

# Lack of Coreceptor Allows Survival of Chronically Stimulated Double-negative $\alpha/\beta$ T Cells: Implications for Autoimmunity

By Abdel Rahim A. Hamad,\* Ananth Srikrishnan,\* Paria Mirmonsef,\*  
Chris P.M. Broeren,<sup>§</sup> Carl H. June,<sup>||</sup> Drew Pardoll,<sup>‡</sup>  
and Jonathan P. Schneck\*

From the \*Department of Pathology and the <sup>‡</sup>Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; the <sup>§</sup>Department of Immunology, Institute of Infectious Diseases and Immunology, University of Utrecht, 3584 CL Utrecht, The Netherlands; and <sup>||</sup>Stellar Chance Laboratories, University of Pennsylvania, Philadelphia, Pennsylvania 19104

## Abstract

Lymphoproliferative diseases are characterized by massive accumulation of CD4<sup>-</sup>CD8<sup>-</sup>B220<sup>+</sup> (double-negative [DN]) T cells in peripheral organs. Although evidence indicates these cells are derived from mature autoreactive  $\alpha/\beta$  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<sup>+/+</sup> and CD4<sup>-/-</sup> T cells from AND T cell receptor (TCR) transgenic mice exhibited similar phenotypes after antigenic stimulation, but the CD4<sup>-/-</sup> T cells survived in much larger numbers than the CD4<sup>+/+</sup> cells upon primary and secondary major histocompatibility complex (MHC)/peptide stimulation. Enhanced survival of CD4<sup>-/-</sup> 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<sup>+/+</sup> cells than when stimulated with MHC/peptide. Finally, we generated DN B220<sup>+</sup> T cells using an in vitro model system and showed they were more tolerant to chronic stimulation than CD4<sup>+/+</sup> 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),<sup>1</sup> which serves as a negative feedback mechanism controlling proliferation of activated T cells (1, 2). In CD4<sup>+/+</sup> 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 sys-

temic 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  $\alpha/\beta$  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-

C.H. June's present address is Abrameson Family Cancer Research Institute and Dept. of Molecular and Cellular Engineering, University of Pennsylvania, Philadelphia, PA 19104.

Address correspondence to Abdel Rahim Hamad, Dept. of Pathology, Johns Hopkins School of Medicine, 720 Rutland Ave., Ross 664G, Baltimore, MD 21211. Phone: 410-614-0642; Fax: 410-614-3548; E-mail: ahamad@jhmi.edu

<sup>1</sup>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.

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<sup>+/+</sup> or CD4<sup>-/-</sup> 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<sup>-/-</sup> AND T cells survived in much large numbers regardless of the TCR ligand used. Enhanced survival of the CD4<sup>-/-</sup> cells was found to be dependent on reduced apoptosis rather than enhanced proliferation. On the other hand, survival of activated CD4<sup>+/+</sup> 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<sup>+/+</sup> T cell cultures, using a novel in vitro model system, and found that DN T cells survived chronic stimulation whereas the CD4<sup>+/+</sup> 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<sup>-</sup>CD8<sup>-</sup>B220<sup>+</sup> 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.

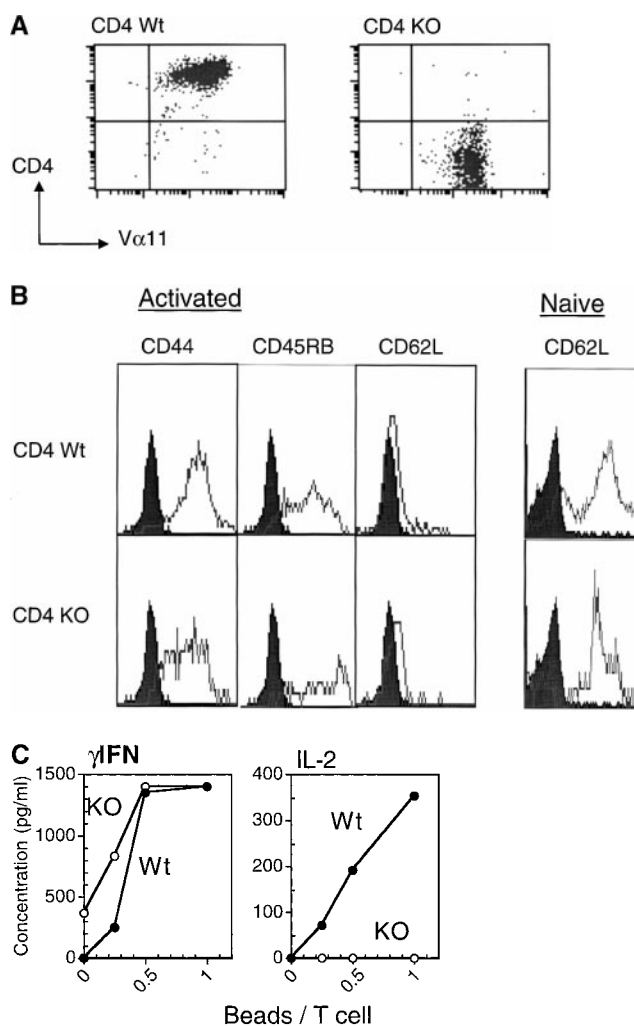
## Materials and Methods

**Mice.** AND V $\alpha$ 11/V $\beta$ 3  $\alpha/\beta$  TCR tg mice specific for moth cytochrome c (MCC) in the context of I-E<sup>k</sup> (23) backcrossed to B10.BR mice (H-2<sup>k</sup>) for 6–8 generations (24) were a gift of Dr. S. Swain (Trudeau Institute, Saranac, NY). CD4<sup>-/-</sup> 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-E<sup>k</sup>/MCC/anti-CD28 beads were prepared using soluble monomeric I-E<sup>k</sup> 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-E<sup>k</sup>/MCC was purified from culture supernatant of infected hi five insect cells (Invitrogen) as described previously (26). I-E<sup>k</sup>/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<sup>TM</sup> flow cytometer (Becton Dickinson) and CELLQuest<sup>TM</sup> software (Becton Dickinson).

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



**Figure 1.** Phenotype and cytokine production by activated CD4<sup>-/-</sup> and CD4<sup>+/+</sup> AND T cells. (A) Phenotype of purified CD4<sup>+/+</sup> and CD4<sup>-/-</sup> AND T cells. AND T cells on CD4<sup>+/+</sup> or CD4<sup>-/-</sup> background were isolated using negative selection as described in Materials and Methods and then analyzed for V $\alpha$ 11 (AND TCR transgene bears V $\alpha$ 11 and V $\beta$ 3) and CD4 expression. Wt, wild-type; KO, knockout. (B) Activation marker expression by stimulated and naive AND T cells. CD4<sup>+/+</sup> and CD4<sup>-/-</sup> 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<sup>+/+</sup> and CD4<sup>-/-</sup> cells. Shaded histograms represent unstained controls. (C) Cytokines production by activated CD4<sup>-/-</sup> and CD4<sup>+/+</sup> 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- $\gamma$  and IL-2 by ELISA.

tion. Purity and specificity of isolated T cells were determined by flow cytometry using mAbs specific for murine V $\alpha$ 11, 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  $2 \times 10^6$  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.

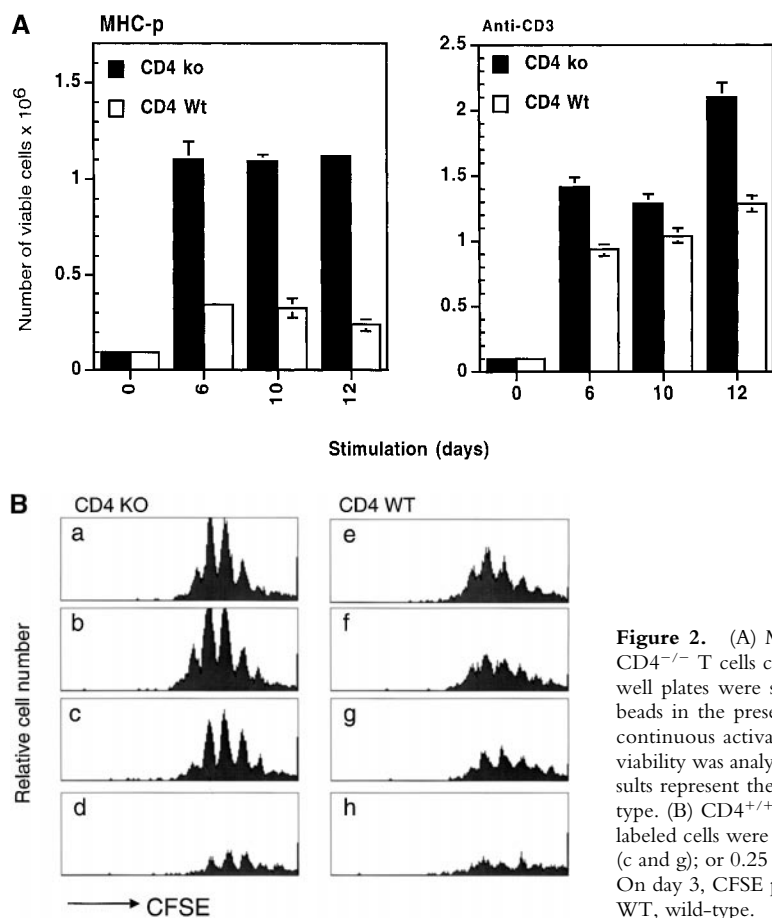
**Cytokine Analysis.** Cells were removed from stimulation cultures, washed, and incubated with MHC/peptide beads for 24 h. Supernatants were collected and assayed for indicated cytokines using captured ELISA according to the manufacturer's instructions (Endogen).

**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  $\mu$ m 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 $\alpha$ 11 $^{+}$  CD4 $^{-}$ CD8 $^{-}$  T cells as described previously (25). Using negative selection, we isolated V $\alpha$ 11 $^{+}$ CD4 $^{-}$ CD8 $^{-}$  T cells from spleens of CD4 $^{-/-}$  AND/B10.BR mice or V $\alpha$ 11 $^{+}$ 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  $\pm$  SEM. ko, knockout; Wt, wild-type. (B) CD4 $^{+/+}$  and CD4 $^{-/-}$  AND cells proliferate at comparable rates. CFSE-labeled cells were stimulated with:  $2 \times 10^5$  (a and e);  $1 \times 10^5$  (b and f);  $0.5 \times 10^5$  (c and g); or  $0.25 \times 10^5$  (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.

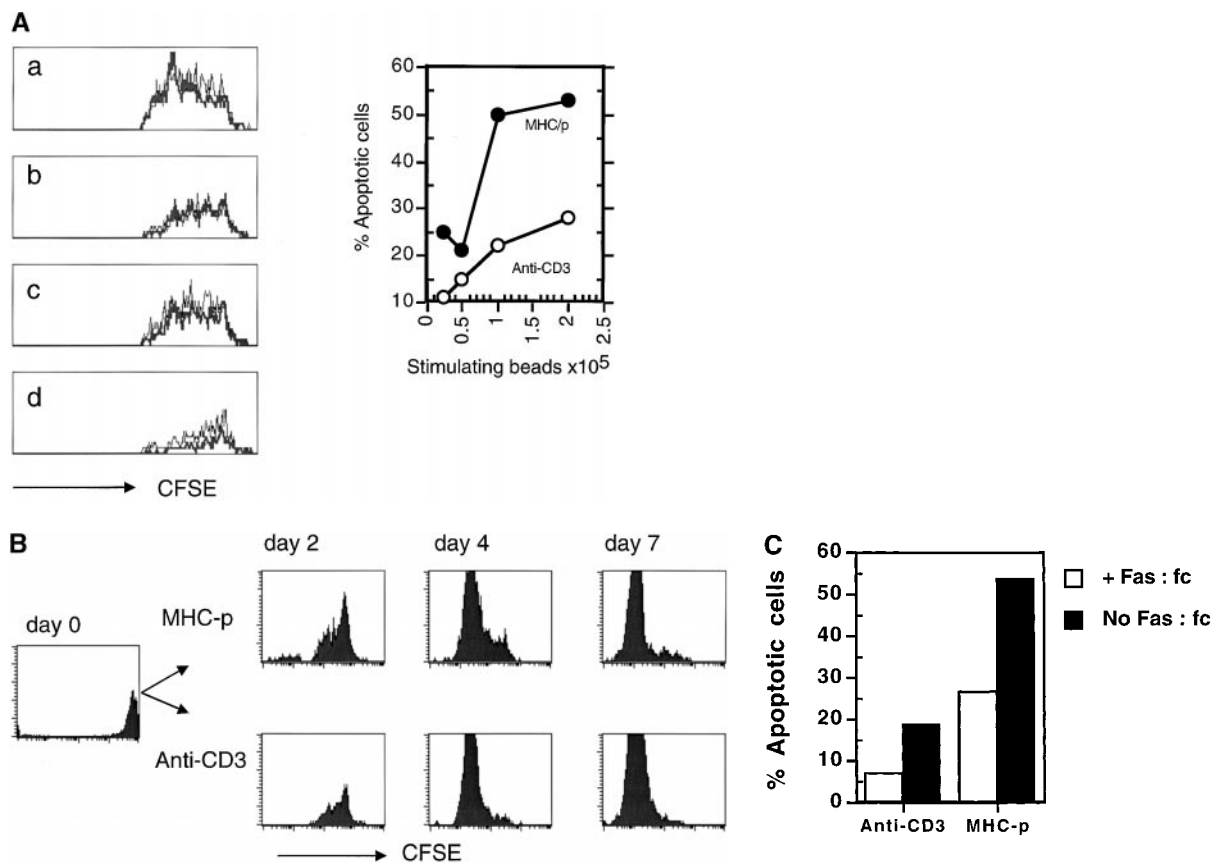
CD44, intermediate level of CD45RB, and downregulated CD62L (Fig. 1 B). These cells were clearly activated as indicated by low levels of CD62L expression, which were high on naive T cells (Fig. 1 B). In addition, both CD4<sup>+/+</sup> and CD4<sup>-/-</sup> cells produced large amounts of IFN- $\gamma$ ; however, only CD4<sup>+/+</sup> cells produced IL-2 (Fig. 1 C). Although CD4<sup>-/-</sup> 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<sup>+/+</sup> cells in the presence of exogenous IL-2, as described previously (25). Thus, CD4<sup>-/-</sup> cells were activated as indicated by cellular activation profiles, IFN- $\gamma$  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<sup>+/+</sup> and CD4<sup>-/-</sup> 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<sup>-/-</sup> T cells

substantially increased upon stimulation and remained high upon restimulation whereas the number of viable CD4<sup>+/+</sup> T cells showed a more limited increase followed by a decline after restimulation (Fig. 2 A). There was  $\sim$ 10-fold increase in the viability of CD4<sup>-/-</sup> cells relative to  $\sim$ 3-fold increase in CD4<sup>+/+</sup> cells after 6 d of stimulation. Cell numbers increased from  $10^5$  to  $10^6$  for CD4<sup>-/-</sup> cells but only from  $1 \times 10^5$  to a maximum of  $3 \times 10^5$  for CD4<sup>+/+</sup> cells.

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

In contrast to the limited expansion of CD4<sup>+/+</sup> T cells stimulated with MHC/peptide, substantial expansion of



**Figure 3.** Normal proliferation but reduced apoptosis of CD4<sup>+/+</sup> 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<sup>+/+</sup> AND T cells were labeled with CFSE and stimulated with: (a)  $2 \times 10^5$ ; (b)  $1 \times 10^5$ ; (c)  $0.5 \times 10^5$ ; or (d)  $0.25 \times 10^5$  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<sup>+/+</sup> 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<sup>+/+</sup> cells in response to 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<sup>+/+</sup> cells were stimulated with an equivalent number of either MHC/peptide or anti-CD3 beads in the presence or absence of 5  $\mu$ g/ml recombinant human Fas:Fc and 1.25  $\mu$ g/ml enhancer protein (Alexis). 6 d later, apoptosis was analyzed using annexin V assay.

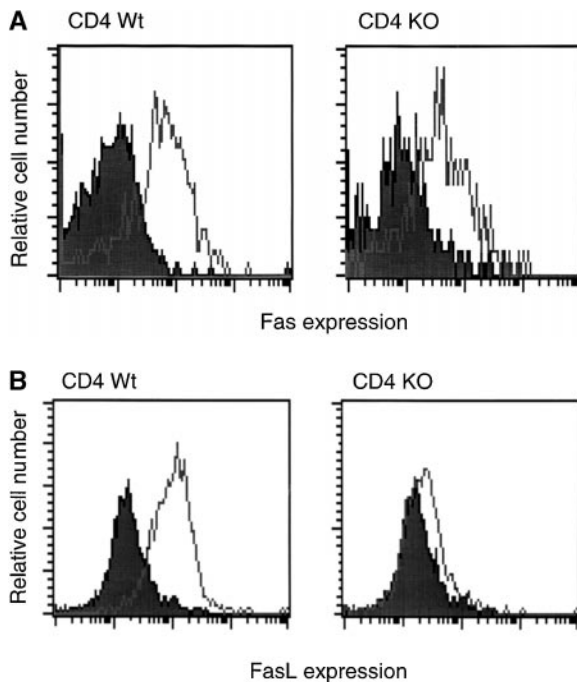
CD4<sup>+/+</sup> T cells occurred when stimulated with anti-CD3/anti-CD28 beads (hereafter referred to as anti-CD3; Fig. 2 A). As this treatment leads exclusively to TCR engagement and circumvents the CD4/MHC interaction, it also implicates CD4 in regulating survival of activated T cells.

**Excluding CD4 during TCR Stimulation Reduces AICD of CD4<sup>+/+</sup> T Cells.** Increased expansion of CD4<sup>+/+</sup> 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<sup>+/+</sup> cells stimulated with various doses of MHC/peptide or anti-CD3. CD4<sup>+/+</sup> 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

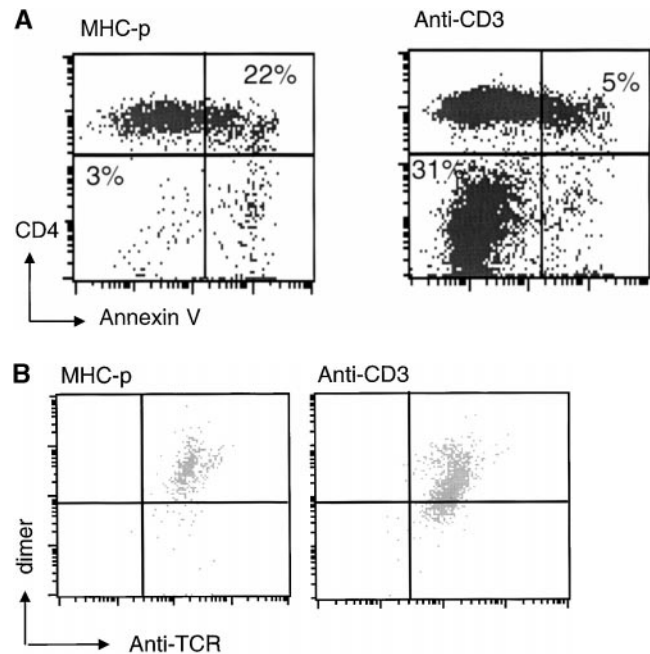
limited apoptosis. On the other hand, stimulation of the CD4<sup>+/+</sup> cells with MHC/peptide complex, the natural ligand for CD4, led to similar proliferation rates but a more limited T cell expansion due to increased apoptosis.

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<sup>+/+</sup> T cells expressed Fas and significant amounts of FasL (Fig. 4). On the other hand, CD4<sup>-/-</sup> T cells showed significant Fas expression but only minimal amounts of FasL expression.

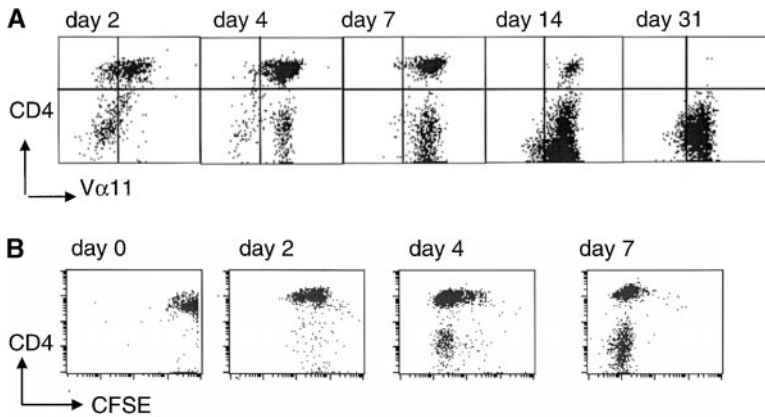
**Suboptimal Apoptosis of Activated CD4<sup>+/+</sup> T Cells Leads to Appearance and Accumulation of DN T Cells.** The accumulation of DN T cells is seen most prominently in autoimmune diseases characterized by defects in Fas-mediated AICD (28) but noticeably absent in other autoimmune diseases. This 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<sup>+/+</sup> T cells on the generation of DN T cells. We stimulated highly purified CD4<sup>+/+</sup> 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<sup>-/-</sup> 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<sup>+/+</sup> T cells leads to generation of DN T cells. (A) Purified CD4<sup>+/+</sup> 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<sup>+</sup> cells and determined the percentage of CD4<sup>+</sup> and apoptotic (annexin V<sup>+</sup>) 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<sup>k</sup>-MCC. Cells were collected from MHC/peptide (left) or anti-CD3 (right) cultures and stained simultaneously with I-E<sup>k</sup>-MCC dimer and anti-TCR $\beta$  (H597) mAb and analyzed by flow cytometry. All cells including DN T cells in anti-CD3 culture stained positive with I-E<sup>k</sup>-MCC indicating they were all expressing AND TCR transgene.



**Figure 6.** DN but not CD4<sup>+/+</sup> T cells tolerate chronic stimulation. (A) Kinetic analysis of DN cells accumulation. CD4<sup>+/+</sup> 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<sup>-/-</sup> 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<sup>+/+</sup> T cells. CD4<sup>+/+</sup> 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<sup>+/+</sup> and DN T cells was visualized by flow cytometry.

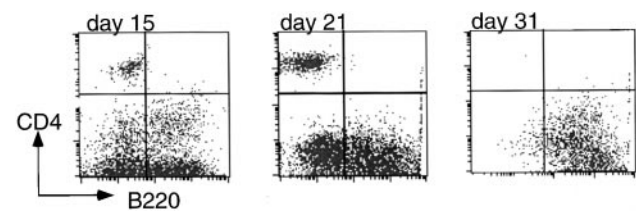
each culture. The MHC/peptide stimulation resulted in a significant number (22%) of apoptotic T cells but few (3%) CD4-negative T cells (Fig. 5 A). Conversely, a significant number (31%) of CD4-negative T cells and few (5%) apoptotic cells were detected in the anti-CD3-stimulated culture. Thus, it appears 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<sup>-</sup>CD8<sup>-</sup> (DN) T cells (data not shown). The CD4<sup>+/+</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>+/+</sup> cells and study their survival. Highly purified CD4<sup>+/+</sup> 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<sup>+/+</sup> 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. Vα11<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T cells began to appear on day 2 and gradually expanded at the expense of

the Vα11<sup>+</sup>CD4<sup>+</sup> T cells (Fig. 6 A). After 5 wk of stimulation, all surviving cells were Vα11<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> 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<sup>-/-</sup> 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<sup>+/+</sup> T cells were stimulated with anti-CD3 to generate and visualize proliferation of both CD4<sup>+/+</sup> and CD4<sup>-/-</sup> cells over a period of 7 d. Emerging DN T cells displayed similar intensity of fluorescence as the CD4<sup>+/+</sup> 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<sup>+/+</sup> T cells express B220. CD4<sup>+/+</sup> 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<sup>+/+</sup> 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<sup>-</sup>CD8<sup>-</sup>B220<sup>+</sup> cells retained their antigen specificity as determined by homogenous expression of V $\alpha$ 11 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<sup>-/-</sup> T cells survived in much larger numbers than CD4<sup>+/+</sup> cells regardless of the TCR ligand used. Second, CD4<sup>+/+</sup> 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<sup>+/+</sup> 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<sup>-</sup>CD8<sup>-</sup>B220<sup>+</sup> 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- $\gamma$  production. Second, MHC/peptide and anti-CD3 induced comparable proliferation but differential apoptosis of CD4<sup>+/+</sup> T cells (Fig. 3). Finally, DN and CD4<sup>+/+</sup> cells present in the same culture proliferated at a similar rate but the DN T cells survived chronic stimulation whereas their CD4<sup>+/+</sup> 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- $\alpha$ -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<sup>+</sup> 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<sup>+</sup> cells of known antigenic specificity (Fig. 6 B, and unpublished data). Using this system to understand the effector function of DN B220<sup>+</sup> T cells may lead to new insights into the *in vivo* function of this important subset of T cells.

We thank Dr. John Kappler and Dr. Philippa Marrack for I-E<sup>k</sup>/MCC construct; Dr. Susan Swain for AND × B10.BR, and Dr. Kim Bottomly for CD4 knockout AND × B10.BR tg mice. We thank Emily Yuan, Lawrence Siew, and Bonifacio S. Casbag, Jr. for excellent technical assistance. We thank Drs. Todd Armstrong, Mark Soloski, and Joan Bieler for critical reading of the manuscript.

This work is supported by National Institutes of Health grants AI2975-05 and AI44129. For details about the laboratory please visit our web site: [www.pathology2.jhu.edu/schnecklab](http://www.pathology2.jhu.edu/schnecklab).

Submitted: 13 October 2000

Revised: 21 February 2001

Accepted: 9 April 2001

## References

1. Van Parijs, L., and A. Abbas. 1998. Homeostasis and self tolerance in the immune system: turning lymphocytes off. *Science*. 280:243–248.
2. Lenardo, M., F. Chan, F. Hornug, H. McFarland, R. Siegel, J. Wang, and L. Zheng. 1999. Mature T lymphocytes apoptosis-immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* 17:221–253.
3. Dhein, J., H. Walczak, C. Baumler, and K. Debatin. 1995. Autocrine T cell suicide mediated by APO-1/(Fas/CD95). *Nature*. 373:438–441.
4. Ju, S., D. Panka, H. Cui, R. Ettinger, M. El-khatib, D. Sherr, B. Stanger, and A. Marshak-Rothstein. 1995. FasL interactions required for program mediated cell death after T cell activation. *Nature*. 373:444–448.
5. Brunner, T., R. Mogil, D. LaFace, N. Yoo, A. Mahboubi, F. Echeverri, S. Martin, W. Force, D. Lynch, C. Ware, and C. Ware. 1995. Cell autonomous Fas (CD95)/Fas-ligand interaction mediates activation induced apoptosis in T cell hybridoma. *Nature*. 373:438–441.
6. Watanabe-Fukunaga, R., C. Brannan, N. Copeland, N. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature*. 356:314–317.
7. Takahashi, T., M. Tanaka, C. Brannan, N. Jenkins, N. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice caused by a point mutation in the fas ligand. *Cell*. 76:969–976.
8. Craft, J., S. Peng, T. Fujii, M. Okada, and S. Fatenejad. 1999. Autoreactive T cells in murine lupus: origin and roles of autoantibody production. *Immunol. Res.* 19:245–257.
9. Fisher, G., F. Rosenberg, S. Straus, J.K. Dale, L.A. Middleton, A.Y. Lin, W. Strober, M. Lenardo, and J.M. Puck. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*. 81:1935–1946.
10. Rieux-Laucat, F., F. Le Deist, C. Hivroz, I.A. Roberts, K. Debatin, A. Fischer, and J.P. de Villartay. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science*. 268:1347–1349.
11. Wang, J., L. Zheng, A. Lobito, F. Chan, J. Dale, M. Sneller, X. Yao, J. Puck, S. Straus, and M. Lenardo. 1998. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell*. 98:47–58.
12. Cohen, P., and R. Eisenberg. 1991. Lpr and gld: Single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243–269.
13. Morse, H., W. Davidson, R. Yetter, E. Murphy, J. Roths, and R. Coffman. 1982. Abnormalities induced by the mutant gene lpr: expansion of a unique lymphocytes subset. *J. Immunol.* 129:2612–2615.
14. Mixter, P., J.Q. Russell, G.J. Morrisette, C. Charland, D. Aleman-Hoey, and R.C. Budd. 1999. A model for the origin of TCR  $\alpha\beta$  CD4<sup>-</sup>CD8<sup>-</sup> B220<sup>+</sup> cells based on high affinity TCR signals. *J. Immunol.* 162:5747–5756.
15. Gay, D., W. Coeshott, W. Golde, J. Kappler, and P. Marrack. 1986. The major histocompatibility complex restricted antigen receptor on T cells. *J. Immunol.* 136:2026–2032.
16. Konig, R., Li-Yun Huang, and R.N. Germain. 1992. MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. *Nature*. 356:796–798.
17. Cammarota, G., A. Scheirle, B. Takacs, D.M. Doran, R. Knorr, W. Bannwarth, J. Guardiola, and F. Sinigaglia. 1992. Identification of a CD4 binding site on the  $\beta$ 2 domain of HLA-DR molecules. *Nature*. 356:799–801.
18. Newell, M.K., L.J. Haughn, C.R. Maroun, and M.H. Julius. 1990. Death of mature T cells by separate ligation of CD4 and the T cell receptor for antigen. *Nature*. 347:286–288.
19. Banda, N.K., J. Bernier, D.K. Kurahara, R. Kurrel, N. Haigwood, R.P. Sekaly, and T.H. Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation induced apoptosis. *J. Exp. Med.* 176:1099–1106.
20. Badley, A., J. McElhinny, P. Leibson, D. Lynch, M. Alderson, and C. Paya. 1996. Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected lymphocytes. *J. Virol.* 70:199–206.
21. Oyaizu, N., Y. Adachi, F. Hashimoto, T. McCloskey, N. Hosaka, N. Kayagaki, H. Yagita, and S. Pathwa. 1997. Monocytes express fas ligand upon CD4 crosslinking and induce CD4<sup>+</sup> T cells apoptosis. *J. Immunol.* 158:2456–2463.
22. Algeciras, A., D.H. Dockrell, D. Lynch, and C.V. Paya. 1998. CD4 regulates susceptibility to Fas ligand and tumor necrosis factor mediated apoptosis. *J. Exp. Med.* 187:711–720.
23. Kaye, J., M. Hsu, M. Sauron, S. Jameson, N. Gascoigne, and S. Hedrick. 1989. Selective development of CD4<sup>+</sup> T cells in transgenic mice expressing a class II restricted antigen receptor. *Nature*. 341:746–749.
24. Cauley, L., K. Cauley, F. Shub, G. Huston, and S. Swain. 1997. Transferable anergy: superantigen treatment induces CD4<sup>+</sup> T cell tolerance that is reversible and requires CD4<sup>-</sup>CD8<sup>-</sup> cells and interferon  $\gamma$ . *J. Exp. Med.* 186:71–81.
25. Leitenberg, D., Y. Boutin, S. Constant, and K. Bottomly. 1998. CD4 regulation of TCR signaling and T cell differentiation following stimulation with peptides of different affinities for the TCR. *J. Immunol.* 161:1194–1203.
26. Kozono, H., J. White, J. Clements, P. Marrack, and J. Kappler. 1994. Production of soluble MHC class II proteins with covalently bound single peptides. *Nature*. 330:256–259.
27. Levine, B.L., J.D. Mosca, J.L. Riley, R.G. Carroll, M.T. Vahey, L.L. Jagodzinski, K.F. Wagner, D.L. Mayers, D.S. Burke, O.S. Weislow, et al. 1996. Anti-viral effect and ex vivo CD4<sup>+</sup> T cell proliferation in HIV positive patients as a result of CD28 costimulation. *Science*. 272:1939–1943.
28. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science*. 267:1449–1456.
29. Hamad, A.R., S.M. O'Herrin, M.S. Lebowitz, A. Srikrish-



- nan, J. Bieler, J. Schneck, and D. Pardoll. 1998. Potent T cell activation with dimeric peptide-major histocompatibility complex class II ligand: the role of CD4 coreceptor. *J. Exp. Med.* 188:1633–1640.
30. Altman, A., A. Theofilopoulos, R. Weiner, D. Katz, and F. Dixon. 1981. Analysis of T cell function in autoimmune murine strains. *J. Exp. Med.* 154:791–808.
  31. Fischbach, M. 1984. Defective T cell response to presented antigen in autoimmune mice. *J. Immunol.* 133:2365–2368.
  32. Davignon, J., P. Cohen, and R. Eisenberg. 1988. Rapid T cell receptor modulation accompanies lack of in vitro mitogenic responsiveness of double negative T cells to anti-CD3 monoclonal antibody in MRL/Mp-lpr/lpr mice. *J. Immunol.* 141:1848–1854.
  33. Budd, R., J. Russel, N. Van Houten, S. Cooper, H. Yagita, and J. Wolfe. 1991. CD2 expression correlates with proliferative capacity of ab<sup>+</sup> or CD4<sup>-</sup>CD8<sup>-</sup> T cells in lpr mice. *J. Immunol.* 148:1055.
  34. Giese, T., J. Allison, and W. Davidson. 1993. Functionally anergic lpr and gld B220<sup>+</sup> T cell receptor (TCR)- $\alpha\beta$  double negative T cells express CD28 and respond to costimulation with phorbol myristate acetate and antibodies to CD28 and the TCR. *J. Immunol.* 151:597–609.
  35. Clements, J., G. Winslow, C. Donahue, S. Cooper, J. Allison, and R. Budd. 1993. Co-stimulation via CD28 induces activation of a refractory subset of MRL-lpr/lpr T lymphocytes. *Int. Immunol.* 5:1451–1460.
  36. Theofilopoulos, A., and F. Dixon. 1985. Murine model of systemic lupus erythematosus. *Adv. Immunol.* 37:269–290.
  37. Davidson, W., F. Dumont, H. Bedigian, B. Fowlkes, and H. Morse. 1986. Phenotypic, functional and molecular genetic comparisons of the abnormal lymphoid cells of C3H-lpr and C3h-gld/gld mice. *J. Immunol.* 136:4075–4084.
  38. Tateyama, M., N. Oyaizu, T. McCloskey, S. Than, and S. Pahwa. 2000. CD4 T lymphocytes are primed to express Fas ligand by CD4 crosslinking and to contribute to CD8 T cell apoptosis via Fas/FasL death signaling pathway. *Blood.* 96:195–202.
  39. Fleury, S.E.A. 1995. HLA-DR polymorphism affects the interaction with CD4. *J. Exp. Med.* 182:733–741.
  40. Hall, M., H. Lanchbury, J. Lee, K. Welch, and J. Ciclitira. 1993. HLA-DQ2 second-domain polymorphism may explain increased trans-associated risk in celiac disease and dermatitis herpetiformis. *Hum. Immunol.* 38:284–292.
  41. Johansen, B., T. Jensen, C. Thorpe, F. Vartdal, E. Thorsby, and L. Sollid. 1996. Both  $\alpha$  and  $\beta$  chain polymorphisms determine the specificity of the disease-associated HLA-DQ molecules, with  $\beta$  chain residues being the most influential. *Immunogenetics.* 45:142–150.
  42. Marrack, P., J. Bender, D. Hilderman, M. Jordan, T. Mitchell, M. Murakami, A. Sakamoto, B. Schaefer, S. Swanson, and J. Kappler. 2000. Homeostasis of  $\alpha\beta$  T cells. *Nat. Immunol.* 1:107–111.