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Development and function of T cells in mice with a disrupted CD2 gene

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CD2 is a T cell surface glycoprotein that mediates cellular adhesion and can participate in signal transduction. It is expressed early in thymocyte ontogeny and consequently has been proposed to participate in T cell development. To study the *in vivo* function of CD2, the murine gene was inactivated using the technique of homologous recombination in embryonic stem cells. Homozygous mutant mice are healthy and have an apparently normal complement of lymphocytes. They mount effective immune responses similar to those of wild type controls. In particular, the generation and function of cytotoxic T cells was found to be normal as was the production of antibodies following immunization. Selection of thymocytes expressing either MHC class I- or class II-restricted transgenic T cell receptors was also grossly normal in the absence of CD2. Thus, CD2 may be dispensable for the development and function of T cells. Within the context of other targetted mutations, these mice should be useful in defining the precise roles of various cell surface molecules involved in T cell responses.

Key words: CD2/gene targetting/T cell activation/T cell development/T cells

Introduction

The CD2 glycoprotein is an adhesion molecule that has been shown to transduce mitogenic signals in T cells (Springer *et al.*, 1987; Moingeon *et al.*, 1989; Dustin and Springer, 1991). Numerous studies have demonstrated inhibition of T cell function and adhesion by monoclonal antibodies specific for CD2, suggesting that this molecule may have an important role in the generation of an immune response (Howard *et al.*, 1981; Kamoun *et al.*, 1981; Van Wauwe *et al.*, 1981; Bernard *et al.*, 1982; Sanchez-Madrid *et al.*, 1982). It has also been speculated that CD2 plays a key role in T cell differentiation, since it is expressed early in thymocyte ontogeny and may be involved in thymocyte binding to thymic epithelium (Denning *et al.*, 1987; Vollger *et al.*, 1987; Owen *et al.*, 1988; Duplay *et al.*, 1989; Law *et al.*, 1989).

The CD2 molecule, also known as T11, LFA-2 or the E-receptor, binds to the widely expressed CD58 (or LFA-3) glycoprotein (Hunig, 1985; Dustin *et al.*, 1987; Selvaraj *et al.*, 1987a). In mice, CD2 is a 55-60 kDa glycoprotein expressed on the surface of T cells, B cells and NK cells (Sewell *et al.*, 1987; Yagita *et al.*, 1989). In humans, CD2 is not expressed on B cells; this permits selective adhesion of human T cells to sheep erythrocytes expressing a high density of CD58 and forms the basis of the widely used E-rosetting technique for T cell purification (Selvaraj *et al.*, 1987b). It has been proposed that binding of CD2 to CD58 is important in the stabilization of conjugates between T cells and their targets, thereby facilitating the generation of an effective immune response (Williams and Beyers, 1992). Recently, two additional ligands for CD2 have been identified: the broadly expressed CD59 molecule (Hahn *et al.*, 1992) and the CD48 molecule expressed on leukocytes and endothelium (L.Kato, M.Koyanagi, H.Okada, T.Takanashi, Y.W.Wong, A.N.Barclay, A.F.Williams, K.Okumura and H.Yagita, submitted). No distinct roles have yet been assigned to these various interactions.

Appropriate combinations of antibodies against CD2 cause T cell mitogenesis (Meuer *et al.*, 1984). Intracellular signals delivered through CD2 mimic those observed when the T cell antigen receptor (TCR-CD3) is engaged by antibodies or antigen-MHC (Moingeon *et al.*, 1989; Ley *et al.*, 1991). However, unlike activation elicited with anti-TCR antibodies, CD2-mediated T cell activation requires the coordinate ligation of two separate epitopes on the molecule (Meuer *et al.*, 1984; Clark *et al.*, 1988). Single antibodies against either epitope alone are not mitogenic even when cross-linked, but CD58, sheep erythrocytes or PMA can effectively substitute for one of the antibodies (Holter *et al.*, 1986; Hunig *et al.*, 1987). It has been postulated that physiologic stimulation through the CD2 molecule may likewise require simultaneous engagement of two separate ligands (Kato *et al.*, submitted).

T cell activation through CD2 may involve direct interactions with components of the TCR-CD3 complex. This follows from the observation that TCR^- cells do not respond to anti-CD2 monoclonal antibodies unless CD2 expression is raised above a critical threshold (Ohno *et al.*, 1991b; Spruyt *et al.*, 1991). Conversely, variants of the Jurkat T leukemic cell line lacking CD2 expression show a diminished capacity to produce IL-2 in response to TCR or CD3 ligation (Makni *et al.*, 1991). This defect can be corrected by transfection of the CD2 gene. Signals delivered by the cytoplasmic domain of the CD2 molecule have also been shown to render cells refractory to further stimulation through the TCR (Ohno *et al.*, 1991a). The most striking example of this is the induction of T cell non-responsiveness following injection of anti-CD2 monoclonal antibodies into mice (Guckel *et al.*, 1991). Co-precipitation of the TCR with antibodies against CD2 (Brown *et al.*, 1989; Beyers *et al.*, 1992) further suggests that CD2 may be an integral participant modulating the recognition of antigen by the TCR-CD3 complex.

Several observations have prompted speculation that CD2 may be involved in T cell development. CD2 expression commences early in thymocyte ontogeny (Owen *et al.*, 1988;

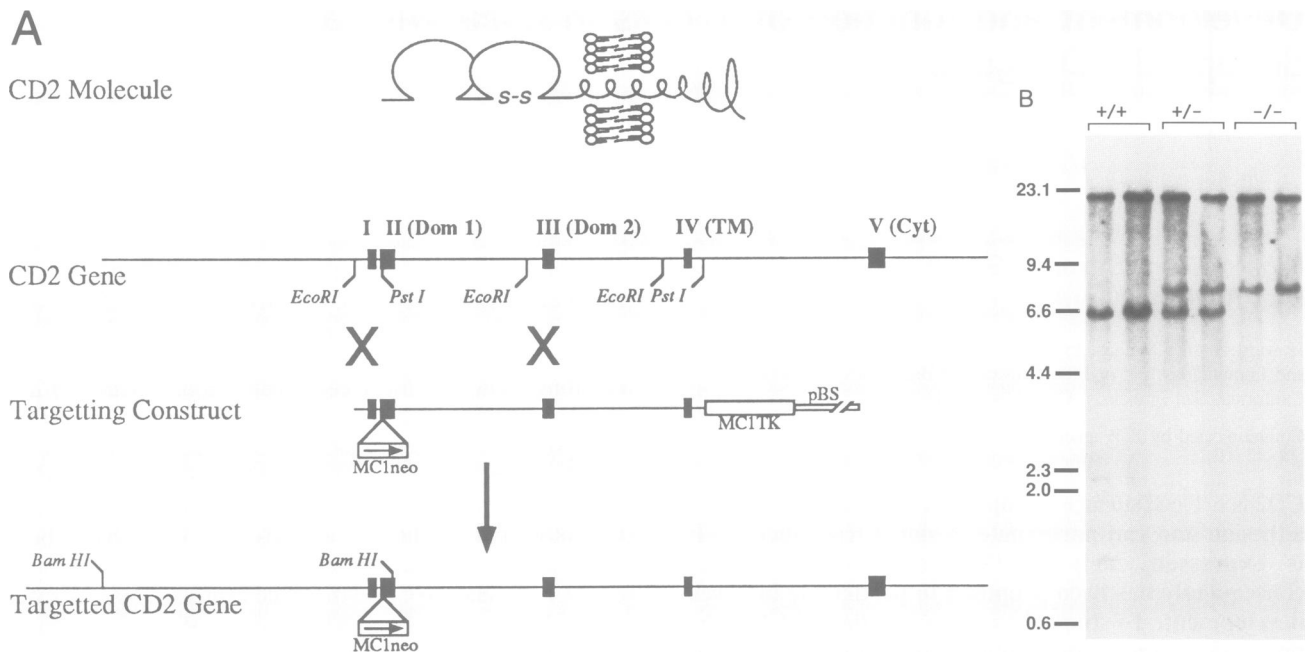

Fig. 1. Disruption of the mouse CD2 gene. **A.** Diagram of the strategy used to disrupt the mouse CD2 gene by homologous recombination. Genomic structure is based on Diamond *et al.* (1988). **B.** Genomic Southern blot analysis of DNA from $CD2^-$ mutant and wild type mice. The 1.2 kb insertion event was detected with a cDNA probe which spans the entire coding region and hybridizes to two *Bam*HI fragments (see A).

Duplay *et al.*, 1989; Law *et al.*, 1989) and surface density of CD2 is greater on mature thymocytes than on their immature precursors (Duplay *et al.*, 1989). Antibodies against CD2 can perturb interactions between thymocytes and $CD58^+$ thymic epithelial cells (Denning *et al.*, 1987; Vollger *et al.*, 1987). Furthermore, thymocytes can respond to mitogenic pairs of anti-CD2 monoclonal antibodies by upregulating their IL-2 receptors and proliferating (Fox *et al.*, 1985). However, monoclonal antibodies against murine CD2 fail to alter the course of T cell development *in vivo* or in thymic organ culture (Kyewski *et al.*, 1989; Yagita *et al.*, 1989), suggesting that CD2 does not have a critical role in positive selection of thymocytes.

To explore further the role of CD2 in thymocyte development and peripheral T cell function, we have generated CD2-deficient mice using homologous recombination in embryonic stem cells (Cappechi, 1989). These mice have a grossly normal complement of T cells and B cells, which exhibit no obvious developmental or functional defects.

Results

Generation of CD2-deficient mice

To inactivate the CD2 gene, D3 embryonic stem (ES) cells (Doetschman *et al.*, 1985) were transfected with the linearized construct shown in Figure 1A. This vector introduces a neomycin resistance gene into a *Pst*I site in the second exon of the CD2 gene, thereby disrupting the sequence encoding the amino-terminal V-like domain. PCR was employed to identify colonies with the expected genomic structure. Approximately one in 50 neo^r colonies was found to have the disrupted gene. These PCR-positive clones were further subjected to Southern blot analysis to confirm that no unpredicted rearrangements had occurred and were then injected into C57BL/6 blastocysts. Chimeric males derived from the mutated ES cells were back-crossed to (C57BL/6×DBA/2) F1 females and one male was found that produced agouti progeny carrying the CD2 mutation. Inter-crosses generated mice homozygous for the mutant allele at the expected Mendelian frequency. The structure of the mutant locus was examined by Southern analysis using a CD2 cDNA probe (Figure 1B), a pMC1neo probe (not shown) and a probe upstream of the region encompassed by the targetting construct (not shown) on several different digests of genomic DNA from ES cells and mice of various genotypes. In all cases the observed bands were consistent with the pattern expected from the map shown in Figure 1A.

Loss of CD2 cell surface expression

Mice homozygous for the CD2 disruption (−/−) remained consistently healthy for >55 weeks. Lymphocytes from these mice did not stain with a panel of five different anti-CD2 monoclonal antibodies and cells from heterozygous mutant mice (+/−) showed a 2-fold drop in fluorescence intensity relative to wild type (+/+) CD2 expression (Figure 2A). To address further the efficacy of the mutation, cells from mutant mice were examined by Northern blot hybridization for the presence of CD2 transcripts. As shown in Figure 2B, strong hybridization to a CD2 cDNA probe was obtained with RNA from +/+ thymocytes and +/+ peripheral lymphocytes, but no detectable signal was observed with cells from homozygous mutant mice, despite control hybridization to a GAPDH probe. This loss of CD2 hybridization may be due to efficient utilization of the pMC1neopolyA addition signal which would terminate any mRNA before the bulk of the CD2 sequence. Alternatively, the pMC1 promoter/enhancer may interfere with transcription initiating at the endogenous CD2 promoter or may cause mRNA instability.

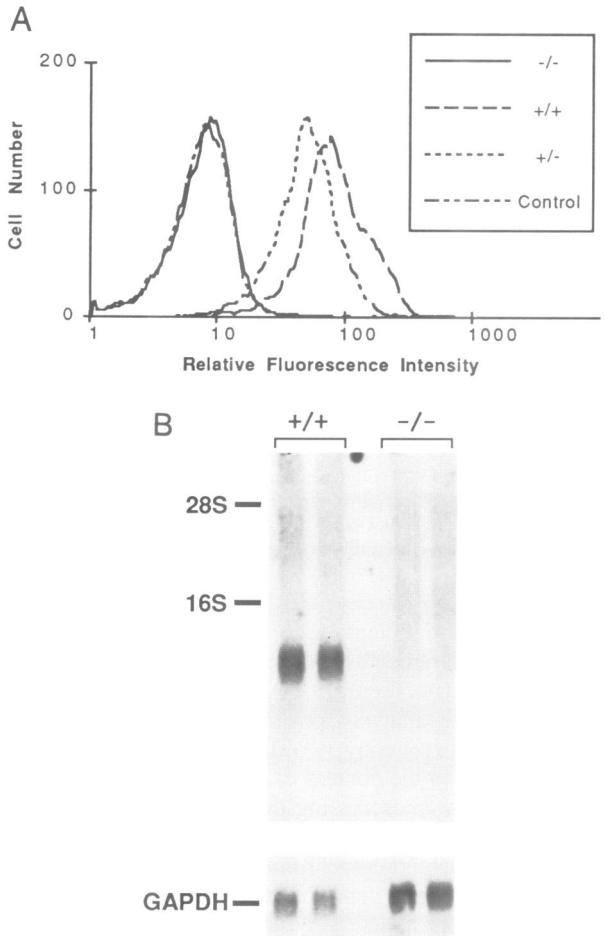

Fig. 2. Loss of CD2 expression in mice homozygous for the CD2 mutation. **A.** Absence of cell surface CD2 in mutant mice. Thymocytes were stained with the anti-CD2 monoclonal antibody RM2-5 (Yagita *et al.*, 1989) and fluorescein-conjugated goat anti-mouse IgG-Fc. Ten thousand gated events were collected using the FACScan. Control background fluorescence was determined using no first antibody. Peripheral lymphocytes from $CD2^-$ mutant mice also showed no detectable staining with anti-CD2 (not shown). **B.** Absence of CD2 mRNA in CD2 −/− mice. RNA was isolated from thymuses and lymph nodes of CD2 +/+ and −/− mice. Northern blots were probed with a murine CD2 cDNA identical to that used in Figure 1B.

Lymphocyte populations in CD2-deficient mice

CD2-deficient mice have normal numbers of T cells and B cells in their peripheral lymphoid organs and have thymuses of typical size and constitution. Cells from these organs were analysed by flow cytometry with a panel of monoclonal antibodies recognizing CD4, CD8, CD3ϵ, Thy-1, CD45R (B220), CD45RB, TCRαβ, TCRγδ, heat stable antigen, CD5, Ly-6C, LFA-1, ICAM-1, MHC Class I (D and K), PGP-1 and IL-2Rα. No obvious peculiarities were apparent with any of these reagents. For example, Figure 3 shows staining profiles with CD3, CD4 and CD8 for thymocytes and Thy-1 and B220 for lymph node cells from CD2 −/−, +/− and +/+ mice. The appropriate mature ($CD3^{hi}CD4^+CD8^-$, $CD3^{hi}CD4^-CD8^+$) and immature ($CD3^-CD4^-CD8^-$, $CD3^{lo}CD4^+CD8^+$) subsets are present in the thymuses of adult and newborn (not shown) mice, suggesting a grossly normal pattern of development. Similarly, the peripheral lymphoid organs contain either T cells ($Thy\text{-}1^+$) or B cells ($B220^+$) in normal proportions.

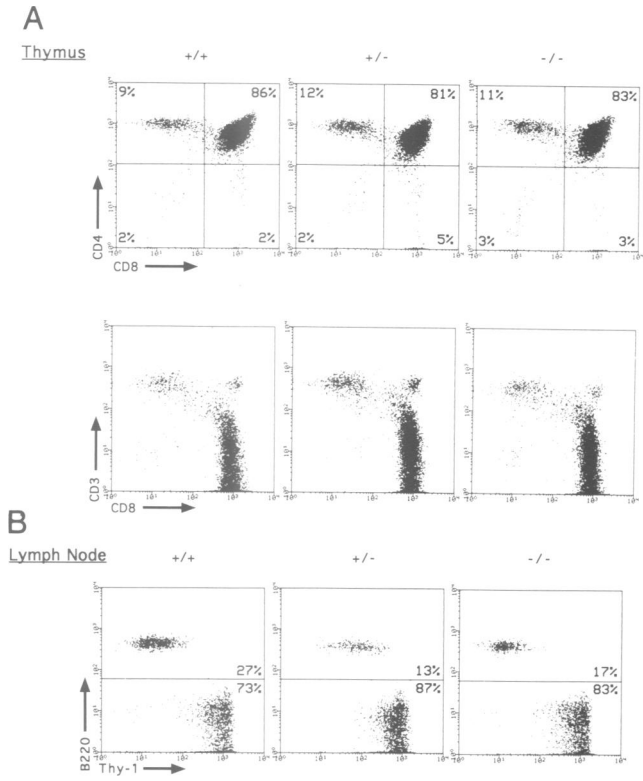

Fig. 3. Cell surface phenotype of CD2 −/− thymocytes and lymphocytes. **A.** Expression of CD3, CD4 and CD8 on thymocytes from CD2 +/+, +/− and −/− mice. Cells were stained with fluorescein−anti-CD8, phycoerythrin−anti-CD4 and biotinylated anti-CD3 followed by streptavidin-tandem conjugate. Ten thousand gated events were analysed on a Becton Dickinson FACScan flow cytometer. CD8 versus CD4 fluorescence is shown in the upper panel and CD8 versus CD3 fluorescence is shown in the lower panel. **B.** Distribution of B and T lymphocytes in lymph nodes from CD2 +/+, +/− and −/− mice. Cells were stained with fluorescein−anti-Thy-1 and phycoerythrin−anti-CD45 (B220). Five thousand gated events were analysed using the FACScan flow cytometer. Differences in the relative proportions of lymphocyte subsets reflect individual variability not correlated with the CD2 genotype.

Lymphocyte function in CD2-deficient mice

T cell function in CD2 −/− mice was initially analysed in mixed lymphocyte reactions (MLR). Because of the hybrid genetic background, donors of responder cells were first typed for MHC haplotype by FACS analysis of peripheral blood mononuclear cells, using monoclonal antibodies specific for K^b and K^d. B cell-depleted lymph node cells from H-2^b-haplotype mice were stimulated *in vitro* with T cell-depleted, irradiated spleen cells from Balb/c (H-2^d), C57BL/6 (H-2^b) and C3H/HeJ (H-2^k) mice (Figure 4A). In several experiments, [^{3}H]thymidine uptake in response to allogeneic stimuli was comparable using cells from CD2 −/− and CD2 +/+ mice. Other experiments showed that proliferative responses of CD2 −/− T cells to H-2^s and to isolated Class I (K^{bm1}, K^{bm11}) and Class II ($I\text{-}A^{bm12}$) mutants were not notably different from controls (not shown). Similarly, DNA synthesis in response to anti-CD3ϵ monoclonal antibody and concanavalin A was normal (not shown).

CD2-deficient mice were further tested for their ability to mediate a cytotoxic T cell response. Following *in vitro* challenge with irradiated DBA/2 (H-2^d) stimulators, P815 (H-2^d) but not EL-4 (H-2^b) target cells were efficiently

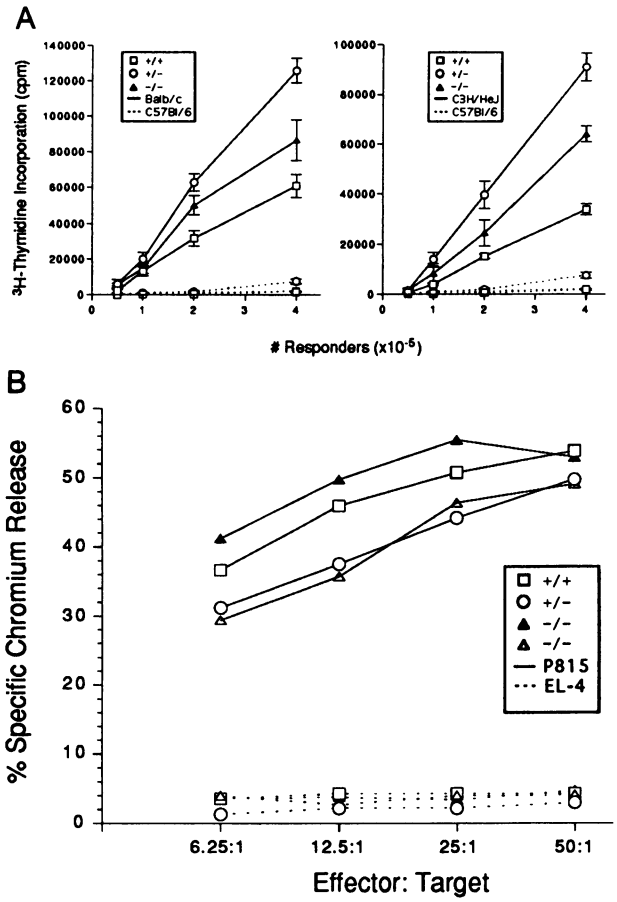

Fig. 4. Analysis of *in vitro* function of CD2-deficient lymphocytes. **A.** Proliferative responses to allogeneic stimuli. Responder T cells were prepared from the lymph nodes of H-2^b CD2 +/+, +/− and −/− mice and were cultured for 4 days in the presence of Thy-1-depleted spleen cells from Balb/c (H-2^d), C3H/HeJ (H-2^K) or C57Bl/6 (H-2^b) mice. Incorporation of [^{3}H]thymidine was used as a measure of cellular proliferation. The left and right panels show responses to Balb/c and C3H stimuli, respectively. In several different experiments, variability in the magnitude of proliferative responses was not found to correlate with CD2 expression. **B.** Generation of cytotoxic T cell responses. Lymph node cells from H-2^b CD2 +/+, +/− and −/− mice were stimulated *in vitro* with irradiated DBA/2 spleen cells. Cytotoxic activity against EL-4 (H-2^b) and P815 (H-2^d) ^{51}Cr-labelled target cells was measured after 5 days and is presented according to the formula: % specific release = (observed release−spontaneous release)/(maximal release−spontaneous release).

lysed by cytotoxic cells from both CD2-negative and control mice (Figure 4B).

It has been proposed that CD2 may be important in B cell−T cell collaboration (Rutschmann and Karjalainen, 1991); we therefore measured the humoral response to a T cell-dependent antigen. Mice were immunized with the hapten TNP conjugated to keyhole limpet hemocyanin (TNP-KLH) and specific anti-TNP antibody titres were measured by ELISA after 10 and 14 days and also following a secondary immunization. As shown in Figure 5, total IgG, IgG1 and IgM responses specific for TNP were similar in CD2 −/− mice and wild type control animals.

Positive and negative selection of thymocytes in CD2-deficient mice

Several studies have suggested a role for CD2 in thymopoiesis (Meuer *et al.*, 1984; Fox *et al.*, 1985; Denning *et al.*, 1987; Vollger *et al.*, 1987). However, both newborn

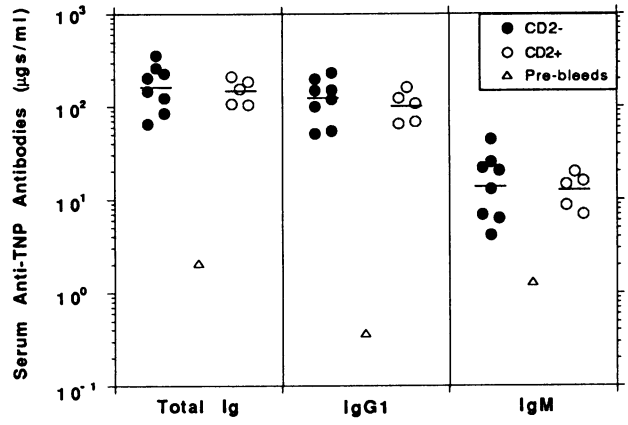

Fig. 5. Generation of a T cell-dependent humoral response in CD2-deficient mice. Mice were immunized with TNP-KLH and the levels of anti-TNP antibodies were determined by ELISA after 10 days. The average level of antibody in preimmune sera is depicted by the triangle.

and adult CD2-deficient mice have apparently normal populations of thymocytes and mature T cells as determined by flow cytometry (Figure 3). As a means to improve the resolution of this analysis, positive and negative selection of thymocytes expressing two distinct TCR heterodimers were examined in the context of the CD2 deficiency. This was acheived by intercrossing the CD2 mutant mice with previously described TCR transgenic lines, one encoding specificity for pigeon cytochrome *c* (PCC) presented by I-E^K (Kaye *et al.*, 1989) and the other producing a receptor specific for the male H-Y antigen presented by D^b (Kisielow *et al.*, 1988; Teh *et al.*, 1988). Figure 6A and B shows that positive and negative selection are not significantly affected in CD2-deficient mice expressing the D^b-restricted receptor. Male H-2^b CD2 −/− mice carrying the transgenic TCR had thymuses of reduced size populated by an increased number of CD4$^-$CD8$^-$ cells expressing both chains of the transgenic receptor (Figure 6A). This deletion phenotype, due to the presence of the male self antigen and the appropriate restricting element, was also observed in the periphery, where CD4$^-$CD8$^-$ and CD8^{lo} cells expressing the complete transgenic heterodimer predominated (not shown). The process of positive selection, observed in female H-2^b mice bearing this transgenic receptor, was not obviously perturbed by the lack of CD2 protein (Figure 6B). As in control mice, there was a high ratio of CD8:CD4 cells and the majority of CD4$^-$CD8$^+$ thymocytes retained expression of the transgenic α chain. Finally, in the absence of a selecting environment, i.e. in the absence of D^b, T cell development in mice lacking CD2 proceeded as in controls, with no selection for retention of the transgenic TCR-α chain (Figure 6C).

Development of CD2-deficient T cells expressing the PCC-specific Vβ3/Vα11 TCR was examined in H-2^d and H-2$^{d/b}$ mice (Figure 6D and E). I-A^b drives positive selection of CD4$^+$ cells expressing this TCR (C.B.Davis, S.M.Hedrick, D.R.Littman and J.Kaye, submitted), whereas I-E^d plus the superantigen MTV-6, provided by the DBA/2 genetic background, induce clonal deletion. This negative selection was apparent from a 5-fold reduction in thymocyte yield and a decrease in the relative proportion of CD4$^+$CD8$^+$ thymocytes compared to non-transgenic mice. The deletion phenotype was also apparent in peripheral

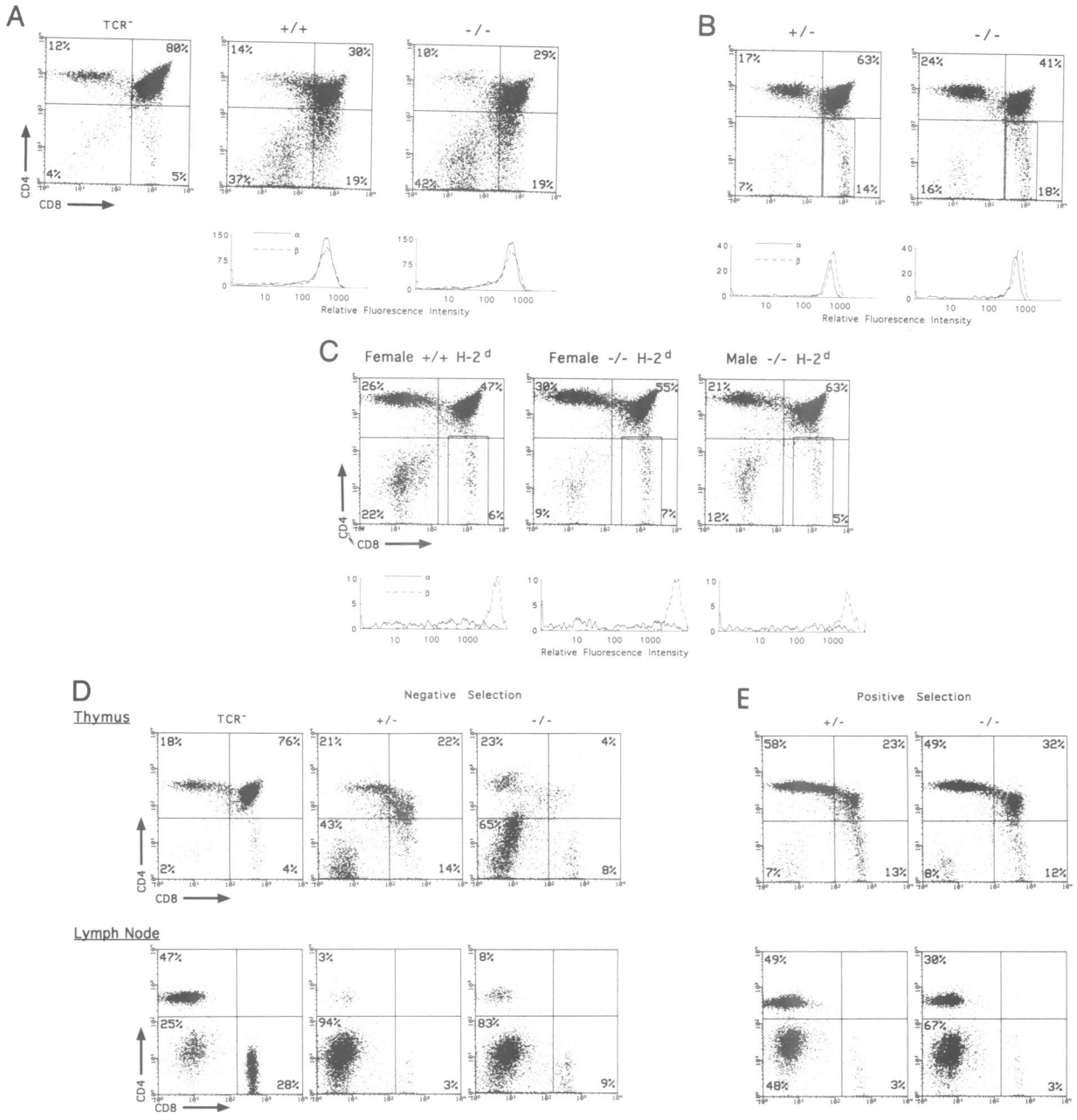

Fig. 6. Positive and negative selection of CD2-deficient T cells analysed in T cell receptor transgenic mice. Class I-restricted TCR transgenic mice (A–C). Thymocytes from male (**A**) and female (**B**) H-2^b mice carrying the D^b-restricted H-Y-specific transgenic TCR were stained with fluorescein–anti-CD8, phycoerytherin–anti-CD4 and either T3.70–biotin (transgenic TCR α chain) or F23.1–biotin (transgenic TCR β chain) followed by streptavidin-tandem conjugate. Male H-2^b TCR transgenic thymuses contained 2- to 6-fold fewer cells than non-transgenic controls. Ten thousand gated events were analysed on a Becton Dickinson FACScan flow cytometer. Dot plots show the expression of CD4 and CD8 on representative TCR transgenic and non-transgenic (TCR$^-$) cells of the indicated CD2 genotype. Histograms show the expression of the transgenic α and β chains on either total thymocytes (A) or CD8$^+$ thymocytes (gated within the rectangle in B). Control H-2^d mice expressing the transgenic TCR (**C**). Lack of staining for the transgenic α chain in the gated CD8$^+$ thymocytes indicates absence of positive selection. Instrument settings for C differed from those used in A and B. FACS profiles for peripheral lymphocytes from CD2-deficient mice were also indistinguishable from those of CD2-expressing control mice (data not shown). Class II-restricted TCR transgenic mice (D and E). Thymocytes and peripheral lymphocytes from mice expressing the pigeon cytochrome C-specific transgenic TCR were analysed. Cells were stained with fluorescein–anti-CD8 and phycoerytherin–anti–CD4. Five thousand gated events were analysed on a Becton Dickinson FACScan flow cytometer. **D.** Negative selection in mice expressing I-E^d and the MTV-6 endogenous murine retrovirus (detected by Southern blot on a *Pvu*II digest of tail DNA using an MMTV LTR probe (data not shown; Frankel *et al.*, 1991). **E.** Positive selection of CD4$^+$ cells in MTV-6$^-$ H-2$^{b/d}$ mice.

lymph nodes, which were depleted in both CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells and were dominated by CD4$^-$CD8$^-$ Vβ3$^+$Vα11$^+$ T cells (Figure 6D and data not shown). Both control and CD2$^-$ mice showed a similar pattern of deletion. Likewise, strong positive selection of CD4$^+$CD8$^-$ cells was observed in mice lacking MTV-6 regardless of whether CD2 was expressed (Figure 6E). Positive and negative selection of T cells bearing either MHC class I- or MHC class II-restricted TCRs were therefore not grossly affected by the absence of CD2.

Discussion

A large body of data has suggested that CD2 plays an important role in lymphocyte function. Two key findings

are the demonstration that CD2 binds to several widely expressed cell surface ligands and that antibody cross-linking of the CD2 molecule activates a mitogenic pathway. The mice described in this paper carry a null allele of the CD2 gene, permitting a direct *in vivo* evaluation of CD2 function. These mice have normal numbers of T cells in their peripheral lymphoid tissues and display no obvious developmental abnormalities. Furthermore, in CD2 −/− mice expressing transgenic T cell receptors, the patterns of positive and negative selection of thymocytes were no different from those observed in mice that expressed CD2. T cells from CD2-deficient mice were also shown to be competent in providing specific help to antibody-producing B cells, in proliferating in response to foreign MHC proteins, and in generating allo-specific cytotoxic T cells. In other experiments, CD2 −/− mice displayed normal NK cell activity and had normal CTL responses against lymphocytic choriomeningitis virus (N.Killeen, W.Seaman, M.Oldstone and D.R.Littman, unpublished results).

A possible interpretation of these results is that murine CD2 mediates a redundant adhesive function that can be effectively performed by other T cell surface components in its absence. Adhesion between T cells and their targets involves several different ligand pairs, including CD11a/CD18 binding to ICAM-1/ICAM-2 and CD5 binding to CD72 (Dustin and Springer, 1991; van de Velde *et al.*, 1991). It seems plausible that for many T cell interactions, these other molecules may adequately substitute for the CD2 deficiency. Alternatively, other T cell surface components may interact with the same ligands as CD2, providing a means for compensation in the CD2-deficient mouse. Both possibilities are consistent with the observation that unlike human T cell clones, a number of murine clones lack CD2 expression, yet retain functional activity (Duplay *et al.*, 1989). Other experiments have shown that following transfection into the murine $CD2^-$ hybridoma 2B4, CD2 confers novel reactivity to low doses of specific peptide (Ohno *et al.*, 1991a). Thus, while CD2 may not be essential for all T cell responses, its presence may endow the responding T cell with a relatively lower activation threshold and permit responses when antigen dose is limiting. Development in the absence of CD2 may select for a repertoire of T cells with qualities that compensate for this otherwise lower sensitivity to antigen. This compensation may take the form of increased TCR affinity or upregulation of other cell adhesion molecules.

Signal transduction through CD2 appears to involve components of the TCR−CD3 complex (Moingeon *et al.*, 1989, 1992; Ohno *et al.*, 1991b; Spruyt *et al.*, 1991). Several studies have shown that CD2 can be co-precipitated with the TCR−CD3 complex (Brown *et al.*, 1989) as well as CD4, CD8, CD5 (Beyers *et al.*, 1992) and CD45 (Schraven *et al.*, 1990; Dianzani *et al.*, 1992); these putative interactions of CD2 with other cell surface components may be important for CD2-mediated mitogenesis. Furthermore, antibodies against CD2 exert a negative influence on antigenic activation of the 2B4 hybridoma; this effect is dependent on the presence of the CD2 cytoplasmic tail (Ohno *et al.*, 1991a). The finding of adequate immune responses in CD2-deficient mice suggests that the function of CD2 in T cell surface complexes is not essential and that its absence can be compensated for by other molecules, which may themselves be members of the multi-component T cell activation complex (Moingeon *et al.*, 1992; Wegener *et al.*, 1992). In light of these results, the inhibition of T cell responses by anti-CD2 monoclonal antibodies could be interpreted as a steric effect involving indirect disruption of complexes required for signal transduction.

In different species, monoclonal antibodies against CD2 have distinct effects on T lymphocyte function. For example, human and rat CD2 transduce activation signals when cross-linked appropriately (Meuer *et al.*, 1984; Clark *et al.*, 1988), but this has not been observed for mouse CD2. Furthermore, inhibition of *in vitro* T cell function is profound for monoclonal antibodies recognizing human CD2 (Springer *et al.*, 1987), but is comparatively weaker with antibodies against the mouse counterpart (Nakamura *et al.*, 1990). Together, these observations suggest that relative to other species, the immune system of the mouse may be less dependent on CD2 function, perhaps due to more extensive uilization of other pairs of adhesion and signal transduction molecules. Thus, a CD2 deficiency may affect humans and rats more markedly than is shown here for mice.

In conclusion, we have so far been unable to detect any developmental or functional defect in the immune system of mice that lack expression of CD2. This result may indicate a redundancy in CD2 function or suggest a subtle function which would not be detected by the experiments described here (Rajewsky, 1992). Further studies of mice bearing mutations in CD2 and in additional components involved in T cell adhesion and signal transduction will be required to resolve this problem.

Materials and methods

Homologous recombination and embryonic stem cells

The CD2 targetting vector was constructed using a CD2 genomic clone isolated from a murine genomic library provided by Dr Glen Evans (The Salk Institute, La Jolla) and plasmids pMC1neopolyA (Stratagene) and pIC19R/MC1-TK (from Dr Kirk Thomas, University of Utah). D3 ES cells (Doetschmann *et al.*, 1985) were kindly provided by Dr Thomas Doetschmann (University of Cinncinnati). These were grown according to the protocols described by Robertson (1987) except that the culture medium was 15% fetal calf serum with no additional newborn calf serum. 2×10^7 cells were transfected by electroporation of 20−25 μg *Cla*I-linearized CD2-targetting vector in 0.8 ml of PBS at 250 V/500 μF. 5×10^6 cells were seeded on 10 cm plates containing 4×10^6 mitomycin C-inactivated neo^r STO feeder cells (gift of Dr Elizabeth Robertson, Columbia University, New York). Selection with 150 μg/ml of G418 was imposed after 36 h. Gancyclovir was not used. Colonies were transferred into 96 well plates containing feeder cells after 12−14 days, at which time selection was removed. Two days later, each colony was split in two parts; half of each clone was returned to culture while the rest was used in pools of 10−30 clones for PCR analysis. DNA was extracted from these pools according to a modification of the protocol described by Bowtell (1987). Briefly, the cells were pelleted and resuspended in a small volume of PBS. 7−10 vol of 6 M guanidine hydrochloride−0.1 M sodium acetate were added and the mixture was rotated for 1 h before precipitating with ethanol and resuspending in TE at 55°C (1−2 h) and 95°C (10 min). PCR was performed for 35 cycles with denaturation for 1 min at 94°C, annealing at 55°C for 1 min and extension at 72°C for 3 min. PCR-positive pools were expanded and reanalysed in smaller pools until the individual clones contributing the PCR signal were identified. DNA was extracted for Southern blot analysis and the cells were frozen at the earliest possible passage.

Blastocysts were harvested from C57BL/6 mice ~3.5 days post coitus. These were injected with targetted ES cells as described by Bradley (1987) and Hogan *et al.* (1986). Injected blastocysts were reimplanted into the uteri of pseudopregnant C57BL/6×DBA/2 females. Chimeric progeny were identified by coat color and the males were mated to C57BL/6×DBA/2 females. Germline transmission of the agouti marker and the neo^r gene identified mice carrying the CD2 disruption, and these were intercrossed to produce homozygous null mice. Screening of mice for the CD2 mutation

was achieved by PCR, Southern blot or routinely by FACS analysis of peripheral blood, which can identify all genotypes.

RNA and Northern blot analysis

RNA was prepared from disaggregated thymocytes and lymph node cells according to a modification of the guanidinium thiocyanate–CsCl procedure described by Chirgwin *et al.* (1979). Total RNA was electrophoresed in formaldehyde gels and transferred to GeneScreen Plus membranes prior to hybridization according to the manufacturer's instructions.

Antibodies and flow cytometry

$1-2\times10^6$ cells were stained with saturating levels of antibodies and 5–10 000 gated events were acquired and analysed using a Becton Dickinson FACScan flow cytometer and Lysys software. Unconjugated antibodies were detected with FITC-labelled goat anti-mouse reagents from Becton Dickinson or Jackson ImmunoResearch. FITC-conjugated anti-CD8 (53-6.7) and PE-conjugated anti-CD4 (GK1.5) were from Becton Dickinson. Biotinylated, FITC- and PE-conjugated antibodies against CD2 (RM2-5), CD3ϵ (2C11), CD5 (53-7.3), CD45R (16A), Ly6C (AL-21), TCR-$\alpha\beta$ (H57-597), TCR-$\gamma\delta$ (GL3), PGP-1 (IM7), Vβ3 (KJ-25), Vα11 (RR8-1) and IL-2Rα (7D4) were purchased from PharMingen. PE-anti-B220 (RA3-6B2), FITC-anti-Thy1.2 and biotin–anti-CD8 (YTS 169.4) were from Caltag. Biotinylated F23.1 (Anti-Vβ8.1,8.2,8.3) and T3.70 (anti-clonotypic TCR-Vα) were a gift of Dr Hung-Sia Teh (UBC, Canada). Anti-CD2 monoclonal antibodies RM2-1, RM2-2, RM2-3, RM2-5 and RM2-6 (Nakamura *et al.*, 1990) were provided by Dr Hideo Yagita (Juntendo University, Japan). H141-31 (anti-D^b) was purchased from BioProducts for Science. M1/69 (anti-Heat Stable Antigen), AF6-88-5.3 (anti-K^b) and SF1-1.1.1 (anti-K^d) tissue culture supernatants were obtained from ATCC cell lines. Biotin groups were detected with streptavidin conjugated to PE (Becton Dickinson), PE coupled to Texas Red (Southern Biotechnology Associates) or PE coupled to Cy5 (Caltag).

Mixed lymphocyte and cytotoxic T cell assays

Responder cells were isolated from cervical, brachial, axillary and mesenteric lymph nodes. For some experiments, B cells were removed by panning for 45 min at 4°C on dishes coated with goat anti-mouse Ig. Stimulator splenocytes were treated for 30 min with anti-Thy-1 monoclonal antibody and guinea pig complement (Gibco) followed by irradiation (~2000 Rad) from a ^{137}Cs source. Culture medium was RPMI supplemented with 10% fetal calf serum, 100 μM non-essential amino acids, 110 μg/ml pyruvate, 5×10^{-5} M β-mercaptoethanol, penicillin and streptomycin. Proliferation assays were set up in a volume of 200 μl in round-bottom wells of microtiter plates and incubated for 3–4 days before pulsing for ~18 h with 1 μCi of [^{3}H]thymidine per well. Incorporated radioactivity was detected following harvesting using a Pharmacia Microtiter plate Cell Harvester. MLRs for generating cytotoxic T cells were set up in T25 tissue culture flasks in 10 ml containing 2×10^7 responder and 2×10^7 stimulator cells. After 5 days, the cells were harvested and tested for cytotoxic activity in standard 4 h ^{51}Cr-release assays using P815 and EL-4 cells as targets.

Immunizations and ELISAs

Reagents for immunizations and ELISAs were kindly provided by Dr Robert Coffman of DNAX (TNP-KLH, TNP-FγG, IgM and IgG1 anti-TNP control antibodies) or purchased from PharMingen (biotin–rabbit anti-mouse Igs and biotin–anti-IgM), Southern Biotechnology Associates (biotin–goat anti-mouse IgG1), Jackson ImmunoResearch (streptavidin-conjugated horseradish peroxidase) and Sigma (ABTS, i.e. 2′,2′-Azinobis(3-ethylbenz-thiazoline sulfonic acid)). Mice were immunized intraperitoneally with 10 μg TNP-conjugated KLH mixed with 2 mg potassium alum in a total volume of 200 μl and bleeds were taken at 0, 10 and 14 days, and also 7 days after secondary immunization (+2 months). Sera were analysed for TNP-specific antibodies by indirect isotype-specific ELISA (Coffman and Carty, 1986) calibrated using known concentrations of U7.6 (IgG1) and U13.6 (IgM) anti-TNP monoclonal antibodies. Plates were coated with 10 μg/ml TNP-FγG. Optical Density determinations were made with a Molecular Devices ELISA reader and SoftMax software.

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