Lack of Coreceptor Allows Survival of Chronically Stimulated Double-negative α/β T Cells: Implications for Autoimmunity

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Abstract

Lymphoproliferative diseases are characterized by massive accumulation of $CD4^{-}CD8^{-}B220^{+}$ (double-negative [DN]) T cells in peripheral organs. Although evidence indicates these cells are derived from mature autoreactive α/β T cells, the significance of coreceptor downregulation is not known. In this study, we examined the role CD4 coreceptor plays in the survival of repeatedly stimulated T cells. $CD4^{+/+}$ and $CD4^{-/-}$ T cells from AND T cell receptor (TCR) transgenic mice exhibited similar phenotypes after antigenic stimulation, but the $CD4^{-/-}$ T cells survived in much larger numbers than the $CD4^{+/+}$ cells upon primary and secondary major histocompatibility complex (MHC)/peptide stimulation. Enhanced survival of $CD4^{-/-}$ T cells was due to decreased apoptosis rather than enhanced proliferation. Similarly, circumvention of the CD4/MHC interaction by using a surrogate TCR ligand that does not engage CD4 led to significant enhancement of $CD4^{+/+}$ cells than when stimulated with MHC/peptide. Finally, we generated DN $B220^{+}$ T cells using an in vitro model system and showed they were more tolerant to chronic stimulation than $CD4^{+/+}$ cells. Together, these results indicate that coreceptor engagement controls expansion of normal T cells. In the absence of coreceptor, T cells survive chronic stimulation and express B220 as seen in autoimmune lymphoproliferative diseases.

Key words: CD4 coreceptor • double-negative T cell • lymphoproliferation • B220 • apoptosis

Introduction

Immune system homeostasis is tightly regulated by activation-induced cell death (AICD),[1] which serves as a negative feedback mechanism controlling proliferation of activated T cells (1, 2). In $CD4^{+/+}$ T cells, AICD results from coexpression of the death receptor, Fas, and its ligand, FasL (3–5). Mouse strains carrying a defect in the expression of either Fas (lpr) or FasL (gld) suffer from a generalized systemic lymphoproliferative disease and autoantibody production (6–8). Patients with mutations in Fas or caspase 10 have a similar phenotype highlighting the importance of this pathway in T cell homeostasis (9–11). The predominant immunologic abnormality of the lymphoid system in these diseases is progressive accumulation of a peculiar subset of double-negative (DN) T cells that lack both CD4 and CD8 coreceptors and express the B cell surface molecule B220 (12). Biochemical and genetic evidence suggests that DN T cells seen in these disorders are derived from mature α/β T cells that have downregulated their coreceptors (12–14). However, the role of coreceptor downregulation in the development of these cells is not known.

CD4 and CD8 coreceptors are well known for their roles in the development and activation of helper and cytotoxic T cells, respectively (15–17). Recent studies suggest that engagement of coreceptors may also prime T cells for apop-

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[1]*Abbreviations used in this paper:* AICD, activation-induced cell death; CFSE, 5- and 6-carboxyfluorescein diacetate succinimidyl ester; DN, double-negative; MCC, moth cytochrome c; tg, transgenic.

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tosis possibly by transducing a negative signal (18, 19). This signal seems to be mediated by FasL, as CD4 cross-linking on T cells or CD4-expressing monocytes leads to FasL expression in a TCR-independent fashion (20–22). Taken together, these observations have led to the idea that if CD4 engagement plays a specific role in priming activated T cells for AICD, absence of such signals during TCR stimulation may render T cells less susceptible to apoptosis. Investigating this possibility can shed further light on the physiology of CD4 coreceptor and the role of coreceptor downregulation on the survival of autoreactive T cells.

We therefore investigated the consequences of coreceptor engagement on the survival of activated T cells. This was analyzed using AND TCR transgenic (tg) mice bred onto either a wild-type $CD4^{+/+}$ or $CD4^{-/-}$ background (23–25) and a set of TCR ligands, MHC/peptide and anti-CD3, that differentially engage CD4. We found that TCR stimulation in the absence of coreceptor led to significantly enhanced survival of activated T cells. $CD4^{-/-}$ AND T cells survived in much large numbers regardless of the TCR ligand used. Enhanced survival of the $CD4^{-/-}$ cells was found to be dependent on reduced apoptosis rather than enhanced proliferation. On the other hand, survival of activated $CD4^{+/+}$ T cells was dependent on the TCR ligand used. When stimulated with MHC/peptide, the natural ligand for CD4, significant apoptosis occurred, whereas anti-CD3 led to significant survival of T cells. Finally, we generated DN T cells from initially $CD4^{+/+}$ T cell cultures, using a novel in vitro model system, and found that DN T cells survived chronic stimulation whereas the $CD4^{+/+}$ T cells died out within 4–5 wk of stimulation. Interestingly, the DN T cells also upregulated B220, which is phenotypically similar to the $CD4^{-}$ $CD8^{-}B220^{+}$ T cells seen in lymphoproliferative diseases. These results indicate that coreceptor engagement limits expansion of normal T cells by enhancing apoptosis, whereas lack of coreceptor allows T cell to tolerate chronic stimulation.

Materials and Methods

Mice. AND Vα11/Vβ3 α/β TCR tg mice specific for moth cytochrome c (MCC) in the context of $I\text{-}E^k$ (23) backcrossed to B10. BR mice ($H\text{-}2^k$) for 6–8 generations (24) were a gift of Dr. S. Swain (Trudeau Institute, Saranac, NY). $CD4^{-/-}$ AND TCR tg mice on B10.BR background were produced using a breeding scheme and mice provided by Dr. K. Bottomly (Yale University, New Haven, CT) (25). All mice were bred and maintained at the Cancer Center of the Johns Hopkins School of Medicine.

Stimulatory Beads. $I\text{-}E^k$/MCC/anti-CD28 beads were prepared using soluble monomeric $I\text{-}E^k$ covalently linked to the MCC 91–103 peptide, a gift of Drs. J. Kappler and P. Marrack (National Jewish Medical and Research Center, Denver, CO). Soluble $I\text{-}E^k$/MCC was purified from culture supernatant of infected hi five insect cells (Invitrogen) as described previously (26). $I\text{-}E^k$/MCC and anti-CD28 mAb were mixed at 1:1 ratio and coupled to tosyl-activated 450 Dynabeads (27). Anti-CD3 (145-2C11)/anti-CD28 (37.51) beads were prepared using the same protocol.

Flow Cytometry. All mAbs were purchased from BD PharMingen. Cells labeled with fluorescent Abs were analyzed using FACScan™ flow cytometer (Becton Dickinson) and CELLQuest™ software (Becton Dickinson).

Cell Preparations. T cells were isolated from spleens of $CD4^{+/+}$ or $CD4^{-/-}$ AND × B10.BR mice by negative selection using Dynabeads (Dynal). In brief, splenocytes were incubated with a cocktail of biotin-conjugated rat mAbs specific for murine CD8, $I\text{-}E^k$, CD16/CD32, and B220 surface molecules. Streptavidin-conjugated beads from Dynal were used to capture and remove mAb-coated cells using the manufacturer's instruc-

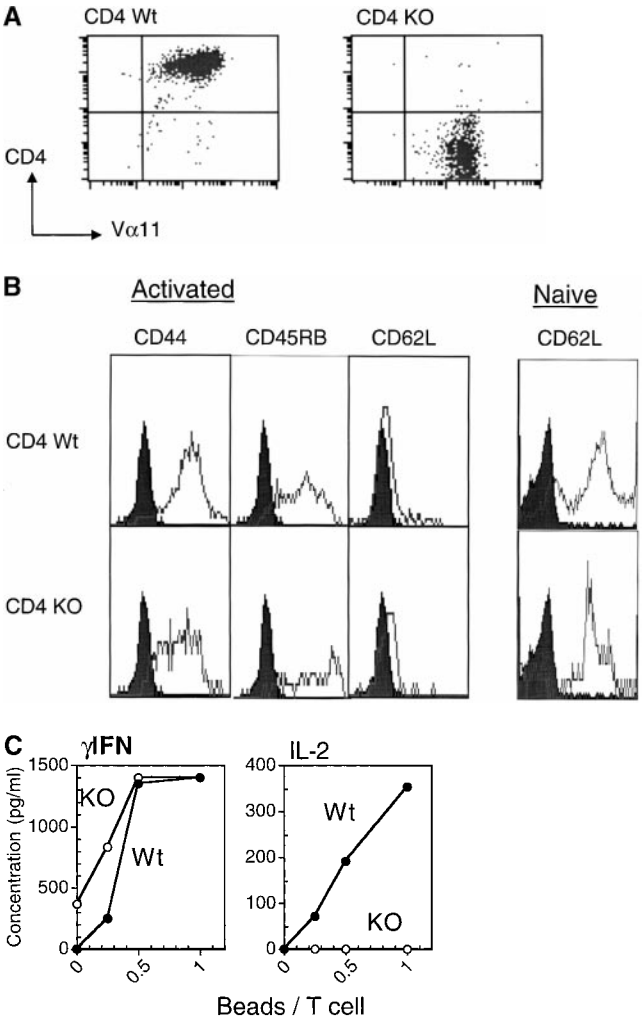

Figure 1. Phenotype and cytokine production by activated $CD4^{-/-}$ and $CD4^{+/+}$ AND T cells. (A) Phenotype of purified $CD4^{+/+}$ and $CD4^{-/-}$ AND T cells. AND T cells on $CD4^{+/+}$ or $CD4^{-/-}$ background were isolated using negative selection as described in Materials and Methods and then analyzed for Vα11 (AND TCR transgene bears Vα11 and Vβ3) and CD4 expression. Wt, wild-type; KO, knockout. (B) Activation marker expression by stimulated and naive AND T cells. $CD4^{+/+}$ and $CD4^{-/-}$ AND T cells were stimulated with MHC/peptide beads for 6 d in 24-well plates and then assayed for CD44, CD45RB, and CD62L expression. Right panel shows CD62L expression on unstimulated $CD4^{+/+}$ and $CD4^{-/-}$ cells. Shaded histograms represent unstained controls. (C) Cytokines production by activated $CD4^{-/-}$ and $CD4^{+/+}$ T cells. T cells were collected from day 6 cultures, washed, and stimulated overnight with various doses of MHC/peptide beads and culture supernatants were assayed for IFN-γ and IL-2 by ELISA.

tion. Purity and specificity of isolated T cells were determined by flow cytometry using mAbs specific for murine Vα11, CD8, and CD4 molecules. Usually ~85–90% of isolated cells were of the desired phenotype. Stimulation assays were conducted as described below.

Stimulation Assay. Typically, 10^5 tg T cells were stimulated with an equivalent number of beads in 96-well round-bottomed microtiter plates or in the case of long-term assays with 2×10^6 beads in a 24-well microtiter plate in the presence of irradiated feeders. Viability was followed for the indicated time period. Recombinant IL-2 (20 U/ml) was added on day 0 and thereafter every 4–5 d in experiments involving $CD4^{-/-}$ cells to compensate for the inability of the $CD4^{-/-}$ cells to produce IL-2. Media was changed and cells were split as required. Fresh beads were added as indicated in Results and the figure legends. Viable cell counts were performed on indicated days using standard trypan blue exclusion.

Cytokine Analysis. Cells were removed from stimulation cultures, washed, and incubated with MHC/peptide beads for 24 h. Supernatants were collected and assayed for indicated cytokines using captured ELISA according to the manfacturer's instructions (Endogen).

5- and 6-Carboxyfluorescein Diacetate Succinimidyl Ester Analysis. Purified T cells were labeled with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS at a final concentration of 2 μm for 3 min at room temperature. CFSE was purchased from Molecular Probes. Labeled cells were stimulated with either MHC/peptide/CD28 or anti-CD3/CD28 beads as described in the figure legends. Cells were analyzed on indicated days by flow cytometry. In some experiments, cells were also stained with CyChrome-conjugated anti-CD4 (GK1.5) mAb to visualize proliferation rate among $CD4^{+/+}$ and DN T cell subsets.

Results

Lack of Coreceptor Enhances Survival of Repeatedly Activated AND T Cells. The tight link between coreceptor downregulation and lymphoproliferative disorders suggests that coreceptor downregulation contributes to the ability of autoreactive T cells to avoid peripheral tolerance mechanisms. To examine this idea, we analyzed the consequences of antigenic stimulation of T cells from $CD4^{+/+}$ and $CD4^{-/-}$ AND tg mice. Splenocytes from $CD4^{-/-}$ AND tg mice contained significant numbers of $V\alpha11^+$ $CD4^-CD8^-$ T cells as described previously (25). Using negative selection, we isolated $V\alpha11^+CD4^-CD8^-$ T cells from spleens of $CD4^{-/-}$ AND/B10.BR mice or $V\alpha11^+CD4^{+/+}$ T cells from spleens of $CD4^{+/+}$ AND/B10.BR mice, respectively (Fig. 1 A). Isolated T cells were activated with beads coated with IE^k/MCC and anti-CD28 (hereafter referred to as MHC/peptide) in the presence of exogenous IL-2. $CD4^{+/+}$ and $CD4^{-/-}$ cells activated with MHC/peptide exhibited similar cell surface markers having a profile typical of activated T cells, with upregulated

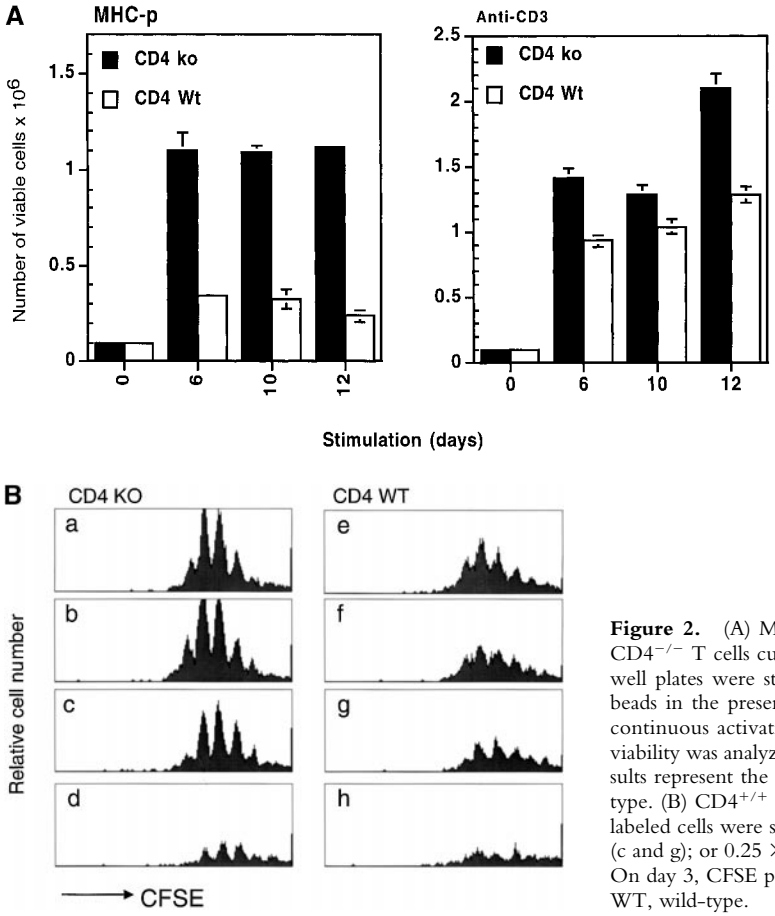

Figure 2. (A) Marked expansion of activated $CD4^{-/-}$ T cells. 10^5 $CD4^{+/+}$ or $CD4^{-/-}$ T cells cultured in triplicate with irradiated syngeneic splenocytes in 24-well plates were stimulated with either MHC/peptide (left) or anti-CD3 (right) beads in the presence of IL-2. Cultures were restimulated on day 7 to maintain continuous activation. Media was changed and cells were split as required. Cell viability was analyzed daily and cell counts were performed on indicated days. Results represent the mean of three determinants ± SEM. ko, knockout; Wt, wild-type. (B) $CD4^{+/+}$ and $CD4^{-/-}$ AND cells proliferate at comparable rates. CFSE-labeled cells were stimulated with: 2×10^5 (a and e); 1×10^5 (b and f); 0.5×10^5 (c and g); or 0.25×10^5 (d and h) of MHC/peptide beads in the presence of IL-2. On day 3, CFSE profiles of activated cells were determined using flow cytometry. WT, wild-type.

CD44, intermediate level of CD45RB, and downregulated CD62L (Fig. 1 B). These cells were clearly activated as indicated by low levels of CD62L expression, which were high on naive T cells (Fig. 1 B). In addition, both $CD4^{+/+}$ and $CD4^{-/-}$ cells produced large amounts of IFN-γ; however, only $CD4^{+/+}$ cells produced IL-2 (Fig. 1 C). Although $CD4^{-/-}$ T cells did not produce IL-2 at any dose tested, they did remain responsive to IL-2 and proliferated at a comparable level to that of $CD4^{+/+}$ cells in the presence of exogenous IL-2, as described previously (25). Thus, $CD4^{-/-}$ cells were activated as indicated by cellular activation profiles, IFN-γ production, and response to exogenous IL-2.

We examined the impact of CD4 engagement by MHC/peptide ligand on the survival and expansion of activated T cells. Viability of $CD4^{+/+}$ and $CD4^{-/-}$ AND T cells stimulated with MHC/peptide beads in the presence of exogenous IL-2 was analyzed during primary and secondary stimulation. The number of viable $CD4^{-/-}$ T cells substantially increased upon stimulation and remained high upon restimulation whereas the number of viable $CD4^{+/+}$ T cells showed a more limited increase followed by a decline after restimulation (Fig. 2 A). There was ~10-fold increase in the viability of $CD4^{-/-}$ cells relative to ~3-fold increase in $CD4^{+/+}$ cells after 6 d of stimulation. Cell numbers increased from 10^5 to 10^6 for $CD4^{-/-}$ cells but only from 1×10^5 to a maximum of 3×10^5 for $CD4^{+/+}$ cells.

To further analyze the preferential accumulation of $CD4^{-/-}$ cells, we compared proliferation rates of MHC/peptide-stimulated $CD4^{+/+}$ and $CD4^{-/-}$ cells using a CFSE-based proliferation assay. Both $CD4^{+/+}$ and $CD4^{-/-}$ cells proliferated at comparable rates to all the MHC/peptide doses tested (Fig. 2 B). Thus, although $CD4^{-/-}$ cells were more tolerant to repeated stimulation than their $CD4^{+/+}$ counterparts, this was not due to differences in the rate of proliferation.

In contrast to the limited expansion of $CD4^{+/+}$ T cells stimulated with MHC/peptide, substantial expansion of

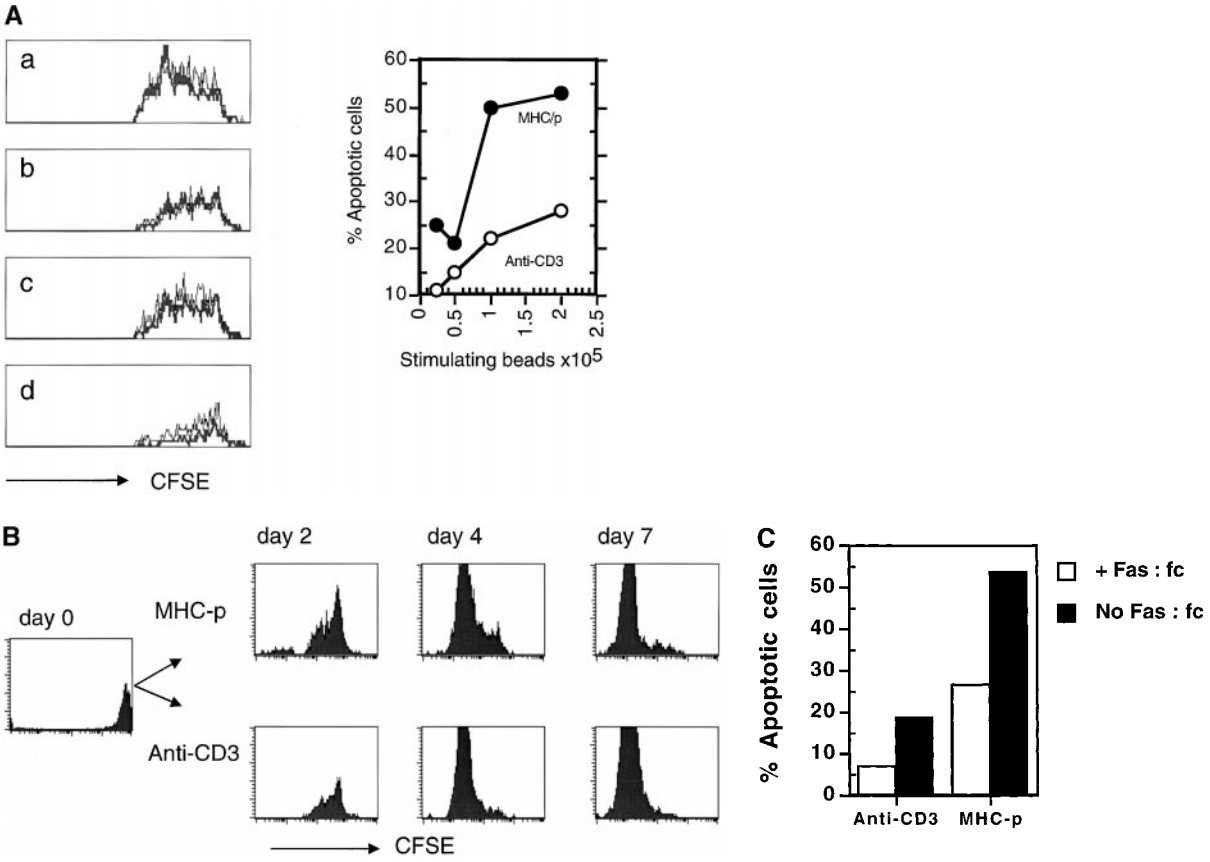

Figure 3. Normal proliferation but reduced apoptosis of $CD4^{+/+}$ T cells stimulated in the absence of CD4 engagement. (A) MHC/peptide and anti-CD3 beads induced similar proliferation but different apoptotic rates. Purified $CD4^{+/+}$ AND T cells were labeled with CFSE and stimulated with: (a) 2×10^5; (b) 1×10^5; (c) 0.5×10^5; or (d) 0.25×10^5 of either MHC/peptide or anti-CD3 beads. Cells were collected on day 3 and CFSE fluorescence of cells stimulated with MHC/peptide (thick line) or anti-CD3 (thin line) was analyzed (left panel). $CD4^{+/+}$ T cells were stimulated with various doses of MHC/peptide or anti-CD3 beads as described above for 6 d and then percentages of apoptotic cells in each culture was determined using annexin V-FITC (right panel). (B) Kinetic analysis of proliferation rates of $CD4^{+/+}$ cells in response to MHC/peptide or anti-CD3 stimulation. 10^5 AND T cells were labeled with CFSE and stimulated with an equal number of either MHC/peptide (top) or anti-CD3 (bottom) beads. Cells were collected and assayed for CFSE fluorescence on indicated days. (C) Soluble Fas:Fc significantly inhibited apoptosis. $CD4^{+/+}$ cells were stimulated with an equivalent number of either MHC/peptide or anti-CD3 beads in the presence or absence of 5 μg/ml recombinant human Fas:Fc and 1.25 μg/ml enhancer protein (Alexis). 6 d later, apoptosis was analyzed using annexin V assay.

$CD4^{+/+}$ T cells occurred when stimulated with anti-CD3/anti-CD28 beads (hereafter referred to as anti-CD3; Fig. 2 A). As this treatment leads exclusively to TCR engagement and circumvents the CD4/MHC interaction, it also implicates CD4 in regulating survival of activated T cells.

Excluding CD4 during TCR Stimulation Reduces AICD of $CD4^{+/+}$ T Cells. Increased expansion of $CD4^{+/+}$ T cells stimulated with anti-CD3 versus MHC/peptide (Fig. 2 A) could be due to either reduced apoptosis or enhanced proliferation. To differentiate between these two possibilities, we compared proliferation and apoptosis of $CD4^{+/+}$ cells stimulated with various doses of MHC/peptide or anti-CD3. $CD4^{+/+}$ T cells stimulated with varying doses of either anti-CD3 or MHC/peptide beads proliferated at comparable rates (Fig. 3 A). Similar results were obtained in a kinetics study tracking proliferation rates of activated cells over a 7-d period (Fig. 3 B). However, a significant difference between stimulation with MHC/peptide and anti-CD3 was seen when apoptosis was analyzed. Stimulation with anti-CD3 induced two- to threefold less apoptosis than stimulation with MHC/peptide (Fig. 3 A). Apoptosis of the activated cells was sensitive to the addition of soluble Fas:Fc (Fig. 3 C), indicating this was Fas-dependent apoptosis. Thus, exclusion of CD4/MHC interaction using a surrogate TCR ligand, anti-CD3, that does not engage CD4, led to significant survival of activated T cells due to limited apoptosis. On the other hand, stimulation of the $CD4^{+/+}$ cells with MHC/peptide complex, the natural ligand for CD4, led to similar proliferation rates but a more limited T cell expansion due to increased apoptosis.

As T cell expansion is regulated mainly by Fas-mediated death (3, 4, 5; Fig. 3 C), we determined whether expression of Fas and FasL on AND T cells was affected by the absence of CD4-dependent signals. Activated $CD4^{+/+}$ T cells expressed Fas and significant amounts of FasL (Fig. 4). On the other hand, $CD4^{-/-}$ T cells showed significant Fas expression but only minimal amounts of FasL expression.

Suboptimal Apoptosis of Activated $CD4^{+/+}$ T Cells Leads to Appearance and Accumulation of DN T Cells. The accumulation of DN T cells is seen most prominently in autoimmune diseases characterized by defects in Fas-mediated AICD (28) but noticeably absent in other autoimmune diseases. This suggests a link between impaired AICD and development of DN T cells. The differential ability of MHC/peptide and anti-CD3 to induce apoptosis allowed us the opportunity to study the impact of reduced apoptosis of activated $CD4^{+/+}$ T cells on the generation of DN T cells. We stimulated highly purified $CD4^{+/+}$ T cells for 6 d with either MHC/peptide or anti-CD3 beads and determined the percentages of CD4-negative and apoptotic T cells in

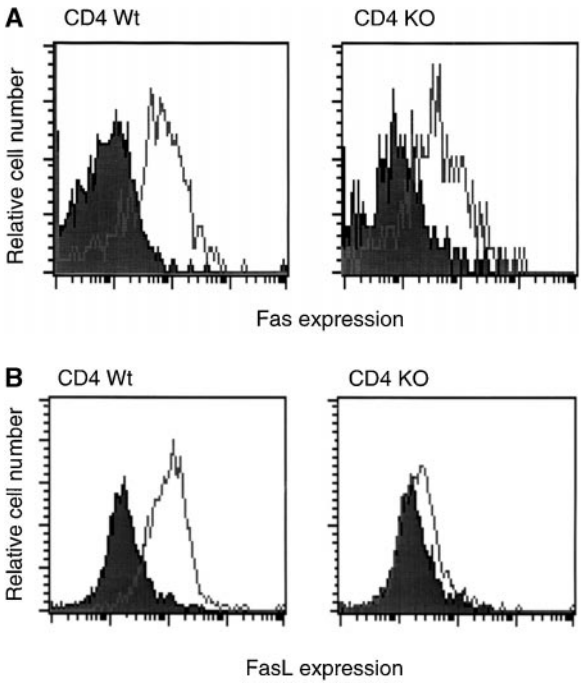

Figure 4. $CD4^{-/-}$ T cells did not upregulate FasL. T cells were activated with MHC/peptide beads as described in the legend to Fig. 1 B for 6 d and then analyzed for (A) Fas and (B) FasL expression. Fas expression was detected using biotinylated anti-Fas Ab followed by streptavidin-PE (BD PharMingen). FasL expression was detected using hamster anti–mouse FasL Ab (MFL3) followed by biotin-conjugated anti–hamster IgG and streptavidin-PE (BD PharMingen). Shaded histograms represent control cells stained with secondary Ab and streptavidin-PE. Wt, wild-type; KO, knockout.

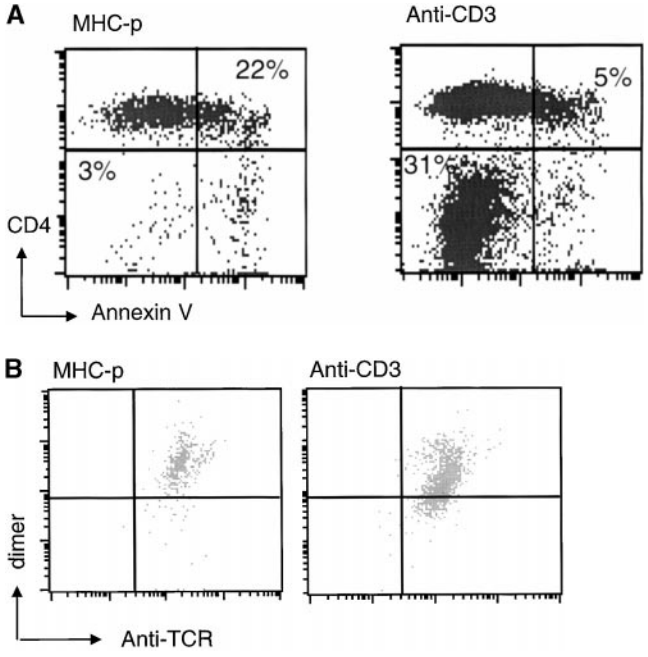

Figure 5. Reduced apoptosis of activated $CD4^{+/+}$ T cells leads to generation of DN T cells. (A) Purified $CD4^{+/+}$ T cells were stimulated with MHC/peptide (left) or anti-CD3 (right) beads for 6 d and then stained and analyzed by flow cytometry. We gated on TCR^+ cells and determined the percentage of $CD4^+$ and apoptotic (annexin V^+) cells in each culture. Cells in the left lower quadrant were DN T cells, as they lack both CD4 and CD8 (data not shown) coreceptors. (B) DN T cells are specific for I-E^k-MCC. Cells were collected from MHC/peptide (left) or anti-CD3 (right) cultures and stained simultaneously with I-E^k-MCC dimer and anti-TCRβ (H597) mAb and analyzed by flow cytometry. All cells including DN T cells in anti-CD3 culture stained positive with I-E^k-MCC indicating they were all expressing AND TCR transgene.

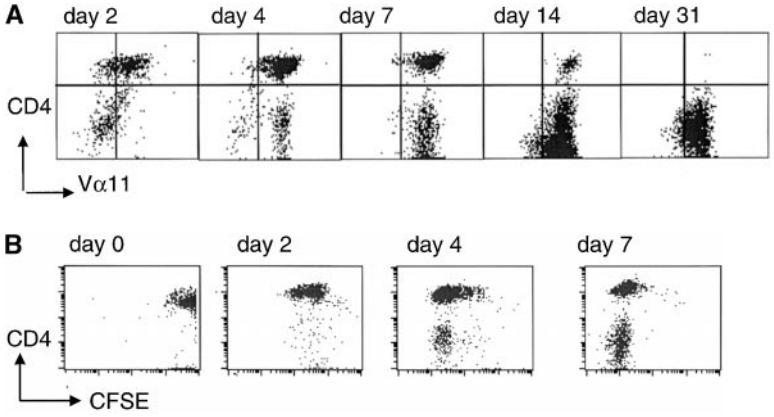

Figure 6. DN but not $CD4^{+/+}$ T cells tolerate chronic stimulation. (A) Kinetic analysis of DN cells accumulation. $CD4^{+/+}$ T cells isolated by negative selection from spleens of AND tg mice were continuously stimulated with anti-CD3 beads. CD4 and Vα11 expression on T cells was determined at various time points. Vα11 expression was used to verify specificity of AND T cells, which express Vα11 and Vβ3. The $CD4^{-/-}$ cells in the lower left quadrant were not antigen specific and eventually disappeared around day 7. (B) DN T cells proliferated at a similar rate as $CD4^{+/+}$ T cells. $CD4^{+/+}$ T cells were purified and labeled with CFSE and then stimulated with anti-CD3 beads. On indicated days, T cells were removed and stained with anti-CD4. The level of CFSE among $CD4^{+/+}$ and DN T cells was visualized by flow cytometry.

each culture. The MHC/peptide stimulation resulted in a significant number (22%) of apoptotic T cells but few (3%) CD4-negative T cells (Fig. 5 A). Conversely, a significant number (31%) of CD4-negative T cells and few (5%) apoptotic cells were detected in the anti-CD3–stimulated culture. Thus, it appears that reducing apoptosis of activated T cells by excluding direct CD4 engagement leads to accumulation of CD4-negative T cells.

The CD4-negative cells were further analyzed for TCR antigenic specificity. All CD4-negative T cells expressed Vα11 and Vβ3 and could be detected with dimeric MHC/peptide (29), confirming that anti-CD3 stimulation did not lead to expansion of nonspecific T cells (Figs. 5 B and 6 A). In addition, these cells did not express CD8 coreceptor, confirming they were $CD4^{-}CD8^{-}$ (DN) T cells (data not shown). The $CD4^{+/+}$ cells are the probable precursors for the DN T cells that accumulated in the chronically stimulated cultures. Were it that these cells originated from the initial low frequency DN T cells in the original population, we would have expected to see them in culture stimulated with either anti-CD3 or MHC/peptide (MHC/peptide–stimulated $CD4^{-/-}$ cells from CD4 knockout mice; Fig. 2). Although we cannot completely rule out that accumulation of DN cells was due to selection of preexisting $CD4^{-/-}$ cells, the fact that DN cells were primarily seen in the anti-CD3–stimulated cultures indicates that they probably arise specifically under conditions that caused suboptimal CD4 recruitment and limited apoptosis. However, both scenarios are consistent with the basic hypothesis that the lack of coreceptor allows accumulation of activated T cells.

Chronic stimulation with anti-CD3 therefore provided a novel and simple technique to generate DN T cells from $CD4^{+/+}$ cells and study their survival. Highly purified $CD4^{+/+}$ T cells isolated from spleens of AND tg mice (Materials and Methods) were stimulated with anti-CD3 beads to generate DN T cells. Beads were added on day 0, 7, 12, 19, and 26 so that the effect of chronic stimulation on the survival of $CD4^{+/+}$ T cells and DN T cells could be studied. The appearance and fate of DN T cells was monitored by analysis of CD4, CD8, and Vα11 expression among surviving cells. $V\alpha11^{+}CD4^{-}CD8^{-}$ T cells began to appear on day 2 and gradually expanded at the expense of the $V\alpha11^{+}CD4^{+}$ T cells (Fig. 6 A). After 5 wk of stimulation, all surviving cells were $V\alpha11^{+}CD4^{-}CD8^{-}$ T cells. The coreceptor reexpression was not seen in cells rested for up to 2 wk. The finding that DN T cells tolerated chronic stimulation is consistent with the ability of $CD4^{-/-}$ knockout T cells to survive repeated stimulation. Thus, the data in all the above experiments indicate that T cells stimulated through the TCR without involvement of the coreceptor are more tolerant to repeated stimulation than when the CD4 coreceptor is engaged.

DN T cells from lpr or gld mice are usually refractory to most TCR stimuli (30–32). Only a small fraction of these cells can proliferate and secrete IL-2 if both TCR and CD28 are engaged (33–35). However, isolating and studying the functional characteristics of these cells has been hampered by their low frequency and technical difficulties. Using the CFSE assay, we analyzed the proliferative potentials of DN T cells generated in vitro to determine if they were functionally competent. CFSE-labeled $CD4^{+/+}$ T cells were stimulated with anti-CD3 to generate and visualize proliferation of both $CD4^{+/+}$ and $CD4^{-/-}$ cells over a period of 7 d. Emerging DN T cells displayed similar intensity of fluorescence as the $CD4^{+/+}$ T cells, as both subsets lost fluorescence at a similar rate (Fig. 6 B). Pure DN T cells were able to grow for up to 10 wk before they became hyporesponsive and died (data not shown). Thus, DN T cells generated in vitro are capable of proliferating in response to stimulation for a considerable period of time.

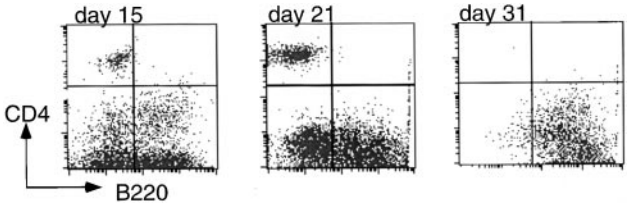

Figure 7. DN T cells generated by chronic stimulation of $CD4^{+/+}$ T cells express B220. $CD4^{+/+}$ T cells were stimulated with anti-CD3 as indicated above. On the indicated days, samples were collected and analyzed for CD4 and B220 expression. Eventually all survivors downregulated CD4 and expressed B220. All cells retained their antigen specificity as determined by Vα11 expression (data not shown).

This feature is critical for studying their function and determining if it has implications for understanding the function of DN T cells seen in vivo.

Expression of B220 on DN T Cells. DN T cells seen in vivo express B220 (36, 37). To determine whether B220 is expressed on DN T cells generated in vitro, we analyzed B220 expression on 15-d cultures that contained both $CD4^{+/+}$ T cells and DN T cells. About 50% of the total DN T cells in these cultures expressed B220 and their percentage gradually increased over time. By the 5th week, all cells that survived downregulated CD4 and acquired B220 (Fig. 7). All $CD4^{-}CD8^{-}B220^{+}$ cells retained their antigen specificity as determined by homogenous expression of Vα11 among these cells (data not shown). Together, these results show, at least in this model system, that chronic in vitro stimulation of T cells that produces suboptimal AICD leads to accumulation of long-lived T cells bearing the two hallmarks (lack of coreceptor and expression of B220) of T cells associated with pathogenic lymphoproliferation.

Discussion

In this study we showed that coreceptor engagement during TCR activation controls T cell survival. This was demonstrated using three different approaches. First, $CD4^{-/-}$ T cells survived in much larger numbers than $CD4^{+/+}$ cells regardless of the TCR ligand used. Second, $CD4^{+/+}$ T cells exhibited limited viability when stimulated with MHC/peptide, the natural ligand for CD4, but enhanced viability when stimulated with a surrogate TCR ligand (anti-CD3) that does not directly engage CD4. Third, $CD4^{+/+}$ T cells completely disappeared during chronic stimulation whereas DN T cells survived. The DN T cells also upregulated B220 and persisted as antigen-specific $CD4^{-}CD8^{-}B220^{+}$ T cells. Together, these data indicate a specific role for the CD4 coreceptor in mediating apoptosis of activated T cells and that a lack of coreceptor allows prolonged survival of activated T cells.

There are at least two mutually nonexclusive mechanisms that may explain the accumulation of activated T cells in the absence of a CD4/MHC interaction. As CD4-dependent signals are required for optimal T cell activation, the absence of CD4 may lead to suboptimal activation. This explanation does not appear likely for several reasons. First, in all instances, T cells appeared activated as indicated by modulation of activation markers and IFN-γ production. Second, MHC/peptide and anti-CD3 induced comparable proliferation but differential apoptosis of $CD4^{+/+}$ T cells (Fig. 3). Finally, DN and $CD4^{+/+}$ cells present in the same culture proliferated at a similar rate but the DN T cells survived chronic stimulation whereas their $CD4^{+/+}$ counterparts died. Therefore, it seems highly unlikely that enhanced survival of T cells in the absence of direct CD4 signals could be explained by inadequate activation. An alternative possibility is that CD4-specific signals are required to prime T cells for apoptosis. In the absence of CD4, these signals are impaired leading to prolonged survival of activated T cells. Our data and previously published reports support this model. CD4 seems particularly important for FasL induction. In our hands, absence of CD4-dependent signals selectively impaired FasL induction (Fig. 4 B) whereas previous reports showed that CD4-dependent signals mediate FasL induction in a TCR-independent fashion on both T cell and nonlymphoid cells. Together, these data support a specific role for CD4 in priming T cells for apoptosis via regulation of FasL (19, 21, 22, 38).

Variations in the interaction of CD4 coreceptor with polymorphic class II MHC complex may affect development of autoimmune diseases. Our results show that if CD4 is not properly engaged, T cells could avoid AICD. By analogy, polymorphism in the CD4 binding site that affects interactions with class II MHC could impair AICD of autoreactive cells, resulting in autoimmune diseases. Although the CD4 binding site is highly conserved (16, 17), allelic MHC polymorphism that effects CD4 binding exists (39). Interestingly, analysis of human MHC class II sequences shows that HLA-DQ isotype, which is integral to several autoimmune diseases, differs from HLA-DR isotype in key residues in the CD4 binding site. Furthermore, two DQ alleles with a single residue difference at position 135 that lies within the CD4 binding site are differentially associated with celiac disease (40, 41). Thus, variations in the CD4 binding ability may represent a previously unrecognized element which contributes to class II MHC association with autoimmune diseases.

Coreceptor downregulation may have a broader role in the pathogenesis of autoimmune diseases. Not only Fas-mediated death, but also TNF-α–mediated AICD has been reported to be impaired in the absence of CD4 (22). In addition, coreceptor downregulation may allow T cells to escape regulatory mechanisms that control the ratio of CD4/CD8 cells, which is very constant in every strain of mouse and regulated by genes other than those involved in regulation of AICD (42). Therefore, it is conceivable that coreceptor downregulation could allow autoreactive CD8 T cells which disproportionately expand in lymphoproliferative diseases to escape such mechanism and persist in vivo as DN cells. Finally, our unpublished data indicate that downregulation of coreceptor correlates with acquisition of novel functions directed towards suppression of ongoing autoreactivity.

In mice with defects in Fas or FasL, most but not all DN $B220^{+}$ T cells are refractory to mitogens and TCR-induced stimulation (34, 35). However, it has been difficult to isolate the responsive subset of DN T cells and study their function in vitro. This together with the unknown specificity of DN cells has hampered progress in understanding the biology and role of DN cells in the pathogenesis of lymphoproliferative disease despite extensive research. The experimental system described in this study provides an alternative source of homogenous functional DN $B220^{+}$ cells of known antigenic specificity (Fig. 6 B, and unpublished data). Using this system to understand the effector function of DN $B220^{+}$ T cells may lead to new insights into the in vivo function of this important subset of T cells.

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