$^+$ Ly5.1$^-$ (DP, squares) or Tva$^+$ CD8$^-$Ly5.1$^-$ (SP, diamonds), or lymph node cells that were Tva$^+$CD8$^-$ Ly5.1$^-$ (LN, circles). The data shown are representative of three separate experiments.

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Increased Apoptosis in CD4$^{-/-}$ Cells and Failure to Thrive after Adoptive Transfer.

Using flow cytometry with Annexin V-FITC, we attempted to determine whether the loss of CD4 expression conferred an enhanced tendency for apoptosis on T helper cells. Peripheral CD4 lineage cells from CD4$^{-/-}$ and CD4-expressing mice were stained immediately after removal from animals. As shown in Figure 5, the frequency of apoptotic cells within the CD4 lineage (identified by expression of the transgenic *cd4-TVA* reporter) was higher in the absence of CD4 than in its presence. No difference was found in CD8 cells from the same mice. Furthermore, when transferred to nonirradiated C57Bl/6 hosts, CD4 lineage cells lacking CD4 declined significantly as a percentage of the total donor population in comparison to cells from wild-type animals (Figure 6); CD8 cells from either donor type persisted effectively. These data indicate that the absence of CD4 has a significant negative effect on the capacity of CD4 lineage cells to survive beyond the thymus.

T cells from CD4-expressing or CD4$^{-/-}$ mice were also tested for their capacity to repopulate peripheral compartments after transfer into RAG-1$^{-/-}$ recipients. In RAG-1$^{-/-}$ mice injected with mixtures of unfractionated spleen and lymph node cells, CD8$^+$ T cells effectively repopulated the recipients regardless of whether they came from CD4-expressing or CD4$^{-/-}$ donors (Figure 7A). However, CD4 lineage cells from the CD4$^{-/-}$ donors were markedly compromised in their repopulation capacity, decreasing four-to-five
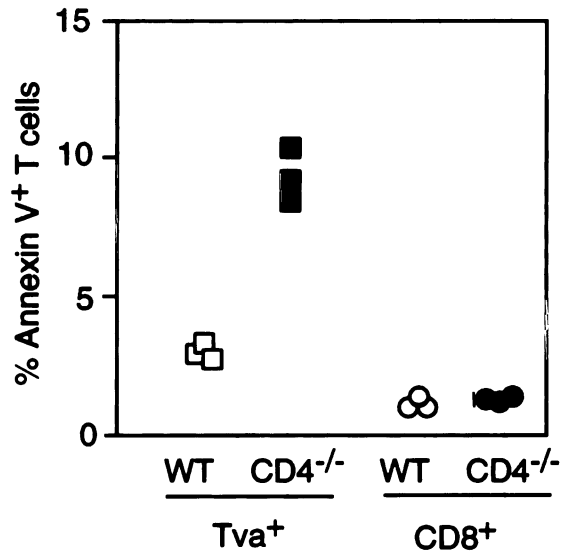
FIG. 5. Apoptosis in CD4 lineage cells from wild-type and CD4$^{-/-}$ mice. Cells from the inguinal, brachial, mesenteric and cervical lymph nodes of CD4$^{+/-}$Tva$^+$ (open symbols) or CD4$^{-/-}$Tva$^+$ (filled symbols) mice were stained using Annexin V, anti-TCRβ and SUA-rIgG or anti-CD8. The cells were analyzed by FACS® using a live cell forward/side scatter gate. The left side of the figure shows the percentage of scatter-gated TCRβ$^+$Tva$^+$ cells that stained with the Annexin V reagent, while the right side shows TCRβ$^+$CD8$^+$ cells. Cells from three animals of each genotype were analyzed, and the data are representative of five independent experiments.
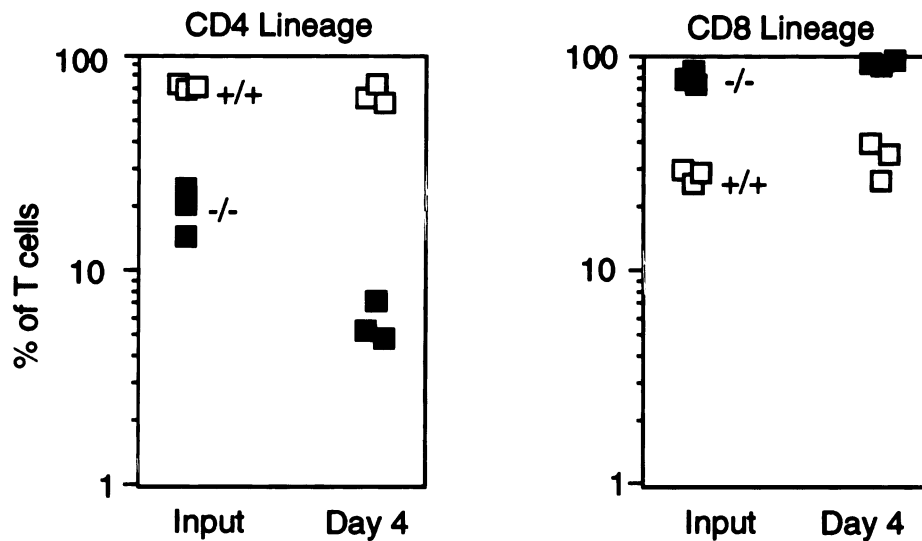
FIG. 6. Survival of cells after transfer to wild-type hosts. 3×10^7 pooled lymph node and spleen cells from wild-type (open squares) or CD4$^{-/-}$ (filled squares) donors were labeled with 0.1μM CFSE and injected into the tail vein of nonirradiated sex-matched C57Bl/6 hosts. The figure shows the percentage of CFSE$^+$ T cells which were CD8$^-$ (left panel) or CD8$^+$ (right panel) found in the recipient lymph nodes four days after transfer. Each symbol represents one recipient from an individual donor animal. The data shown are representative of three separate experiments.

A.

CD4 Lineage

100

% of T cells

$\square$ +/-

-/-

10

1

Input   Day 5

CD8 Lineage

100

-/-

+/-

10

1

Input   Day 5

B.

100

% from CD4$^{-/-}$ donor

10

1

Input   Day 5

FIG. 7. Survival of CD4 lineage cells after transfer to RAG-1$^{-/-}$ mice. (A) Pooled lymph node and spleen cells from individual CD4$^{+/-}$ (open symbols) or CD4$^{-/-}$ (filled symbols) donor animals were labeled with 3.3μM CFSE. 5x10^7 labeled cells were then injected into the tail veins of sex-matched RAG-1$^{-/-}$ recipients. Five days after transfer, lymph node cells were stained with anti-TCRβ and anti-CD8α and examined by FACS® analysis to determine the percentage of TCRβ$^+$ cells that were CD8$^-$ (left panel) or CD8$^+$ (right panel). Each symbol represents one recipient of cells from an independent donor animal of the indicated genotype. (B) 2.5x10^6 purified CD8- T cells from wild-type animals were mixed with an equal number of such cells from CD4$^{-/-}$ animals and injected into the tail veins of RAG-1$^{-/-}$ recipients. The figure shows the percentage of total T cells derived from the CD4$^{-/-}$ donor and each point represents one recipient animal. The data shown are representative of three separate experiments.

fold as a fraction of total T cells over a five-day period (Figure 7A). The same relative decrease in representation was observed when either 5×10^7 or 1×10^7 cells were transferred (data not shown), suggesting that the absolute number of CD4 lineage cells was not the limiting factor. Similar results were also obtained from mixed chimeras in which a 1:1 mix of purified CD4 lineage cells from CD4-expressing and CD4$^{-/-}$ donors was used as the inoculum (Figure 7B). From CFSE labeling experiments, we found that the persisting CD4 lineage cells from the CD4$^{-/-}$ donors divided as much or more than their wild-type counterparts over the five-day period (Figure 8), indicating that the failure to repopulate was not caused by an inability to proliferate. Thus, consistent with their apoptotic phenotype *in vitro*, the adoptive transfer experiments indicated that the absence of CD4 impaired the survival of T helper cells *in vivo*.

Enrichment for memory T helper cells in the absence of CD4.

Although the absence of CD4 can affect the magnitude of immune responses, CD4$^{-/-}$ mice are still capable of robust and effective T helper cell-dependent responses to both primary and recall challenges (Brown et al., 1997; Fowell et al., 1997; Locksley et al., 1993; Rahemtulla et al., 1994). FACS® analysis of the steady-state CD4 lineage in CD4$^{-/-}$ mice showed significant enrichment for cells with a surface phenotype that is normally typical of memory T cells (Figure 9A). Nearly 35% of CD4 lineage cells from CD4$^{-/-}$ mice expressed high levels of CD44, compared to only 2% of the lineage in CD4-expressing mice. A similar enrichment was seen in the fraction of cells expressing low levels of CD62L (29% CD4$^{-/-}$ vs. 5% CD4$^{+/+}$). As a control, we

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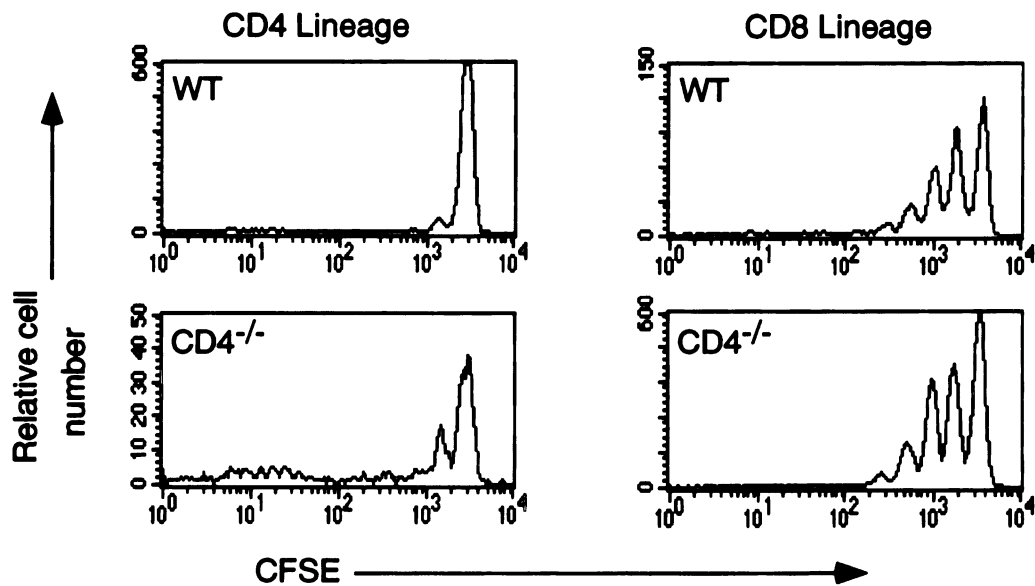FIG. 8. Cell division in CD4 lineage cells transferred to RAG-1$^{-/-}$ mice. Representative CFSE profiles of remaining CD4 lineage cells (left panels) or CD8 lineage cells (right panels) from wild type (top) or CD4$^{-/-}$ (bottom) donors on day five after transfer of bulk lymphocyte and splenocyte populations into RAG-1$^{-/-}$ mice. The data shown are representative of three separate experiments.
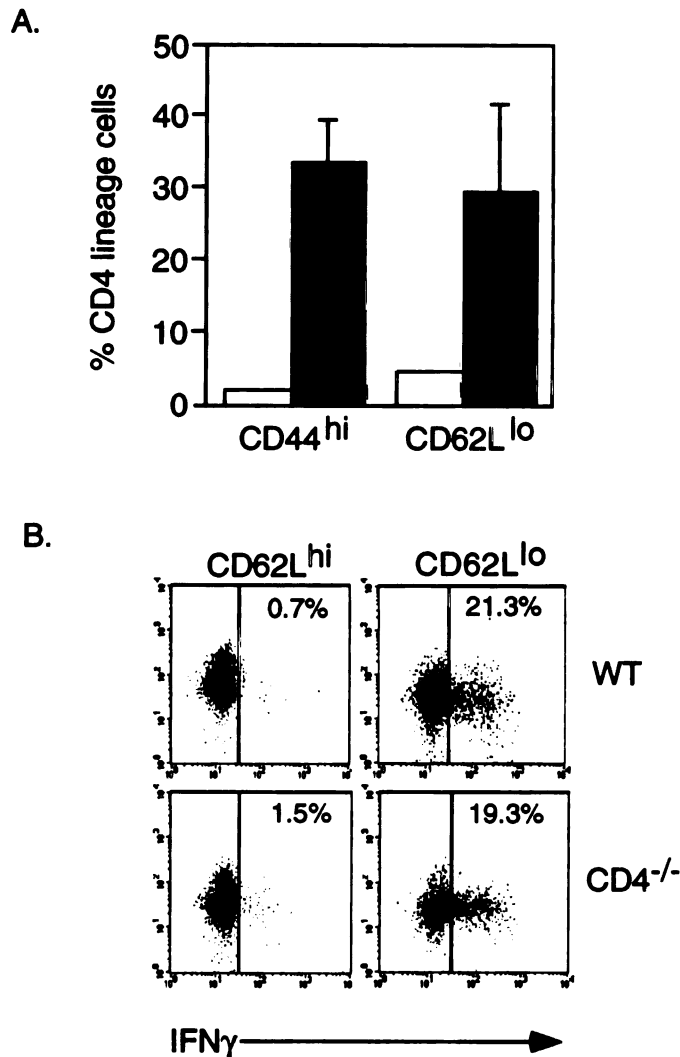
FIG. 9. Frequency and phenotype of memory cells in the CD4 lineage of wild type and CD4$^{-/-}$ mice. (A) Pooled mesenteric, cervical, inguinal and brachial lymph nodes were examined by FACS® for expression of CD4 or Tva, CD8 and the indicated markers. The figure shows the percentage of live CD4 lineage T cells (CD4^{+}, wild-type, open bars or Tva^{+}, CD4$^{-/-}$, filled bars) having the indicated phenotype. Data are from three mice of each genotype, and the experiment shown is representative of three others. (B) Pooled lymph node and spleen cells were sorted by flow cytometry for Tva expression and CD62L level. They were then activated for four hours with PMA and ionomycin, fixed and stained for IFNγ expression. The figure shows cytokine expression in CD62L^{hi} (left) or CD62L^{lo} (right) CD4 lineage cells from wild type or CD4$^{-/-}$ mice, as indicated. The flow cytometry channel represented on the Y-axis is empty. The data are representative of two independent experiments.

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found no difference in the relative expression of any of these markers on CD8$^+$ T cells from the same animals (data not shown).

In contrast to naïve cells, memory T helper cells rapidly secrete cytokines such as γ-interferon (IFNγ) when they are activated (Croft et al., 1994; Rogers et al., 2000). Consistent with their designation as memory T helper cells, we found that CD62L^{lo} CD4 lineage cells from CD4$^{-/-}$ mice exhibited this rapid cytokine release phenotype. As shown in Figure 9B, one fifth of CD62L^{lo} Tva-expressing cells produced IFNγ after brief *in vitro* activation with phorbol ester and ionomycin. This frequency of cytokine-producing cells was very similar to that observed for CD62L^{lo} Tva-expressing cells from C57BL/6 mice and clearly distinct from the very low frequency of cytokine positive CD62L^{hi} (naïve) cells from either type of mouse (Figure 9B). Finally, we noted a much higher frequency of apoptotic cells among naïve versus memory CD4$^{-/-}$ T helper cells (Figure 10). The increase in total apoptosis we noted earlier in CD4$^{-/-}$ mice (Figure 5) is almost entirely confined to the naïve compartment. Taken together, these data suggest that the survival effect conferred by CD4 expression is significantly more pronounced in naïve cells than in the memory compartment. Thus, the absence of CD4 selects against naïve T helper cells and leads to enrichment for cells that have adopted a memory fate.

Expansion of the CD4 lineage in CD4$^{-/-}$CD5$^{-/-}$ doubly-mutant mice.

The CD5 molecule is expressed on the surface of thymocytes as well as mature T cells, and its absence results in an increase in sensitivity to TCR
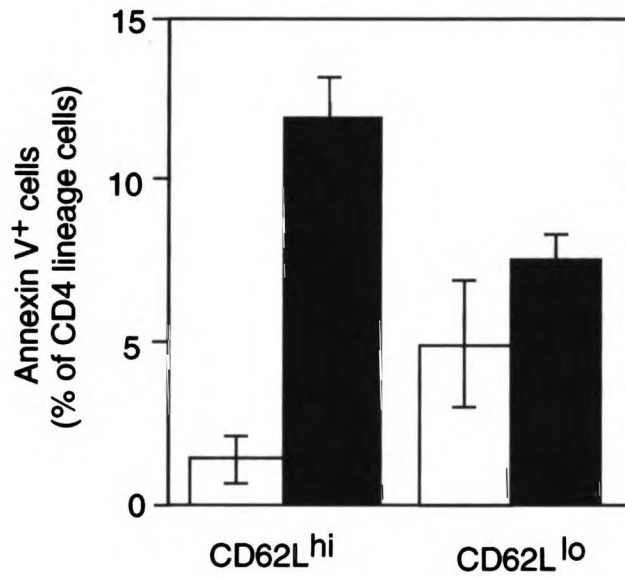
FIG. 10. Apoptosis in naive and memory CD4 lineage cells.
Pooled lymph node cells from wild-type (open bars) or CD4$^{-/-}$
Tva$^+$ (filled bars) mice were stained for expression of CD4 (or
Tva), CD62L and Annexin V. The graph shows the percentage of
CD62L^{hi} (left) or CD62L^{lo} (right) CD4 lineage cells that stain with
the Annexin V-FITC reagent. Three mice of each genotype are
represented by each bar, and the profiles are representative of data
from three independent experiments.

stimulation in thymocytes (Tarakhovsky et al., 1995). It seemed possible, then, that the loss of CD5 expression might cause an augmentation of TCR-derived signals which might at least partially compensate for the defect in thymic selection and peripheral survival found in the absence of CD4. To investigate this possibility we generated mice mutant in both the CD4 and CD5 genes (Pena-Rossi et al., 1999). First, to examine the effects of CD5 on positive selection, we compared singly and doubly-mutant mice using the BrdU pulse-labeling assay described earlier (see Figure 2). As shown in Figure 11A, the loss of CD5 significantly increased the level of positive selection of CD4 lineage cells in $CD4^{-/-}CD5^{-/-}$ mice over that found in $CD4^{-/-}$ singly-mutant animals (31% versus 18% on day 3, for example). This expansion was also evident in the periphery, where the percentage of total T cells derived from the CD4 lineage in doubly-mutant animals was increased as compared to $CD4^{-/-}$ animals (27% versus 11.5%) (Figure 11B). However, the loss of CD5 could not completely compensate for the loss of CD4, as the levels of CD4 lineage cells reached less than half of wild-type levels.

Despite the increase in cellularity of the CD4 lineage compartment, the peripheral lymphoid organs of $CD4^{-/-}CD5^{-/-}$ mice were enriched for memory-phenotype as well as apoptotic cells, similar to mice deficient in CD4 alone. We examined mature lymphocytes by flow cytometry for expression of the activation marker CD69 and the memory markers CD44 and CD62L, as well as for staining with the Annexin V reagent. We found that, similar to CD4 lineage cells from $CD4^{-/-}$ animals, such cells from $CD4^{-/-}CD5^{-/-}$ mice showed elevated levels of activated- and memory-phenotype cells as compared to

A.

B.
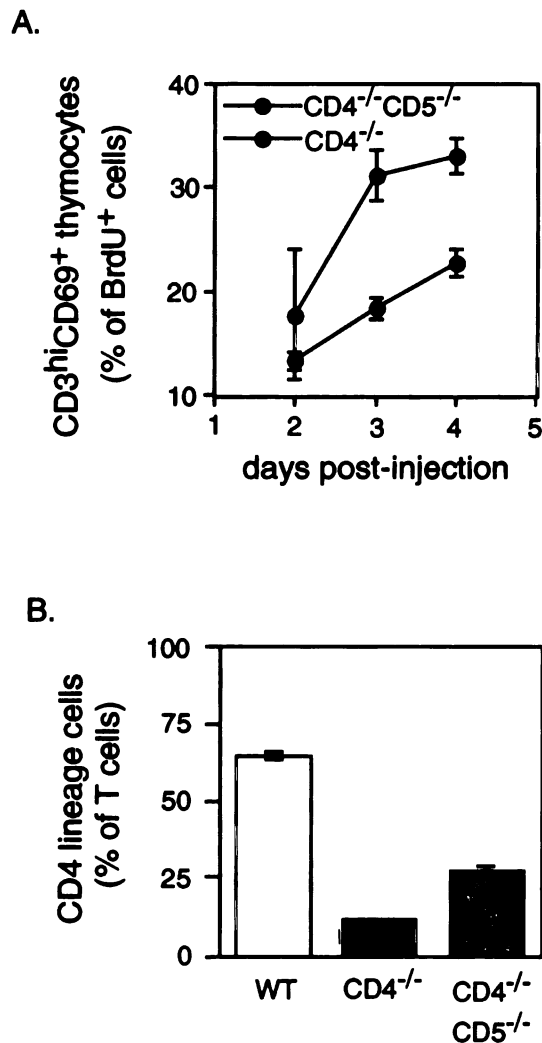
FIG. 11. Increased positive selection and peripheral representation of CD4-deficient helper lineage cells in the absence of CD5. (A) 5-7 week old CD4$^{-/-}$ (shaded circles) or CD4$^{-/-}$CD5$^{-/-}$ (filled circles) mice were injected intraperitoneally with two doses of 1 mg BrdU each, four hours apart. At the indicated times, thymocytes were stained using anti-CD3ε, anti-CD69 and anti-BrdU. The figure shows the percentage of BrdU$^+$ cells that express high levels of CD3 as well as CD69. Each point represents three animals of each strain and the data are representative of three independent experiments. (B) Lymph node cells from adult mice of the indicated genotype were examined by flow cytometry. The figure shows the percentage of TCRβ$^+$ cells that were CD8$^-$ in each strain. The bars represent three mice of each genotype, and the data is representative of three independent experiments.

wild-type counterparts (34.5% vs. 10.3% CD69$^+$, 17.9% vs. 3.6% CD44^{hi}, 15.2%

vs. 7.1% CD62L^{lo}) (Figure 12). Furthermore, CD4 lineage cells from the doubly

mutant animals also showed an increase in apoptotic cells as compared to

wild-type CD4 lineage cells (8% vs. 3.5%) (Figure 12). Interestingly, in each

case cells from the doubly-mutant animals showed slightly lower levels of

cells of the given phenotype than were found in the CD4$^{-/-}$ mice, suggesting a

partial but incomplete compensation for the loss of CD4. This evidence shows

that although the loss of CD5 can partially restore the size of the CD4 lineage

compartment in the absence of CD4 expression, peripheral T cells lacking both

CD4 and CD5 remain significantly compromised in their survival, although

perhaps to a slightly lesser degree than cells from the CD4 singly-mutant

mice. A change in magnitude and/or quality of TCR-derived signaling such

as that generated by the loss of CD5 does not fully compensate for the lack of

surface CD4 expression, either during thymic selection or in the periphery.

Loss of Fas expression does not restore CD4 lineage cell survival in the
absence of CD4.

Apoptosis in T cells can be potentiated by members of the Tumor

Necrosis Factor (TNF) receptor family. In particular, activation-induced cell

death is mediated by FADD-dependent signals transmitted via Fas (Van Parijs

and Abbas, 1998; Van Parijs et al., 1998). To examine the possibility that the

apoptosis of CD4 lineage cells in CD4-deficient mice might be Fas-mediated,

we utilized CD4$^{-/-}$ mice that had been bred onto the MRL/*lpr* background

(Chesnutt et al., 1998). The naturally-occurring *lpr* mutation has been shown
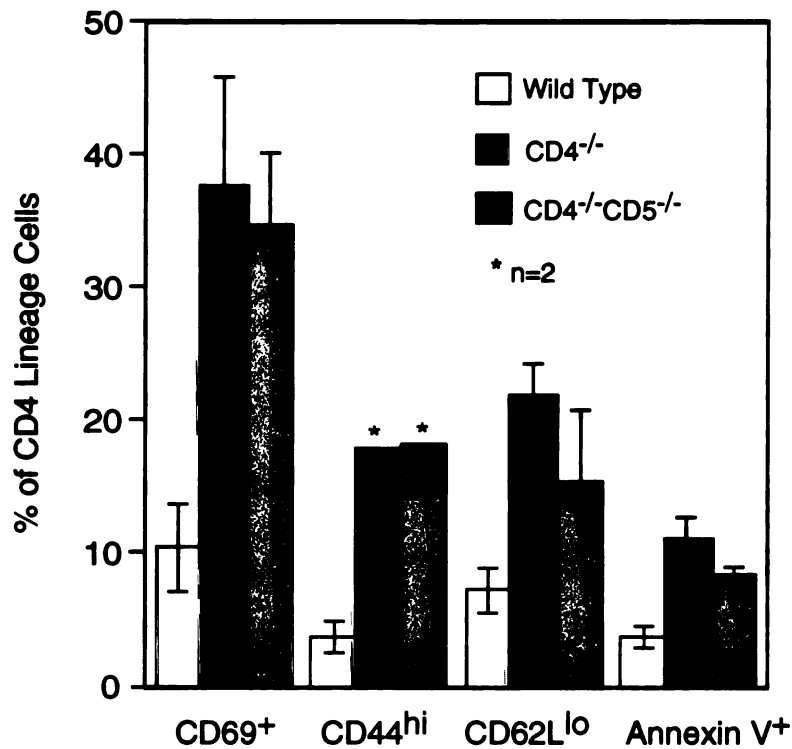
FIG. 12. Activation, memory and apoptotic markers on CD4 lineage cells from CD4$^-$/$^-$CD5$^-$/$^-$ mice. Pooled mesenteric, inguinal, cervical and brachial lymph node cells from adult wild type (white bars), CD4$^-$/$^-$ (black bars) or CD4$^-$/$^-$5$^-$/$^-$ (grey bars) mice were examined by flow cytometry for expression of the indicated markers. The figure shows the percentage of TCRβ$^+$CD8$^-$ cells having the indicated phenotype. Three animals of each genotype are represented by each bar except as noted, and the data shown are representative of two independent experiments.

to abrogate expression of the Fas molecule (Watanabe-Fukunaga et al., 1992). Mice carrying this mutation exhibit an accumulation of CD4⁻CD8⁻ cells as part of the lymphoproliferative disorder (Wofsy et al., 1984). Because this population is difficult to distinguish from the CD4 lineage population normally found in CD4$^{-/-}$ mice, we examined the mice at 3-4 weeks of age, prior to onset of lymphoproliferation. At this stage the size of the lymphocyte compartment in Fas-deficient mice is not increased over that of wild-type mice (data not shown).

We found that the lack of Fas expression did not appear to increase the size of the CD4 lineage compartment to any degree as compared to Fas-expressing CD4$^{-/-}$ animals (Figure 13A). The levels of CD4 lineage cells were, if anything, slightly lower in the presence of the *lpr* mutation than in the Fas-expressing context. Furthermore, the increase in apoptotic cells in the CD4 lineage of CD4-deficient animals was also evident in animals lacking both Fas and CD4 as compared to the CD4 lineage cells of CD4-expressing *lpr* mice (5.1%±0.3 vs. 3.4%±0.4) (Figure 13B). This increase is slightly lower than that seen in the presence of Fas expression (see Figure 5); nonetheless, although a portion of the apoptosis of CD4-deficient helper lineage cells may be due to Fas, at least some of that cell death is independent of the Fas molecule. Taken together, these data strongly suggest that the Fas molecule does not mediate the apoptosis found in CD4-deficient helper lineage cells.

A.
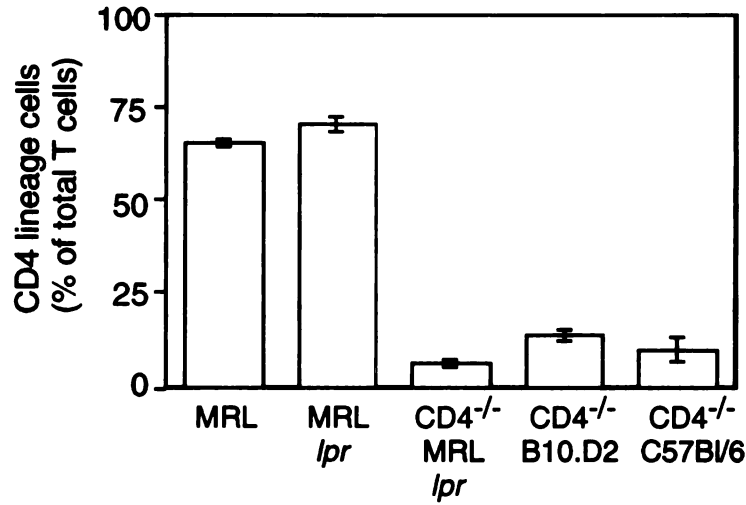
B.
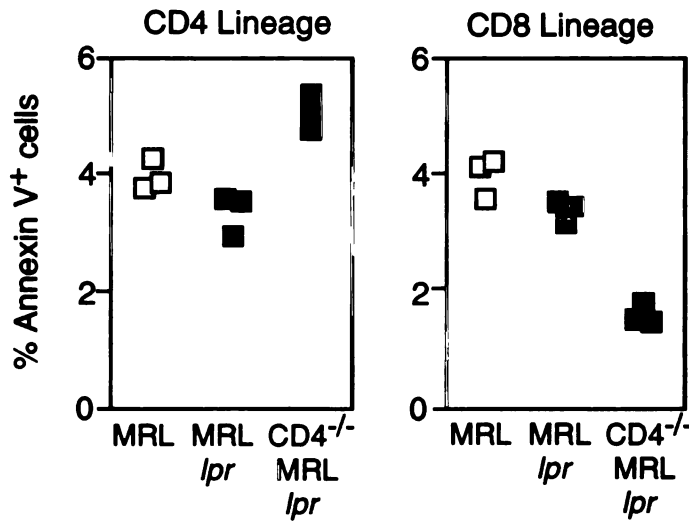
FIG. 13. CD4 lineage cells in the absence of Fas expression. Pooled cervical, mesenteric, inguinal and brachial lymph node cells from 3-4 week-old animals of the indicated genotype were examined by flow cytometry for expression of TCRβ, CD8 and Annexin V. (A) The figure shows the percentage of total TCRβ+ cells that were CD8⁻ in the indicated strains. (B) The figure shows the percentage of TCRβ+CD8⁻ (CD4 lineage, left panel) or TCRβ+CD8+ (CD8 lineage, right panel) from MRL (open squares), MRL/lpr (shaded squares) or CD4⁻/⁻ MRL/lpr (filled squares) mice that stained with the Annexin V reagent. The data shown are representative of three independent experiments.

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Overexpression of Bcl-2 does not rescue helper lineage cells in the absence of CD4 expression.

Constitutive expression of the Bcl-2 protein can protect cells from apoptosis (Strasser et al., 1991), promote survival of cells responding to antigen (Van Parijs et al., 1998) and compensate for mutations that impair survival (Akashi et al., 1997). To examine the possibility that Bcl-2 might prevent apoptosis of CD4-deficient helper cells, we bred mice carrying the CD4-null allele to Eμ-bcl-2-25 mice in which Bcl-2 is highly expressed in thymocytes and T cells.

As shown in Figure 14A, overexpression of the Bcl-2 protein did not result in an increase in the contribution of the helper lineage to the total T cell pool in CD4-deficient mice. Furthermore, the phenotype of CD4 lineage cells was virtually identical in the presence or absence of the *bcl-2* transgene. No significant difference was found in the percentage of Annexin V$^+$ helper-lineage cells between transgenic and nontransgenic animals (8.3% in *bcl-2*$^+$ mice versus 9.1% in *bcl-2*$^-$ mice, Figure 14B and C). Additionally, we found that the increase in memory cells seen in the absence of CD4 remains in the presence of Bcl-2 overexpression. The proportion of helper cells which had low levels of CD62L expression was 24.1% in transgenic mice, a number which was not significantly different from the 28.1% found in nontransgenic littermates (Figure 14B and C). These data suggest that the pathway which results in helper T cell apoptosis in the absence of CD4 expression is not regulated by the Bcl-2 protein.

A.

B.

C.
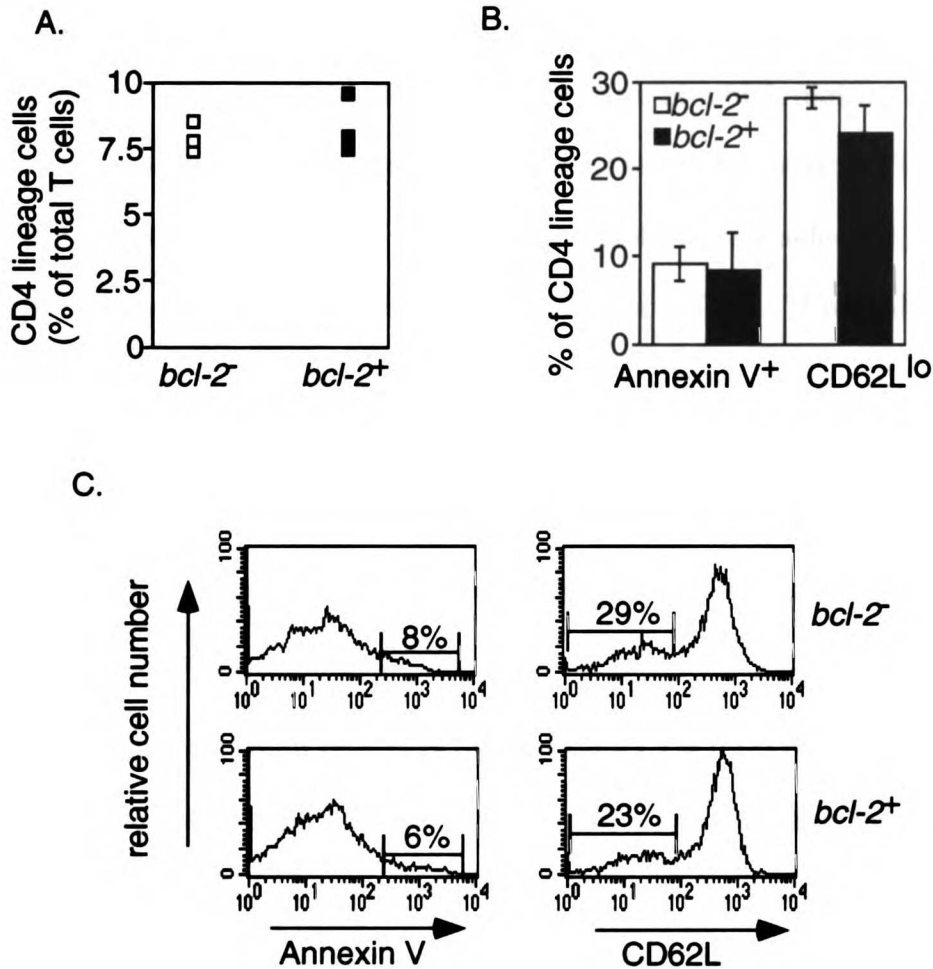
FIG. 14. Effect of *bcl-2* transgene on helper lineage cells in CD4-deficient mice. (A) Pooled lymph node cells from *bcl-2*⁻(open squares) or *bcl-2*⁻ (filled squares) CD4⁻/⁻ mice were examined by flow cytometry for expression of TCRβ and CD8α. The figure shows the percentage of TCRβ⁺ cells which were CD8⁻. (B) CD4 lineage cells as defined in (A) were stained using the Annexin V reagent and anti-CD62L. The figure shows the percentage of CD4 lineage cells from *bcl-2*⁻ (open bars) or *bcl-2*⁺ (filled bars) CD4⁻/⁻ mice which were Annexin V⁺ (left portion) or CD62L^lo (right portion). (C) Representative histograms showing Annexin V staining (left panels) or CD62L staining (right panels) from *bcl-2*⁻ (upper panels) or *bcl-2*⁺ (lower panels) mice.

Discussion

The kinetic and steady state measurements reported here reveal approximately a five-fold reduction in the rate at which mature CD4 lineage thymocytes accumulate in the absence of CD4. An impairment in lineage commitment is evident at a very early stage of positive selection, when wild-type cells would normally express both CD4 and CD8. Thus, mice that lack both CD4 and β_2m have very few CD69$^+$ thymocytes because of the combined effect of loss of MHC class I selecting ligands and inefficient positive selection on class II ligands in the absence of CD4. Such observations highlight the key role that the coreceptor CD4 plays in facilitating the development of CD4 lineage thymocytes.

Although small numbers of wild-type CD4 lineage cells can readily repopulate the T helper cell compartment of T lymphopenic hosts (Rocha et al., 1989), CD4-deficient T helper cells are obviously defective in this respect. This striking finding is made clear by the dominance of the CD8 lineage in mice that lack CD4, by the reduced total number of T cells in CD4$^{-/-}\beta_2$m$^{-/-}$ mice and by the failure of CD4-deficient T helper cells to expand in numbers after adoptive transfer to RAG-1$^{-/-}$ hosts. Furthermore, a defect in peripheral T cell homeostasis in the absence of CD4 was also readily apparent from the low ratio of CD4$^{-/-}$ to CD4-expressing cells in the secondary lymphoid tissues of mixed bone-marrow chimeras. The most likely explanation for this deficiency is that the survival of T helper cells is impaired in the absence of CD4. In support of this, we find that the CD4$^{-/-}$ T helper population is significantly

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enriched for apoptotic cells relative to the wild-type population. Such data suggest that even though T helper cells can emerge from the thymus without CD4, their survival once they reach the periphery is markedly impaired.

Naive versus Memory T Cell Survival

In contrast to normal CD4$^+$ T cells, the surface phenotype of a large proportion of CD4$^{-/-}$ T helper cells resembled that of memory rather than naïve T cells. Importantly, apoptotic cells were also significantly less frequent in the population that had a memory rather than naïve phenotype, suggesting that the survival of memory T helper cells is less CD4-dependent than that of their naïve precursors. These data imply that CD4 lineage cells can be rescued from an apoptotic fate by involvement in an immune response and differentiation into a memory state. This interpretation would be consistent with the demonstration that CD4$^{-/-}$ mice can mount effective T cell recall responses to a variety of antigenic challenges (Brown et al., 1997; Fowell et al., 1997; Locksley et al., 1993).

That memory T cells would be less dependent on a survival signal involving CD4 might be suggested by recent work documenting long-term survival of memory but not naïve T cells after transfer into hosts that lack expression of MHC ligands (Murali-Krishna et al., 1999; Swain et al., 1999). Selection against naïve T cells in the absence of CD4 indicates that naïve T cells must involve CD4 when they engage MHC class II ligands in order for the provision of survival signals to be efficient. Such a role for CD4 in potentiating T cell survival would be consistent with data from Konig and

colleagues (Maroto et al., 1999) showing reduced survival of CD4$^+$ T cells in mice that express a mutant form of MHC class II that does not bind to CD4.

Mechanisms of CD4-Dependent T Helper Cell Homeostasis

When cross-linked with antibodies or HIV gp120, CD4 can induce the activation of associated p56^{lck} molecules (Goldman et al., 1994; Veillette et al., 1989a; Veillette et al., 1989b), it can cause changes in the expression of T cell surface molecules such as CD62L (Marschner et al., 1999) and it can also prime cells for apoptosis (Banda et al., 1992; Bank and Chess, 1985; Desbarats et al., 1996; Foster et al., 1995; Newell et al., 1990). These observations raise the possibility that the provision of survival signals to naïve T helper cells might involve a function of CD4 that could be distinct from its role in facilitating TCR signaling. In this vein, binding between CD4 and either the cytokine IL-16 (Center et al., 1996) or the seminal vesicle protein gp17 (Autiero et al., 1995) can have functional consequences for T cells (Gaubin et al., 1999; Mashikian et al., 1999; Theodore et al., 1996). Although interactions between CD4 and non-MHC-encoded ligands might potentiate T helper cell survival under some circumstances, the most common effect of such interactions appears to be either anti-proliferative (Cruikshank et al., 1996) or pro-apoptotic (Newell et al., 1990). By contrast, signaling through the T cell receptor has a demonstrably critical role in the provision of survival/expansion signals to T cells (Brocker, 1997; Freitas and Rocha, 1999; Markiewicz et al., 1998; Takeda et al., 1996; Tanchot et al., 1997a). The finding that enhancing the TCR signal by eliminating CD5 can cause expansion of the CD4 lineage is consistent with the relevant signal being delivered through the TCR. It seems probable,

therefore, that the main impact of CD4 in promoting naïve T helper cell survival is to enhance the effectiveness of immunological synapse formation and the transduction of pro-survival signals from TCRs engaged by peptide/MHC complexes.

The mechanism for apoptosis in CD4-deficient cells remains unclear. Although Fas and FasL are upregulated on the surface of such cells (Maroto et al., 1999, our observations), loss of Fas expression does not increase the cellularity of the helper lineage or significantly alter the phenotype of CD4-deficient cells. These data strongly argue against a causative role for Fas in the increased helper cell apoptosis in this system. Additionally, overexpression of the Bcl-2 protein failed to restore the numbers or cell-surface phenotype of helper cells lacking CD4, suggesting that the pathway leading to apoptosis is not regulated by Bcl-2. The mechanism of cell death in the CD4-deficient context is therefore at least superficially distinct from previously described activation-induced or passive cell death (Van Parijs and Abbas, 1998; Van Parijs et al., 1998). The mediators of death in these cells will require further investigation.

Conclusions

The proliferation of T cells after transfer into lymphopenic hosts is likely to be driven by the same type of TCR engagements as those that normally regulate extrathymic T cell survival and selection. TCR recognition in such contexts is demonstrably peptide-specific, as it is sensitive to strategies that limit the diversity of peptides presented by MHC class I or class II

molecules (Bender et al., 1999; Ernst et al., 1999; Goldrath and Bevan, 1999a; Viret et al., 1999). Moreover, according to one study, the peptides that govern the proliferation of T helper cells in lymphopenic hosts are distinct from those that allow for the selection of the same cells in the thymus (Bender et al., 1999). Our data reveal a further disconnect between the thymus and the periphery, because successful selection in the former clearly does not ensure selection and survival in the latter. At present, it is unclear whether this disconnect is the result of differences in the TCR signaling behavior of thymocytes versus T cells (Davey et al., 1998; Pircher et al., 1991), or is instead a reflection of variation in the range of peptide/MHC ligands presented to the T helper cells inside and outside the thymus (Marrack et al., 1993). Regardless, the data emphasize that extrathymic T helper cell fate is sensitive to mutations that change the character of TCR signaling. By extension, therefore, the survival of T helper cells must normally depend on TCR signals of appropriate character – themselves dependent on the types of MHC/peptide ligands displayed to the T cells in the periphery.

In conclusion, we show here that naïve T helper cell survival is crucially dependent on CD4 expression and that the absence of CD4 results in a selection for cells with a memory phenotype. While reinforcing previous results that memory and naïve T cells are variably dependent on TCR signals for their survival, these data also emphasize a key role for the CD4 molecule in the regulation of T helper cell homeostasis.

CHAPTER 2:

THE EFFECTS OF CONDITIONAL EXPRESSION OF CD4

Summary

Peripheral T cell survival is dependent on the interaction of T cells with MHC-peptide complexes. We have shown that CD4 participates centrally in the delivery of survival signals to helper lineage T cells. Here we present a novel genetic system for the conditional control of CD4 expression. We examine the effects of extrathymic loss of CD4 from the surface of naïve and previously activated T cells and demonstrate that while naïve helper cells are exquisitely dependent on CD4 for persistence, cells which have been previously activated survive much more effectively in the absence of CD4 expression. This genetic system allows for cell-type specific and temporal control of CD4 expression, and we have used it to dissect the participation of CD4 in the survival of both naive and previously activated T cells.

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Introduction

Through the use of animals carrying a null allele of the CD4 gene, we have demonstrated that the participation of CD4 in interactions between the T cell and the APC has profound effects both on thymic selection and on peripheral survival of the helper lineage. However, any analysis of the phenotype of peripheral T cells in this setting is complicated by the fact that they have undergone selection in the absence of CD4. The T helper population that emerges from the thymus in the absence of CD4 has subtle but important differences from that found in a wild-type setting, particularly in the TCR repertoire expressed (Wang et al., in preparation).

Genetic systems allowing for temporal or cell-type specific control over gene expression have been used successfully in several settings to enable finer dissection of the functions of the gene of interest. In systems using drug-responsive promoters such as the *Escherichia coli* tetracycline-resistance operon, expression of the gene of interest is controlled directly by administration of tetracycline or its derivatives (Furth et al., 1994; Gossen and Bujard, 1992). Systems using steroid hormones such as the *Drosophila melanogaster* hormone ecdysone as the inducing substance have similar properties, in that gene expression is controlled by a transgenic promoter and not by the endogeneous promoter (No et al., 1996). Induction in these systems is reversible.

An alternative method for controlling gene expression involves the use of the bacteriophage P1 site-specific recombinase Cre, which catalyzes

recombination between pairs of *loxP* sites (Austin et al., 1981; Orban et al.,

1992; Sauer and Henderson, 1988; Sternberg and Hamilton, 1981). By

targeting placement of *loxP* sites to the genomic locus, the gene of interest can

remain under the control of the endogeneous promoter until Cre is expressed,

at which point expression of the *loxP*-flanked sequences ceases due to the

recombination event (Kuhn et al., 1995). Alternatively, gene expression can be

abrogated until Cre induction by targeting *loxP*-flanked disruptive elements

("Stop" sequences), which prevent expression of downstream sequences, to the

appropriate location (Lakso et al., 1992). In the *cre-lox* system, the induction

event is irreversible because it involves the loss of sequences from the genome.

The cell-type specificity or temporal control over the induction event occurs at

the level of Cre expression, which can be controlled by a cell-type-specific

promoter (Gu et al., 1994; Lakso et al., 1992) or by the addition of a drug (Feil

et al., 1996; Kuhn et al., 1995).

In order to investigate the effects of extrathymic loss of CD4, we have

generated a system in which the expression of CD4 can be shut off via Cre-

mediated recombination of the genomic CD4 locus. Here we utilize this

conditional expression system to demonstrate that the capacity to potentiate

survival signals is an intrinsic and critical function of the CD4 molecule.

Results

Construction of the CD4^{off} allele.

To allow for regulated extinction of CD4 expression, we chose to make use of the *cre/lox* site-specific recombinase system (Sauer and Henderson, 1988; Sternberg and Hamilton, 1981). We designed an allele (CD4^{off}) in which the *loxP* sites flank the region of the CD4 locus that codes for the Class II MHC-binding domain (Bowman et al., 1990; Clayton et al., 1989) (Figure 15A). The 5' recognition site was placed just upstream of the translational start site, while the 3' *loxP* site allowing for deletion of CD4 sequences was placed in intron 3. This arrangement should, upon Cre expression, result in little or no surface expression of CD4; furthermore, should any residual expression remain, the molecule would be unable to bind Class II MHC.

The neomycin resistance gene (*neo^r*) required for selection of transfected colonies *in vitro* was located in the intronic portion and followed by a third *loxP* recognition site, to allow for removal of *neo^r* *in vitro* prior to injection of targeted ES cells into blastocysts. Additionally, we included the cDNA of human CD52 downstream of *neo^r*, after the final *loxP* site; the CD52 coding region was designed to mark CD4 lineage cells following the loss of surface CD4. Correctly targeted clones were identified by Southern blot of genomic DNA using the probes noted in Figure 15A. Neomycin resistance sequences were eliminated by transient transfection of Cre recombinase in the ES cells (Figure 15B), so that cells bearing only a pair of *loxP* sites and the CD52 sequences could be injected into blastocysts to obtain chimeric mice. Germline

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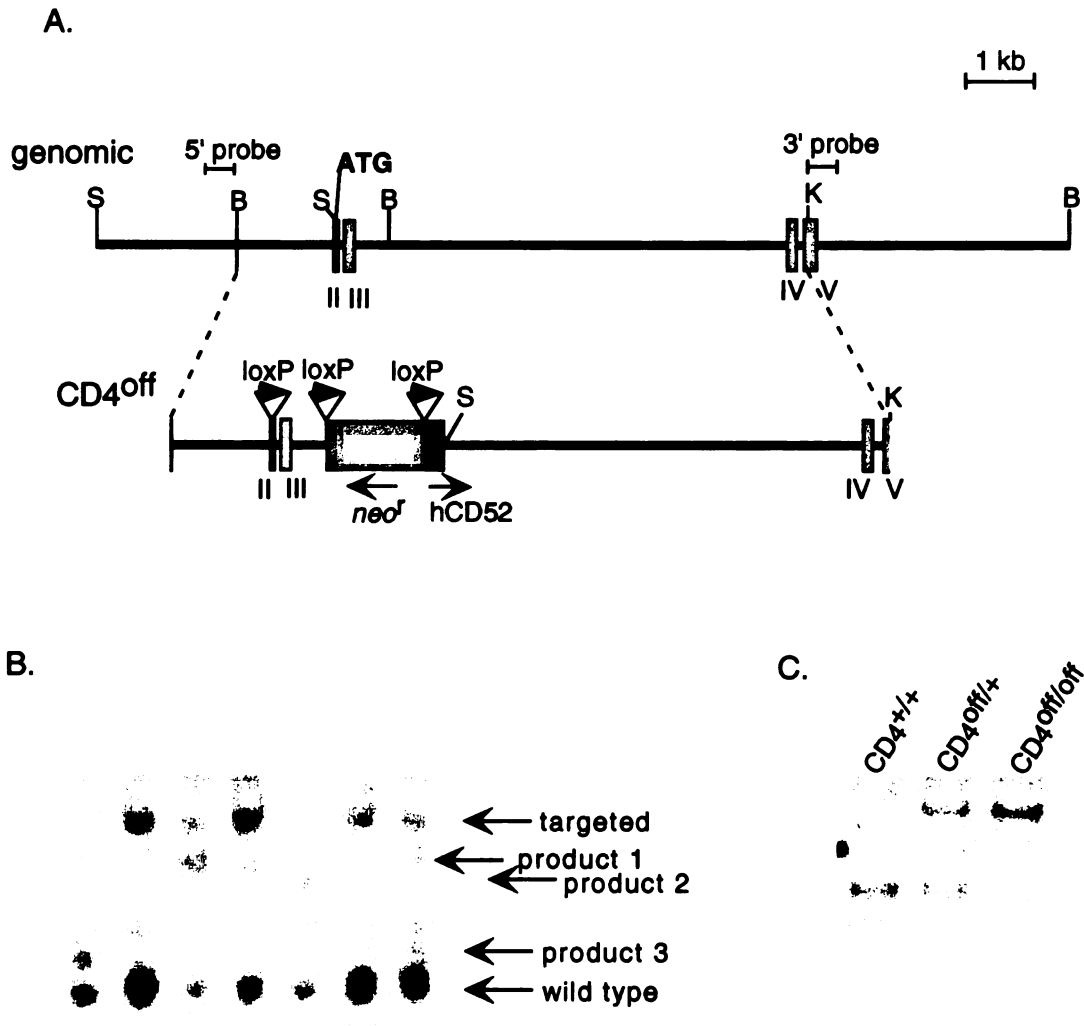
FIG. 15. Generation of the CD4^{off} allele. (A) The upper portion shows the arrangement of the genomic CD4 locus, while the lower part shows the targeting construct created. A series of three directionally aligned loxP sites were inserted into the genomic CD4 sequence. Also inserted were a neomycin resistance gene (neo^r) to allow for selection of homologous recombinants in ES cells, and the human CD52 coding sequence as a marker of Cre-mediated deletion of CD4 sequences. Homologous recombinants were indentified by Southern blot of DNA from neo-resistant ES cell colonies. (B) Correctly targeted clones were transiently transfected with Cre to eliminate the neo^r gene. Three products were obtained, corresponding to loss of CD4 sequences (product 1), loss of the neo^r gene (product 2), or loss of both portions (product 3). Clones missing only the neo^r gene were identified by Southern blot and neomycin sensitivity, and injected into blastocysts. (C) Germline transmission of the allele. Tail DNA from mice shows the targeted (upper) allele and wild-type (lower) allele.

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transmission of the allele was verified by Southern blot with 5' (Figure 15C) and 3' (data not shown) probes.

We bred mice carrying the CD4^{off} allele to those carrying a null allele of CD4 (Killeen et al., 1993) to obtain heterozygous mice (CD4$^{off/-}$) and examined expression levels of CD4 from a single CD4^{off} allele in the absence or presence of Cre. The level of CD4 found on the surface of CD4$^{off/-}$ mice was slightly lower than that found in mice heterozygous for a wild-type and a null allele (CD4$^{+/-}$) (Figure 16A); CD4 lineage cells from both CD4$^{off/-}$ and CD4$^{+/-}$ mice showed reduced levels of CD4 as compared to CD4$^{+/+}$ animals. Interestingly, the reduction in CD4 expression from the CD4^{off} allele resulted in a slight but consistent decrease in the contribution of the CD4 lineage to the peripheral T cell compartment (Figure 16B). In animals in which the only CD4 production came from a single CD4^{off} allele, the CD4 lineage was 54.1% of the total T cell compartment, as compared to 66.2% in wild-type mice or 63.5% in animals carrying a wild-type and a null allele. This restriction likely occurs at the positive selection stage in the thymus, and is consistent with published findings showing that reduced levels of surface CD4 can impair the selection of TCR transgenic T cells (Frank and Parnes, 1998). However, the conditional allele did support the development of near wild-type numbers of CD4 lineage cells.

We then investigated the effects of constitutive Cre expression on CD4 expression from the CD4^{off} allele. For this purpose we examined cells from CD4$^{off/-}$ mice bearing a transgenic *cre* under the control of the distal p56^{lck}

A.

B.
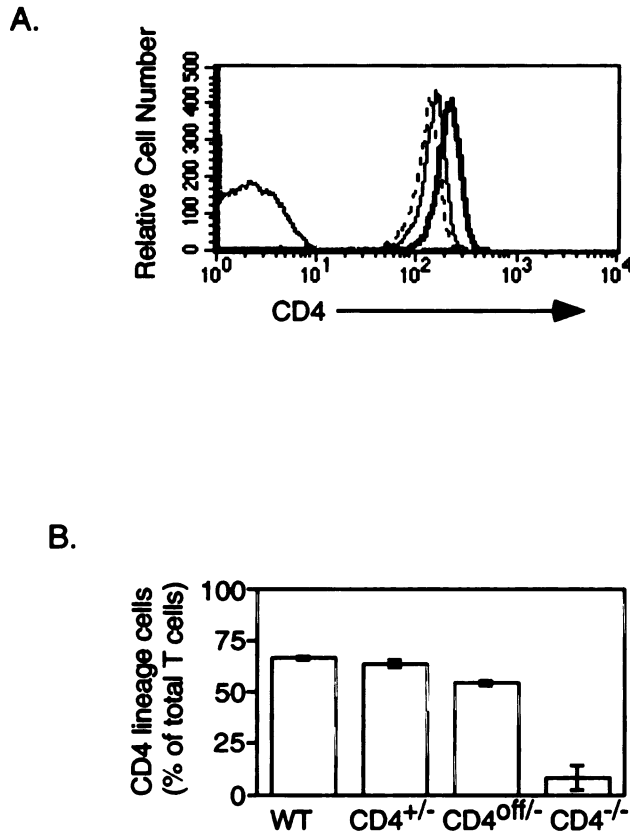
FIG. 16. Expression of CD4 and representation of the CD4 lineage in animals carrying the CD4^{off} allele. (A) Pooled lymph node cells from adult wild type (thick line), heterozygous for a wild type and a null allele (CD4$^{+/-}$, narrow line), heterozygous for CD4^{off} and a null allele (CD4$^{off/-}$, dotted line) and homozygous for the null allele (CD4$^{-/-}$, gray line) were examined by flow cytometry for CD4 expression levels on TCRβ$^+$CD8$^-$ cells. (B) The figure shows the percentage of total T cells which are of the CD4 lineage in lymph nodes of animals from the above experiment. The data shown in parts A and B are representative of three independent experiments.

promoter. These mice will be described in detail later; briefly, Cre is highly expressed in both thymus and lymph node (Figure 20B, line 4425). In the presence of Cre, CD4 expression was completely extinguished, with only background staining (<0.2%) evident in the CD4$^+$ compartment (Figure 17A). The same phenotype was observed when Cre was expressed from a different constitutive allele (data not shown). The percentage of CD8 lineage cells increased significantly in the presence of Cre (91% as compared to 47% in Cre$^-$ lymph nodes), consistent with the reduced positive selection of CD4 lineage cells due to the loss of coreceptor prior to selection. We were unable to detect expression of the CD52 molecule on the surface of lymphocytes (Figure 17B), possibly because the mRNA may not have been processed properly due to the lack of a poly-adenosine signal on the CD52 coding sequence.

Decline of CD4 lineage T cells after inducible extinction of CD4 expression.

In order to obtain temporal control of CD4 extinction, we made use of the Mx-cre transgenic system developed by Rajewsky and colleagues (Kuhn et al., 1995). In this setting, Cre is expressed from a transgene containing the Cre coding sequence under the control of the murine *Mx1* gene. The *Mx1* gene is important in viral immunity and is inducible by Type I interferons or the synthetic double-stranded RNA species polyinosinic-polycytidilic acid (pI-pC) (Arnheiter et al., 1990; Hug et al., 1988). In mice carrying the *Mx-cre* transgene, we were able to induce Cre expression using three doses of pI-pC (Figure 18A). Additionally, we were able to visualize genomic recombination by Southern blot of the CD4 locus in mice carrying the CD4^{off} allele and the

A.

B.
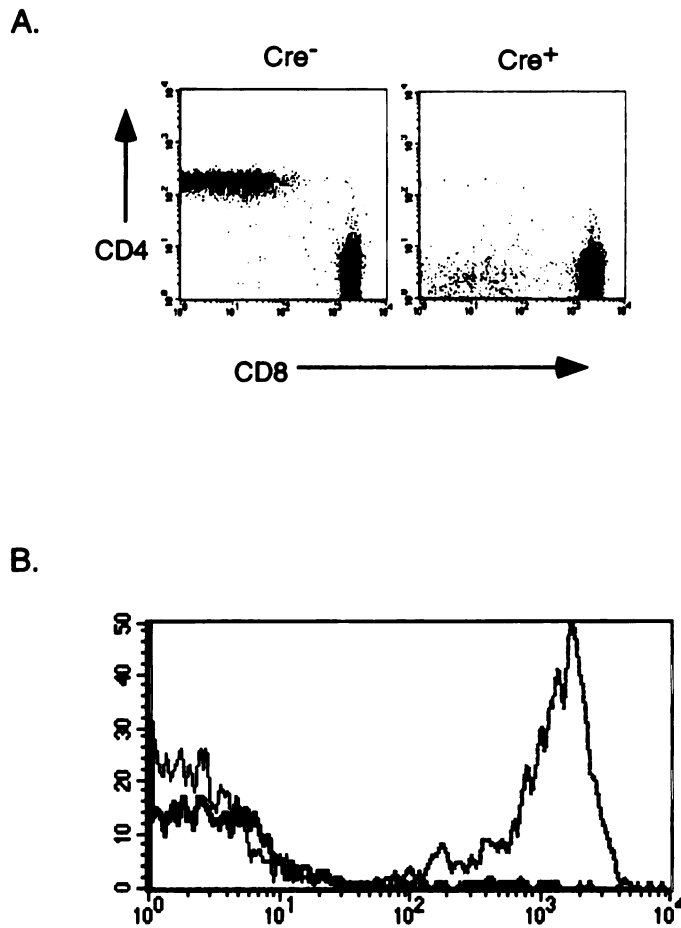
FIG. 17. Extinction of CD4 expression but no expression of CD52 in CD4^{off} mice carrying a constitutive Cre allele. (A) Representative flow cytometry plots of TCRβ$^+$ lymph node cells from CD4$^{off/-}$ mice which express (right) or do not express (left) constitutive Cre. Each plot is representative of three others. (B) CD52 staining on CD4 lineage cells from mice expressing (thick black line) or not expressing (narrow black line) mice Cre. As a positive control, EL-4 cells transfected with CD52 are shown (gray line). The mice are representative of four others of each genotype.

A.

Mx-cre
transgene: -   +   +   +   -

B.

	bulk	CD4 lineage	CD8 lineage	bulk	bulk
Mx-cre	+	+	+	-	+
pI-pC	+	+	+	+	-

←—CD4^{off}
←—recombined CD4^{off}
←—CD4-null
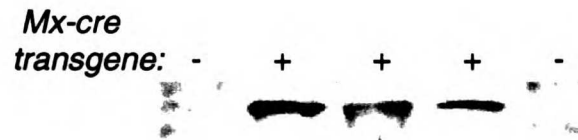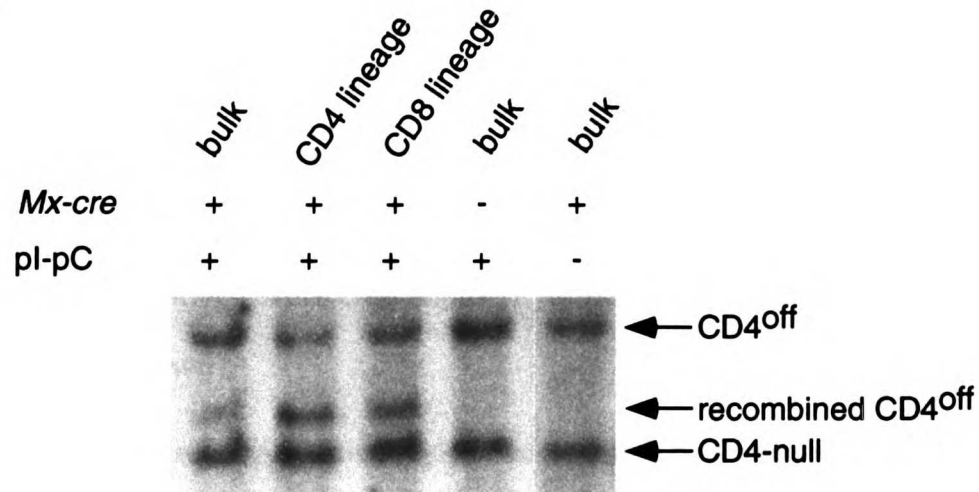
FIG. 18. Expression of Cre and recombination of the CD4^{off} allele in CD4$^{off/-}$*Mx-cre* mice. (A) Cre was immunoprecipitated from whole cell lysate corresponding to 19×10^6 splenocytes from mice bearing (+) or not bearing (-) the *Mx-cre* transgene two days after completion of three doses of 400μg pI-pC each. Cre was detected by Western blot after SDS-PAGE of the immunoprecipitate. (B) Recombination of the CD4^{off} allele detected by Southern blot using the 5' probe indicated in Figure 15A. Data shown is from bulk splenocytes or cells enriched to >70% purity for CD4 lineage or CD8 lineage T cells. Cells were harvested on day 3 after pI-pC induction. All samples are from the same gel.

Mx-cre transgene (CD4^{off}Mx) (Figure 18B). In bulk splenocyte populations, or in splenocytes enriched for CD4 or CD8 lineage T cells, the level of recombination varied somewhat between individuals but was typically approximately 50% at the genomic level three days after the final pI-pC dose.

We then examined the effects of the extrathymic loss of CD4 on the helper lineage in CD4$^{off/-}$ mice in the presence of the *Mx-cre* transgene. First, we found that the extent of recombination seen at the genomic level was rarely if ever reflected in a large increase in CD4⁻CD8⁻ T cells when spleens from treated animals were analyzed by flow cytometry (Figure 19). The percentage of CD4 lineage splenic T cells having the CD4⁻CD8⁻ phenotype after Mx-Cre induction was typically only two-fold elevated over that found in *Mx-cre⁻* animals (for example, 28.9% versus 15.2%, Figure 19B). No evidence of such coreceptor-negative T cells could be found in lymph nodes of treated mice (data not shown).

Despite the relatively low levels of cells which had lost surface CD4, the system still revealed a defect in the ability of those cells to survive in the absence of coreceptor expression. To track the fate of CD4⁻CD8⁻ T cells induced by pI-pC treatment, we removed the spleens of treated animals, labeled the cells with CFSE, and transferred them into sex-matched RAG-1$^{-/-}$ recipients. We found that the percentage of CD4⁻CD8⁻ T cells declined significantly as a percentage of the T helper cell compartment (29% on day 0 as compared to 11% on day 5, Figure 19B). As a control, we found no decline in the CD4⁺ or CD8⁺ compartments in the donor populations. After transfer both
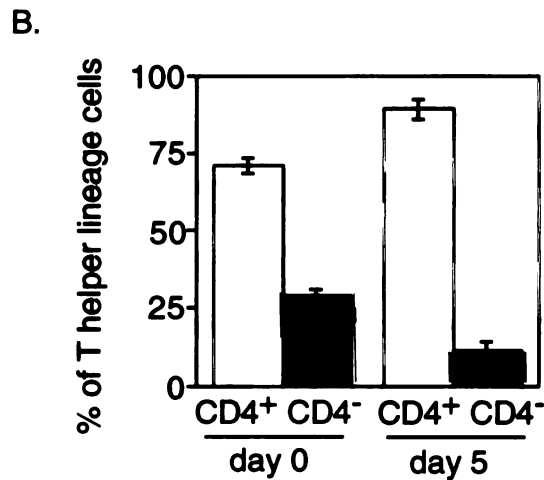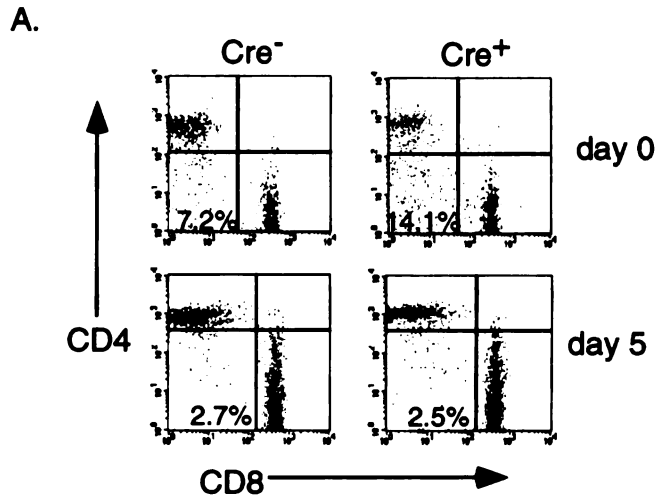
FIG. 19. Loss of CD4 after pI-pC injection, and decay of the CD4⁻CD8⁻ population in the presence of the *Mx-cre* transgene. Three days after the pI-pC induction course was completed, spleens from transgenic and nontransgenic CD4^{off}/⁻ micewere removed and 40x10^6 splenocytes were injected into the tail vein of sex-matched RAG-1$^{-/-}$ recipients. Five days after transfer pooled cells from the inguinal, brachial, mesenteric and cervical lymph nodes of recipients were analyzed by flow cytometry for expression of TCRβ, CD4 and CD8. (A) Representative FACS® plots from Cre⁻ (left panels) and Cre⁺ (right panels) mice on day 0 (upper panels) or day 5 (lower panels) after transfer. Each pair of upper and lower panels shows cells from a single donor animal at the indicated time point. The data shown are representative of three animals of each genotype. (B) The figure shows the percentage of TCRβ⁺CD8⁻ cells that were CD4⁺ (open bars) or CD4⁻ (filled bars) on day 0 or day 5 after transfer. Each bar represents three animals, and the data shown in parts A and B are representative of three independent experiments.

Cre$^+$ and Cre$^-$ donor populations showed a reduction in the total level of CD4$^-$ CD8$^-$ T cells; this decrease occurred consistently in each of the experiments. One possible explanation for this decline is that the cells in the CD4$^-$CD8$^-$ compartment divide slightly more slowly in response to the lymphopenic RAG-1$^{-/-}$ environment than do CD4$^+$ or CD8$^+$ cells. No gross division defect was found in this instance (data not shown) or in the CD4$^{-/-}$ situation (Figure 8) but more data would be necessary to reveal a subtle defect. Regardless, the decline of the CD4$^-$CD8$^-$ cells from Cre$^+$ donor populations over the five day course of the experiment was quite apparent, and revealed a defect in the ability of such CD4 lineage cells to survive without surface coreceptor. These data support the findings in CD4-deficient mice and demonstrate that the effect is not due to any cell-intrinsic alterations resulting from aberrant thymic selection. Instead the failure to survive is a result of inefficient survival signals generated in the absence of the interaction between CD4 and MHC.

This system of Cre induction possessed significant limitations. Type I interferons themselves have significant effects on T cell homeostasis, causing antigen-independent turnover of memory T cells (Tough et al., 1996; Zhang et al., 1998a). Consistent with this property, the pI-pC treatments routinely caused dramatically enlarged spleens (data not shown). We also observed some incidence of toxicity from the injections. Due to these limitations, we chose to develop alternative strategies for Cre expression.

Loss of surface CD4 expression in naïve helper lineage cells.

In order to examine the effects of the loss of CD4 on naïve cells, we have generated mice carrying a transgene that allows for Cre expression under the control of the distal promoter of the tyrosine kinase p56^{lck} (Figure 20A). This promoter is active in mature CD4$^+$ and CD8$^+$ thymocytes as well as in peripheral T cells, but not in more immature thymocytes (Wildin et al., 1991; Wildin et al., 1995). Fourteen founder lines were obtained and bred to CD4^{off} mice so that recombination activity in thymus, lymph node, spleen and tail could be analyzed by Southern blot. Six lines were selected for further breeding, and examined for Cre expression by Western blot. As shown in Figure 20B, these six lines showed significant variation in both the timing and level of Cre expression.

Not surprisingly, these variations were reflected in the surface phenotype of thymocytes and T cells from CD4$^{off/-}$ mice bred with different founder lines. As shown in Figure 21, mice from line 3800 retain CD4 expression on large numbers of cells even in the periphery, although a small percentage of CD4$^-$CD8$^-$ peripheral T cells was present (5% as compared to 1% in control animals). Some thymic expression of Cre was apparent, as evidenced by the increase in CD4$^-$CD8$^+$ thymocytes (14% as compared to 2% in the control). In contrast, neither line 3785 nor 4425 showed any peripheral CD4, although the levels of recombination in the thymus appeared to be different (Figure 21). While in both cases the transgene appeared to express at the CD4$^+$CD8$^+$ stage, the extent of recombination was greater in line 4425, with

69

A.

Distal lck   hCre   hGH
Promoter

B.

4425		3777		3800		3778		3785		4430	
Thy	LN	Thy	LN	Thy	LN	Thy	LN	Thy	LN	Thy	LN
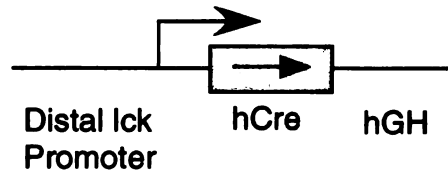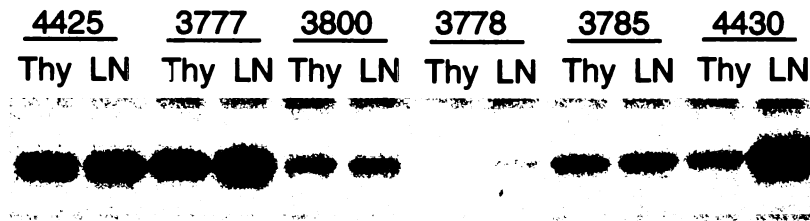
FIG. 20. Generation of *dlck-hcre* transgenic mice. (A) A Cre coding sequence optimized for expression in humans was placed under the control of the distal *lck* promoter, which has been shown to direct gene expression to mature thymocytes and peripheral T cells but not to earlier developmental stages. (B) Western blot of Cre expression in thymus (Thy) and lymph node (LN) of animals descended from five individual transgenic founders. Cre was immunoprecipitated from whole cell lysate corresponding to 19×10^6 cells and detected by immunoblot following SDS-PAGE and electrophoretic transfer.
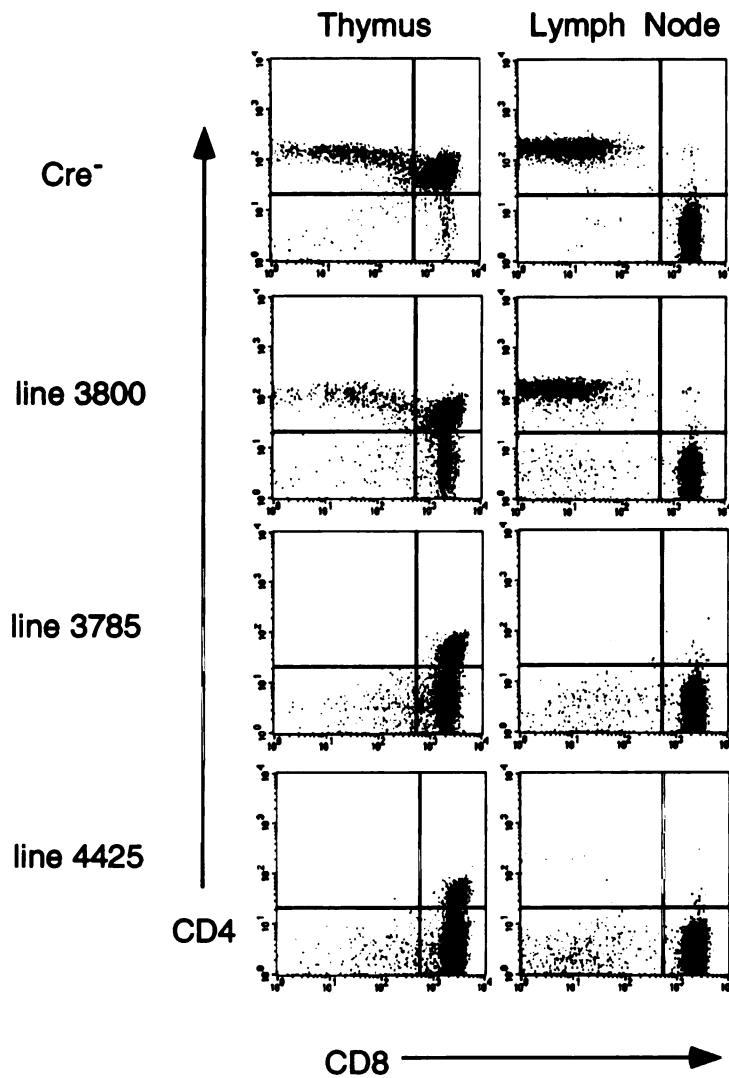
FIG. 21. Phenotype of various *dlck-hcre* lines. Mice carrying the CD4^off allele were bred to lines originating from three different *dlck-hcre* transgenic founders. The figure shows thymocytes (left panels) and TCRβ^+ lymph node cells (right panels) from a Cre^- animal as well as representative plots from lines 3800, 3785 and 4425.

only 15% of thymocytes retaining CD4 expression as compared to 36% of such cells from line 3785 or 86% in the Cre⁻ situation. Both lines had a population of CD4⁻CD8⁻ T cells in the lymph nodes (5% in line 3785 and 9% in line 4425).

Perhaps most strikingly, the three lines varied greatly in the phenotype of the CD4⁻CD8⁻ T cell populations. Whereas both lines 3785 and 4425 showed a significant elevation in the proportion of memory cells among helper cells lacking CD4 (to nearly one-fourth of the lineage, as measured by CD62L downregulation), no such increase was evident in cells from mice of line 3800 (Table 2). As a control, we found no difference in the $CD8^+$ memory compartments. Most likely the distinctions in the helper lineages from the various lines resulted from differences in the developmental stage at which the cells lost CD4. The phenotype of cells from lines 3785 and 4425 was similar to that of helper cells found in $CD4^{-/-}$ mice, for instance, suggesting that the peripheral CD4⁻CD8⁻ cells in lines 3785 and 4425 underwent selection without CD4 and therefore were phenotypically similar to cells found in a CD4-null situation. Further characterization of the timing and levels of Cre expression in these and other lines of Cre-expressing mice will allow for a better understanding of the differential effects of the loss of CD4 on various T cell subsets.

Generation of a system for activation-dependent extinction of CD4.

Since our data and others' have suggested that naive and memory T cells have different survival requirements, we also wished to examine the effects of the loss of surface CD4 on previously activated cells. We therefore

Table 2. Memory markers in *dlck-hcre* lines. Pooled lymph node cells were analyzed by flow cytometry for TCRβ, CD4, CD8 and CD62L or interleukin-2 receptor β chain (IL-2Rβ). The table shows the percentage of the CD4$^+$ compartment or CD4$^-$CD8$^-$ (DN) compartment having the indicated phenotype. A dash (-) indicates no cells are found in that compartment in that strain.

line	CD62L		IL-2Rβ	
	CD4$^+$	DN	CD4$^+$	DN
Cre$^-$	4.7	-	1.8	-
3800	6.9	4.5	1.7	3.5
3785	-	24.8	-	8.9
4425	-	25.5	-	11.6

designed a system in which Cre is controlled by the regulatory elements of the *ox40* gene. OX40, or CD134, is expressed on activated T cells (al-Shamkhani et al., 1996; Baum et al., 1994). In order to obtain an activation-induced Cre, then, we inserted an internal ribosomal entry site (IRES) into exon 3 of the *ox40* gene, followed by the coding sequence of Cre (Figure 22A). The construct also contained an FRT-flanked *neo*[r] gene, which could be removed *in vitro* using the site-specific recombinase FLP (Landy, 1993) (Figure 22A). Mice heterozygous for the *ox40-Cre* allele and a wild-type allele showed high levels of activation-induced Cre protein upon treatment *in vitro* with phorbol ester and ionomycin, whereas little Cre protein was evident in unactivated lymph node or in thymus (Figure 22B). This pattern appeared faithful to the normal expression pattern of OX40, and allowed for the analysis of cells that had undergone Cre-induced recombination at the CD4 locus during the process of activation.

$CD4^{off/-}$ mice carrying the *ox40-Cre* allele showed a significant number of $CD4^-CD8^-$ T cells in the periphery as compared to their Cre-negative counterparts. The lymph nodes of such Cre-expressing mice contained an average of 21.8±5.3% $CD4^-CD8^-$ cells as a proportion of total T cells, or approximately one-third of the CD4 lineage, as compared to 0.3±0.16% for mice not carrying the Cre construct (Figure 23A, lower panels). We found no evidence of significant thymic recombination induced by this Cre expression system (Figure 23A, upper panels): the level of $CD4^-CD8^-$ thymocytes was 1.2±0.5% in Cre-negative mice, as compared to 2.1±0.8% in their *ox40-Cre*[+] counterparts. At the genomic level, the recombination was not significantly

A.

B.
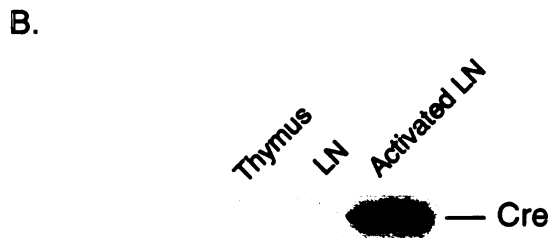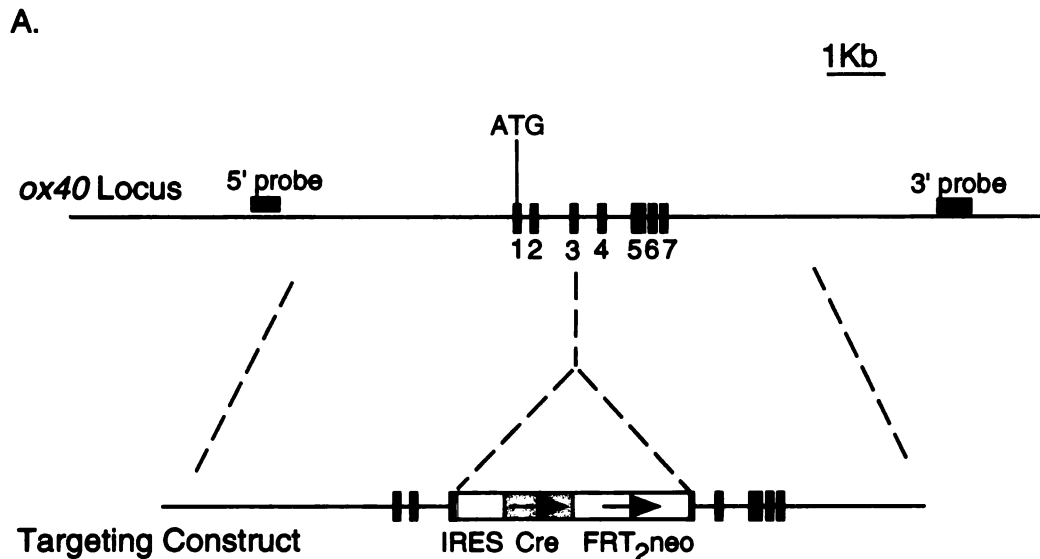
FIG. 22. The *ox40-Cre* allele. (A) The top portion of the figure shows the genomic *ox40* locus, while the lower portion shows the targeting construct used to allow for Cre expression under the control of *ox40* regulatory elements. ES cells were transfected with the construct and appropriately targeted clones were identified by Southern blot using the probes shown. The neomycin resistance gene was removed *in vitro* by transient transfection of FLP into correctly targeted clones prior to injection of the resulting ES cells into blastocysts. Chimeric mice were then bred to obtain germline transmission of the allele. (B) Cre protein was immunoprecipitated from lysate corresponding to 19×10^6 cell equivalents and detected by Western blot after SDS-PAGE. The figure shows Cre expression in thymocytes (Thymus), resting lymphocytes (LN) and PMA/ionomycin-activated (Activated LN) lymphocytes and is representative of two independent experiments.

A.

ox40-cre⁻   ox40-cre⁺

| 10% | 86% | 8% | 88% | thymus |

| 1% | 3% | 1% | 3% | |

CD4 ↑

| 50% | | 27% | | lymph node |

| 1% | 49% | 18% | 55% | |

CD8 ——————→

B.

ox40-cre   -   +   +
PMA/ionomycin   -   -   +

←CD4^off

←recombined CD4^off
←WT
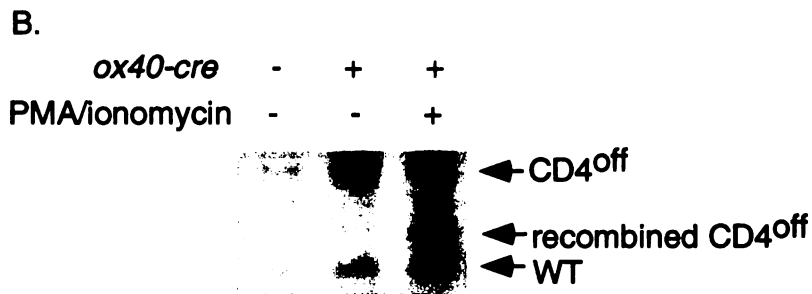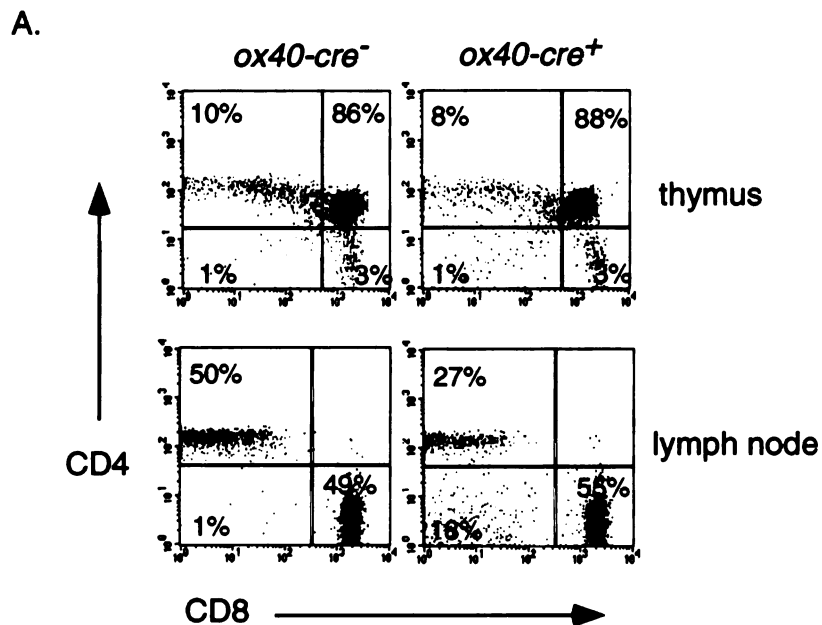
FIG. 23. Recombination of the CD4^off allele in mice carrying the ox40-Cre allele. (A) Representative FACS® plots of thymocytes (upper panels) or TCRβ⁺ lymph node cells (lower panels) from mice carrying the CD4^off allele balanced by a null allele and either expressing (right panels) or not expressing (left panels) Cre. The plots shown are representative of mice from four independent experiments. (B) Southern blot of lymph node cells from resting lymph node (left two lanes) or PMA/ionomycin-activated lymph node (right lane) of animals carrying the CD4^off allele alone (left lane) or in the presence of the ox40-Cre allele (center and right lanes). The data are representative of mice from two independent experiments.

evident in Southern analysis of DNA from lymph node cells except in cells that had been activated *in vitro* with PMA/ionomycin (Figure 23B). The CD4⁻ CD8⁻ population constituted a relatively small percentage of the total lymph node prior to *in vitro* activation, and therefore was not readily detected by this assay. The genomic and flow cytometric analysis, in conjunction with the protein expression pattern shown in Figure 22B, are consistent with the CD4⁻ CD8⁻ cells having arisen from cells which underwent some degree of activation.

Elevated levels of memory-phenotype cells but no alteration in apoptotic levels in CD4⁻CD8⁻ cells in OX40-Cre mice.

To better characterize the phenotype of the coreceptor-negative cells in mice carrying both the CD4^{off} allele and the *ox40-Cre* allele, we performed flow cytometric analysis for a variety of markers. First, we found that the cells showed an enrichment for markers normally associated with memory T cells. The proportion of CD4⁻CD8⁻ cells that expressed low levels of CD62L was 7.1% in the Cre-expressing mice, as opposed to 3.1% of CD4⁺ cells in control mice (Figure 24A, left portion). Interestingly, in the population which retained CD4 in the presence of the *ox40-Cre* allele the percentage of CD62L^{lo} cells was consistently slightly lower than that found in Cre-negative mice (Figure 24A, left portion). This finding suggests a selective loss of memory cells from the CD4⁺ population in the Cre⁺ animals. Most likely this loss occurs because cells which become activated lose CD4 expression in the process of becoming memory cells. At the steady-state level, then, those cells are found in the CD4⁻
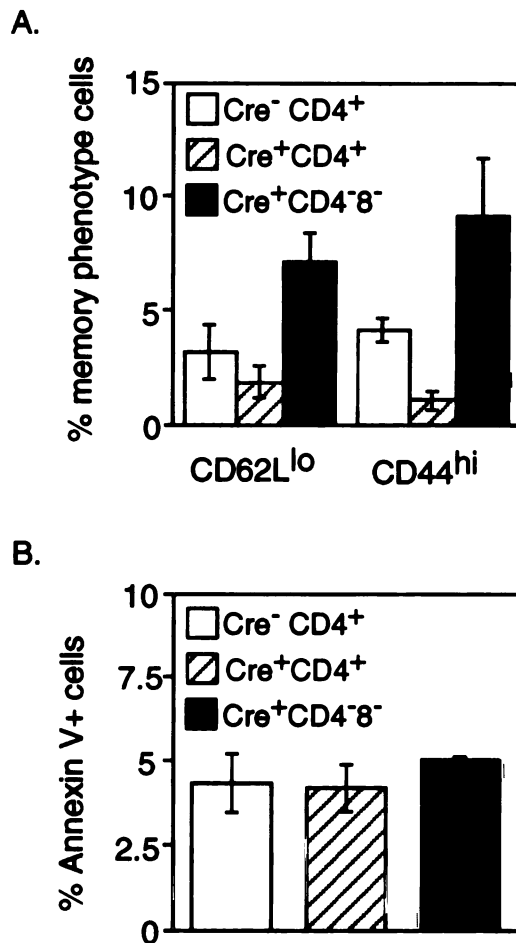
FIG. 24. Enrichment for cells with a memory phenotype but not apoptotic cells in CD4⁻CD8⁻ T cells from $CD4^{off}/^-ox40\text{-}Cre^+$ mice. (A) Lymph nodes of adult Cre-expressing and Cre-negative mice carrying the $CD4^{off}$ allele were analyzed by flow cytometry for expression of TCRβ, CD4, CD8 and the memory markers CD62L and CD44. The figure shows the percentage of either $CD62L^{lo}$ (left portion) or $CD44^{hi}$ (right portion) cells found in the $CD4^+$ compartment of Cre⁻ (open bars) or Cre⁺ (striped bars) mice or in the $TCR\beta^+CD4^-CD8^-$ population (black bars) from Cre-expressing mice. (B) Lymph node cells from the above mice were examined for apoptotic cells by FACS®. The figure shows the percentage of each indicated compartment which stained with the Annexin V reagent. Shown are cells from the $CD4^+$ population of Cre⁻ mice (open bars) and the $CD4^+$ (striped bars) and $CD4^-CD8^-$ (black bars) T cell compartments of Cre-expressing mice.

CD8⁻ population preferentially. Similar results were found when CD44 expression was used as a measure of memory phenotype: 9.1% of CD4⁻CD8⁻ T cells showed high levels of CD44, as opposed to 4.1% of CD4⁺ cells from Cre⁻ animals and only 1.0% of CD4⁺ cells from Cre-expressing animals (Figure 24A, right portion). This elevation in memory-phenotype cells is consistent with the findings from CD4-deficient mice. An enrichment for memory cells in the population lacking CD4 suggests a selective disadvantage for naïve cells in comparison to memory cells. These data support the hypothesis that memory cells are less dependent on TCR-mediated survival signals, and therefore less dependent on CD4, than their naïve counterparts.

We further characterized the coreceptor-negative population by assessing the level of apoptosis in the population using the Annexin V reagent. Strikingly, unlike bulk CD4 lineage cells from CD4-deficient mice, the CD4⁻CD8⁻ cells show little or no elevation in the percentage of cells that stain with Annexin V (Figure 24B). Only 5.0% of coreceptor-negative cells stain with Annexin V, as compared to 4.2% of CD4⁺ cells from Cre⁻ mice or 4.3% of remaining CD4⁺ cells in Cre-expressing mice. The sheer number of coreceptor-negative cells found in these mice may indicate better survival of such cells than CD4⁻CD8⁻ cells that have not been previously activated. We consistently found somewhat higher levels of CD4⁻CD8⁻ cells in the context of the *ox40-cre* allele than in CD4⁻/⁻ mice (Killeen et al., 1993; Rahemtulla et al., 1991) (21.8% in CD4^off/⁻ OX40-Cre mice versus 7.7% in CD4⁻/⁻ mice, see Figure 16B for comparison). The cellularity of the CD4⁻CD8⁻compartment in OX40-Cre mice suggests that the such cells may be less compromised than helper

lineage cells in CD4$^{-/-}$ mice. The fact that these previously-activated cells are so abundant and are not especially apoptotic in the absence of CD4 expression is again consistent with the hypothesis that once a cell has been activated, it is freed from the selection pressure imposed on naïve cells. Such previously activated cells are therefore less dependent on coreceptor than naïve cells, and as such do not acquire a propensity to apoptose upon loss of surface CD4.

Discussion

Here we have generated a conditional allele of CD4 and used that allele in combination with multiple systems for Cre expression to demonstrate a specific role for CD4 in T helper cell homeostasis. Through the use of an inducible Cre, we have shown that cells that lose CD4 extrathymically decline rapidly when transferred to lymphopenic hosts. Additionally, we have generated two systems for stage-specific expression of Cre. Using these systems we have shown that CD4 has a differential effect on the survival of naive and memory cells, such that previously activated cells that lose CD4 do not become apoptotic. Taken together, these data reveal that CD4 participates centrally in the generation of survival signals for T helper cells.

Inducible Loss of CD4: the Mx-Cre System

When Cre was expressed from the inducible *Mx-cre* allele, we observed a rapid decline in the percentage of CD4⁻CD8⁻ T cells in splenocytes transferred following the peak of induction. These data were consistent with our previous observations in the $CD4^{-/-}$ system, and showed that the survival defect found in helper cells from the CD4-null mice was in fact indicative of a specific role for CD4 in peripheral T cell survival.

This system had significant limitations, such as the pleiotropic effects of the pI-pC as well as the low level of CD4⁻CD8⁻ cells generated. The relatively small percentage of such coreceptor-negative cells even at the peak of induction may in itself be indicative of the importance of CD4 for helper cell persistence. Given the extent of recombination seen at the genomic level in

comparison to the number of CD4$^-$CD8$^-$ cells seen by flow cytometry, it seems quite possible that individual cells drop out of the population rapidly after losing CD4, and that this loss is spread over the course of the induction period. These cells would likely be rapidly replaced by those cells that retain CD4, preventing the detection of large numbers of coreceptor-negative cells at any one time. Despite the limitations of the system, the decay of the CD4$^-$ population seen after the inducible extinction of CD4 was remarkably similar to that observed when cells from CD4$^{-/-}$ mice are transferred to RAG-1$^{-/-}$ or wild-type hosts. These data, then, support the findings from CD4-deficient mice and illuminate an intrinsic function of the CD4 molecule in facilitation of survival signals.

Stage-Specific Cre Expression: the ox40-cre and dlck-hcre Systems

In addition to making use of the Mx-cre system, we have demonstrated that the expression of CD4 can be extinguished at specific stages of development, as in the *dlck-hcre* and *ox40-cre* situations. The findings from these systems provide further evidence for the differential effects of CD4 on naïve versus memory cells. In the case of *dlck-hcre* lines 3875 and 4425 in which CD4 expression is lost during thymic development, perhaps even before positive selection occurs, the population of CD8$^-$ T cells is greatly enriched for memory cells. This augmentation is similar to that found in the helper lineage in CD4$^{-/-}$ mice, implying the preferential survival of memory cells over naïve ones. In contrast, when CD4 is lost upon activation as in the *ox40-cre* setting, loss of CD4 does not cause cells to be detectably prone to apoptosis. These data are also consistent with the findings from CD4$^{-/-}$ mice,

in which the increase in apoptosis in the helper lineage is primarily confined to naïve cells. Taken together, the data from the CD4-deficient mice and the conditional systems complement studies examining the MHC requirements for naïve and memory cell survival (Murali-Krishna et al., 1999; Swain et al., 1999; Tanchot et al., 1997a) by identifying CD4 as a key participant in the delivery of an effective survival signal to the T cell.

Conclusions

The evidence presented here suggests that the use of such conditional expression of CD4 can allow for a fine dissection of the involvement of CD4 at specific stages of development. Given the limitations of the Mx-cre system, additional methods may be necessary to examine the role of CD4 in naïve cells. The *dlck-hcre* transgenic mice may provide some information, provided founders can be identified in which the transgene expression is both low at the $CD4^+CD8^+$ thymocyte stage and high in more mature T cells. An inducible system such as the Mx-cre setting is attractive because it allows for extinction of CD4 expression at specific stages of development and/or activation, allowing for a more detailed analysis. Furthermore, such a system can generate a cohort of cells that undergo recombination simultaneously, enabling kinetic studies to be performed. Conditional expression of Notch-1 has been used to demonstrate the importance of Notch-1 in commitment to the T cell lineage (Radtke et al., 1999); Rajewsky and colleagues used Mx-cre-mediated extinction of the B cell antigen receptor to show the necessity for signaling through that receptor for B cell survival (Lam et al., 1997). However, the Mx-cre system is subject to the multiple limitations discussed above.

Other constraints apply to the stage-specific Cre alleles we have described. For example, the use of a transgenic approach such as the *dlck-hcre* system results in some disregulation of the expression of Cre, probably due to the effects of the integration site and transgene copy number. Other systems, such as those driven by cytokines such as Interleukin-4 or IFNγ, or the Granzyme-B-driven Cre used by Baltimore and colleagues to mark memory cells (Jacob and Baltimore, 1999), might allow the functions of CD4 in memory cells to be examined in more detail.

In conclusion, we demonstrate here that cells lacking CD4 exhibit marked defects in homeostasis, and that these deficiencies are much more pronounced in the naive helper cell compartment than in cells that have been previously activated. These data illuminate the central involvement of CD4 in T cell-APC interactions that promote the survival of naïve T cells. The conditional allele of CD4 presented here can be used in many other settings to examine the functions of CD4 in immune responses and T cell homeostasis.

CONCLUSION

In summary, we have demonstrated that CD4 plays a critical role in the delivery of a survival signal to helper lineage T cells, particularly those of the naïve compartment. Using several genetic systems, we have shown that naïve helper T cells lacking CD4 expression fail to persist in adoptive transfers into wild type and lymphopenic hosts and are more apoptotic than wild-type counterparts. We have further shown that memory cells are much less dependent on CD4 than are naive cells, surviving preferentially in the absence of coreceptor. Additionally, we have described the generation and use of an allele that allows for conditional CD4 expression. We have demonstrated that extrathymic loss of CD4 results in a failure to persist in adoptive transfers; however, loss of CD4 from previously activated cells does not result in any detectable survival defect. This system is particularly versatile in that it allows for temporal or cell-type-specific control over CD4 expression, depending on the Cre allele used to induce recombination at the CD4 locus.

Mechanism of Apoptosis

We investigated two potential factors that might influence apoptosis of helper lineage cells lacking CD4 and found that cell death did not appear to be dependent on either one. First, we examined the possibility that CD4-deficient cells might die as a result of Fas signaling. We (unpublished observations) and others (Maroto et al., 1999) have noted an upregulation of Fas and FasL on the surface of helper lineage cells that lack CD4 expression. However, since the lineage is not rescued in the absence of Fas, this change

appears more likely to be an aftereffect rather than a primary cause of apoptosis. Additionally, we tested the hypothesis that increased Bcl-2 activity might rescue CD4-deficient cells. Overexpression of Bcl-2 from a transgene did not increase the cellularity or change the phenotype of helper lineage cells in the absence of CD4. This latter finding is perhaps more surprising than the former. While Fas is centrally involved in apoptosis of activated T cells (Peter and Krammer, 1998; Singer et al., 1994), the Bcl-2 family of proteins seems to be more important in the regulation of cell death in cases where activated or partially stimulated cells are deprived of cytokines or other growth stimuli (Chao and Korsmeyer, 1998; Cory, 1995; Van Parijs and Abbas, 1998). Since the lack of surface CD4 would be expected to result in insufficient survival signals rather than hyperactivated T cells, it follows that pathways that regulate cell death in other situations of insufficient stimuli might be operational in this context. However, it appears that at least the Bcl-2 molecule itself is not a critical component in this process, and more investigation will be necessary to determine the mediators of apoptosis of helper cells in the absence of CD4 expression.

The Survival Signal

The exact nature of the survival signal generated in the T cell also remains to be determined, but our data taken together with others' (Brocker, 1997; Takeda et al., 1996; Tanchot et al., 1997a) imply that the signal is highly likely to be TCR-derived and involve recognition of a self-MHC-peptide complex by both the TCR and the coreceptor. However, the exact nature of the signal remains unknown. The TCR has been demonstrated to transduce

signals which result in widely varying outcomes for the T cell (reviewed in Madrenas and Germain, 1996). The differences in downstream consequences must be due to differences in the quality or quantity of signaling; in support of this hypothesis, differential phosphorylation of the CD3 ζ chain has been shown as a result of interaction of the TCR with agonist or antagonist peptides (Rabinowitz et al., 1996). It seems likely that the frequent interaction with MHC/peptide that is required for naïve T cell survival results in a phosphorylation pattern that acts to prevent apoptosis. Elucidation of any possible differential pattern of phosphorylation at the TCR-CD3 complex in the presence or absence of appropriate survival signals awaits further investigation.

The involvement of the coreceptor in the delivery of a survival signal may shed some light on the nature of the transduction molecules involved. The association of CD4 with p56^{lck} (Barber et al., 1989; Rudd et al., 1988; Veillette et al., 1988) may quantitatively or qualitatively affect the signal transduced in response to ligand. Although some data suggest that CD4 may actually be recruited to the TCR-CD3 complex via interaction with p56^{lck} (Xu and Littman, 1993), once the coreceptor becomes part of the complex CD4 may serve to hold the kinase in close juxtaposition with the signaling complex (Killeen and Littman, 1995). In this way p56^{lck} may participate in signal transduction to a greater extent than would otherwise occur. In the absence of CD4 expression, less p56^{lck} may be associated with the signaling complex; decreased survival in this context may implicate p56^{lck} specifically in the delivery of an efficient survival signal. Alternatively, the critical function of

CD4 in homeostasis may rest primarily in the ability to physically stabilize the interaction between the T cell and the APC, although the low affinity of CD4 for MHC makes this possibility unlikely (Margulies et al., 1996; Ward and Qadri, 1997).

Implications and Future Directions

While the biological effect of the influence of MHC and peptide on the persistence of T cells, and in particular naïve cells, is not completely understood, it seems likely that this dependence on MHC/peptide is in place to continue to shape the repertoire of circulating T cells. The peripheral MHC can have a significant impact on the TCR repertoire, as demonstrated in systems in which the MHC of the thymus and periphery were mismatched (Zinkernagel and Althage, 1999). This influence is likely to act through a very similar, if not identical, mechanism as the survival phenomena documented above, as the repertoire in these situations is thought to be shaped by the preferential expansion of cells that recognize MHC once they have emerged from the thymus (Zinkernagel and Althage, 1999). Elucidating the repertoire differences between recent thymic emigrants, which have not yet been subjected to peripheral selection, and resident naïve cells will require further investigation.

In addition to its contribution to T helper cell homeostasis, CD4 improves the efficiency of T cell reactivity to antigenic peptides (Hampl et al., 1997; Madrenas et al., 1997). More recently, an important role for CD4 has been proposed in the generation of Th2-type responses (Fowell et al., 1997).

When inoculated with Nippostrongylus brasiliensis, CD4$^{-/-}$ mice did not develop effective Th2-mediated immunity to the worms, and helper cells lacking CD4 could not be induced to produce Interleukin-4 *in vitro*, nor could CD4$^+$ cells primed on APCs bearing a mutant Class II molecule lacking the coreceptor binding site. The pathways leading to development of Th1 versus Th2-type responses remain incompletely defined but are of great interest in the design of effective immunomodulatory therapeutics (Abbas et al., 1996). The conditional genetic system described above could be used to examine the requirement for CD4 in greater detail, through the use of other systems for the expression of Cre. Stage-specific Cre alleles, such as the memory-cell allele used by Baltimore and colleagues (Jacob and Baltimore, 1999) may allow the further dissection of the nature and timing of signals which skew the immune response towards a particular path.

In this work we have revealed the central role for CD4 in the complex process of peripheral T cell homeostasis. In clinical settings in which lymphopenia occurs, such as in patients being treated for cancer (Mackall et al., 1995) or autoimmunity (Lindsey et al., 1994) the rebound of CD4$^+$ T cells appears relatively rapid, but consists largely of memory cells responding to antigen and so results in a severely constricted repertoire (Mackall et al., 1996; Mackall and Gress, 1997). The rate of naïve cell regeneration is significantly slower. Similarly, in patients with HIV-1 who are treated with highly active anti-retroviral treatment, the initial increase in the CD4$^+$ compartment primarily consists of memory cells (Autran et al., 1997; Lafeuillade et al., 1997; Pakker et al., 1998; Zhang et al., 1998b) and only later are significant

contributions made by newly generated naïve cells (Zhang et al., 1998b). A better understanding of the pathways for controlling the survival and generation of naïve T cells will provide the basis for new research into methods for modulating the balance between T cell generation, survival and death and allow for the design of new therapies for patients with T cell deficiencies.

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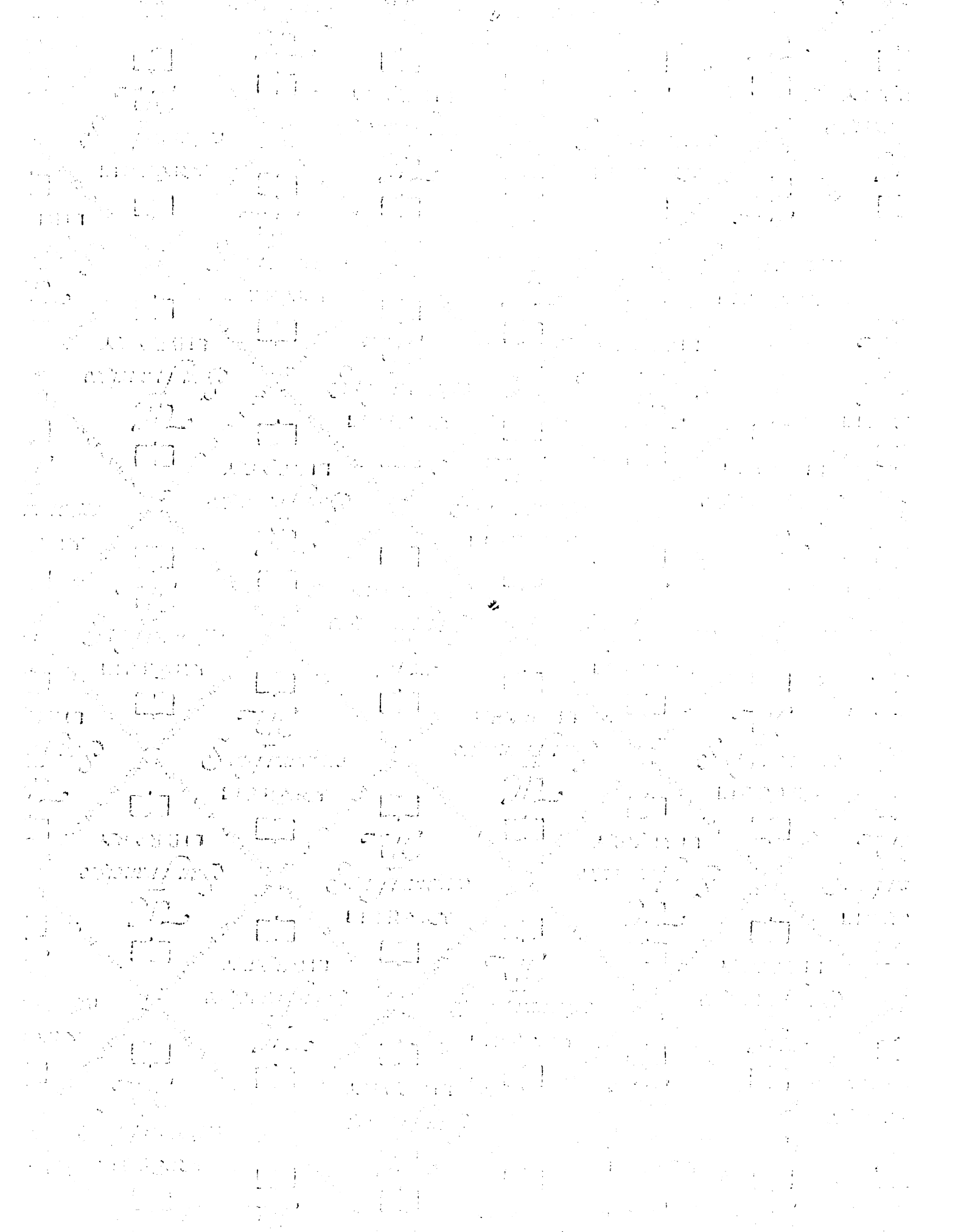
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