CD45-Null Transgenic Mice Reveal a Positive Regulatory Role for CD45 in Early Thymocyte Development, in the Selection of $CD4^+CD8^+$ Thymocytes, and in B Cell Maturation

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Summary

The CD45 transmembrane glycoprotein has been shown to be a protein phosphotyrosine phosphatase and to be important in signal transduction in T and B lymphocytes. We have employed gene targeting to create a strain of transgenic mice that completely lacks expression of all isoforms of CD45. The spleens from CD45-null mice contain approximately twice the number of B cells and one fifth the number of T cells found in normal controls. The increase in B cell numbers is due to the specific expansion of two B cell subpopulations that express high levels of immunoglobulin (IgM) staining. T cell development is significantly inhibited in CD45-null animals at two distinct stages. The efficiency of the development of $CD4^-CD8^-$ thymocytes into $CD4^+CD8^+$ thymocytes is reduced by about twofold, subsequently the frequency of successful maturation of the double positive population into mature, single positive thymocytes is reduced by a further four- to fivefold. In addition, we demonstrate that CD45-null thymocytes are severely impaired in their apoptotic response to cross-linking signals via T cell receptor (TCR) in fetal thymic organ culture. In contrast, apoptosis can be induced normally in CD45-null thymocytes by non-TCR–mediated signals. Since both positive and negative selection require signals through the TCR complex, these findings suggest that CD45 is an important regulator of signal transduction via the TCR complex at multiple stages of T cell development. CD45 is absolutely required for the transmission of mitogenic signals via IgM and IgD. By contrast, CD45-null B cells proliferate as well as wild-type cells to CD40-mediated signals. The proliferation of B cells in response to CD38 cross-linking is significantly reduced but not abolished by the CD45-null mutation. We conclude that CD45 is not required at any stage during the generation of mature peripheral B cells, however its loss reveals a previously unrecognized role for CD45 in the regulation of certain subpopulations of B cells.

CD45 is a transmembrane glycoprotein of variable molecular weight (180–220 kD) that is expressed exclusively by cells of the hemopoietic lineage (for a review see reference 1). Alternative splicing of the nascent RNA transcript generates several different protein isoforms that possess an intrinsic protein phosphotyrosine phosphatase activity in their common cytoplasmic domains (1, 2). CD45 is believed to play an important role in antigen receptor signaling in T and B lymphocytes (2). Mouse and human T cell lines that are deficient in CD45 expression are defective in their ability to respond to signals via their TCR–CD3 complex (2–5). CD45-deficient B cell lines also fail to make responses to surface Ig–B cell receptor (BCR)[1] complex cross-linking (6). Various groups have also demonstrated that TCR–CD3 signaling can be restored in CD45-deficient T cell lines by transfection of phosphatase-active CD45 or of membrane-bound chimeric proteins containing the intact CD45 cytoplasmic domains (7–11), but not by expression of an enzymatically inactive CD45 mutant (12).

[1] *Abbreviations used in this paper:* BCR, B cell receptor for antigen; DN, double negative; DP, double positive; ES, embryonic stem; FTOC, fetal thymic organ culture; PTK, protein tyrosine kinase; SP, single positive.

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T cell development involves two distinct selection checkpoints (13). Early thymocytes rearrange their TCR-β chain genes and, if rearrangement produces a functional TCR-β chain, express a pre-TCR complex in which TCR-β associates with pTα and CD3 (14). Formation of this pre-TCR complex is required for further development of the early precursors into immature $CD4^+CD8^+$ thymocytes (14, 15) that subsequently rearrange their TCR-α chain genes. At least one further positive signal is required by the immature TCR-α/β^+ cells before they complete their maturation and migrate to the periphery. This second positive signal requires an interaction of TCR-α/β with self-MHC complexed with an appropriate peptide (for review see references 13, 16). However, by means still obscure, MHC/peptide ligands can also generate a negative signal giving rise to death by apoptosis, thus permitting maintenance of self-tolerance (16). The negative signal may be mimicked in fetal thymic organ culture (FTOC) by CD3 cross-linking (17).

We have employed gene targeting to generate a transgenic mouse model in order to study the functions of CD45. By specifically targeting an exon common to all isoforms (exon 9) we have achieved the first complete knockout of the CD45 gene. This new line of transgenic mice is hereafter referred to as CD45-null mice. We have analyzed the phenotype of B cells from CD45-null mice and provide evidence for a possible role for CD45 in the regulation of peripheral B cell maturation. We report that CD45 is an important positive regulator of the signals mediated via the pre-TCR and TCR complexes that lead to maturation or to apoptosis. Consequently, T cell development is severely impaired in CD45-null transgenic mice. Apoptosis as a result of CD3 cross-linking in FTOC is almost completely blocked, whereas the apoptotic pathway triggered by calcium ionophore, glucocorticoid, or via the Fas antigen is normal.

We have used this model to study the requirement for CD45 in B cell activation both via the antigen receptor and through other pathways known to trigger tyrosine phosphorylation. In human B cell lines, triggering of CD40 by mAbs or CD40L induces tyrosine phosphorylation of a number of substrates including protein tyrosine kinases (PTKs) Lyn, Fyn, and Syk, phospholipase Cγ2, and phosphatidylinositol-3-kinase (18, 19). The Lyn kinase has been implicated in this process (18). A role for CD45 in the regulation of CD40 signaling has been suggested by the effects of anti-CD45 mAbs on CD40-mediated proliferation (19–21). Murine CD38 is a transmembrane type II glycoprotein with ADP-ribosyl cyclase activity and is expressed on resting and activated B cells (22, 23). Triggering of CD38 with mAbs results in an influx of calcium from the extracellular milieu and an increase in tyrosine phosphorylation (24); it also induces proliferation of small resting B cells and LPS-activated B cell blasts (22). We report that CD45 is differentially required for signaling through the BCR, CD40, and CD38.

Materials and Methods

Generation of Transgenic Mice. Gene targeting was achieved using a replacement-type targeting vector containing an 8.0-kb BamHI-EcoRI fragment of isogenic genomic DNA encompassing exons 5–9 of CD45. The pMC1NeopA cassette, corrected for a small point mutation thought to reduce the transformation efficiency of embryonic stem (ES) cells (25), was inserted into a BsmI site in exon 9 in inverse transcriptional orientation to the CD45 gene. Transfections were performed with passage 13 of the (129/Sv × 129/Sv-CP)F1 ES line R1 (kindly provided by A. Nagy and J. Rossant, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). In a typical experiment, 10^7 cells in 250 μl Hepes buffered saline were transfected with 40–80 μg/ml linearized construct at 0.4 kV, 25 μF. Targeted clones were screened by Southern blotting using a probe external to the targeting construct. The targeting frequency was in the range of 1/32–1/188 $G418^R$ cells. A single clone (5-89) was used to generate, by blastocyst injection, 10 fertile male chimeras, 5 of which gave germline transmission. Genotyping was by Southern blotting of tail DNA. Homozygous mutant animals were generated by intercrossing $(R1 \times C57BL/6)F_1$ $CD45^{+/-}$ heterozygotes or by intercrossing (R1 × B6) mice backcrossed to B6 for four generations (BC4 × BC4). The CD45 genotype of all individuals used in these experiments was determined by Southern blotting. Animals were maintained in a barrier unit. Unless otherwise stated, experiments were performed on 5–9-wk-old animals. Timed matings were performed by pairing a $CD45^{-/-}$ or $^{+/-}$ male with one to two $CD45^{+/-}$ females overnight. The day of plug was considered day 0.

Cell Preparation. Thymuses were obtained by dissection from 5–8-wk-old mice or fetuses. Spleens were obtained from 5–9-wk-old mice. In both cases, tissues were disaggregated by mincing with fine forceps and forced through a fine mesh filter to generate a single cell suspension. Total cell numbers were determined by microscopic observation using a Neubauer hemocytometer.

Western Blotting. Single cell suspensions were prepared from pooled thymuses from homozygous mutant or wild-type mice. Cells were lysed at 2×10^8 cells/ml in 10 mM Tris, pH 7.3, 1 mM $MgCl_2$, 0.5% NP-40, 0.02% sodium azide, and 0.1 mM PMSF. Aliquots of each lysate were electrophoresed through a nonreducing 7% SDS-PAGE gel and electroblotted onto PVDF membranes (Immobilon™-P; Millipore Corp., Bedford, MA). Membranes were blocked for 2 h at 37°C in TSW buffer (10 mM Tris, pH 7.4, 0.1 M NaCl, 0.1% Triton X–100, and 0.2% SDS) containing 3% BSA. Primary antibodies were an anti-CD45 cytoplasmic domain rabbit antiserum (kindly provided by M.L. Thomas; Washington University, St. Louis, MO) and heat-inactivated normal rabbit serum as a negative control, both at 1/1,000 in TSW containing 3% BSA and 0.1% sodium azide. Incubation was performed for 16 h at 4°C with gentle shaking, followed by five 30-min washes in TSW, supplemented with 0.5 M NaCl and 1% BSA for the second and fifth washes, respectively. Secondary antibody was peroxidase-conjugated goat anti–rabbit Ig (Sigma Chemical Co., St. Louis, MO) at a 1/1,000 dilution in TSW 3% BSA. Incubation was performed at room temperature for 2 h with gentle shaking, followed by four 15-min washes in TSW. Specific binding was visualized using enhanced chemiluminescent detection reagents for Western blotting (ECL; Amersham International, Amersham, Bucks., UK).

Flow Cytometry. The following reagents and mAbs were used: 145-2C11 (hamster IgG, anti-CD3ε) was obtained as purified IgG (Cedarlane Laboratories, Ltd., Hornby, ON) or directly biotinylated (PharMingen, San Diego, CA); YTS 191.1 (rat IgG2b; anti-CD4) was obtained as a PE conjugate (Caltag Laboratories, South San Francisco, CA); FITC-conjugated YTS 105.18 (rat IgG2a, anti-CD8), biotinylated YTS 105.18, biotinylated YTA3.1 (rat

IgG2b, anti-CD4) and YBM 42.2 (rat IgG2a, anti-CD45) were kind gifts from S.P. Cobbold, Oxford University, Oxford, UK (26, 27). FITC-conjugated 3C7 (rat IgG2b, anti-CD25) was purchased from Sigma Chemical Co.; PE-conjugated IM7.8 (rat IgG2b, anti-CD44), Jo2 (hamster IgG, anti-Fas), and PE-conjugated R8-140 (rat IgG1, anti IgL κ) were purchased from PharMingen. b7.6 (rat IgG1, anti-IgM) and 1.19 (rat IgG2a, anti-IgD) were kindly provided by R.M.E. Parkhouse (Institute of Animal Health, Pirbright, UK). Single cell suspensions of thymocytes or splenocytes were stained sequentially with appropriate combinations of these reagents in the presence of 2.5% (vol/vol) heat-inactivated normal rabbit serum. Unconjugated antibodies were detected by incubation with an appropriate isotype-specific secondary reagent: biotinylated MARG1 (for IgG1) or FITC-conjugated MARG2a (for IgG2a), both from Serotec Ltd., Kidlington, OX, UK. Biotinylated antibodies were stained with either FITC-Extravidin™ (Sigma Chemical Co.) or with PE-streptavidin or Tricolor™-streptavidin (both from Caltag Laboratories). Samples were fixed after staining with 1% formaldehyde and analyzed by FACS® (Becton Dickinson & Co., Mountain View, CA) using a wide gate incorporating large and small lymphocytes. 20,000 events per sample were collected in list mode, and analyzed using Lysys II software.

FTOC. Thymus lobes from 14-d gestation embryos were grown in organ culture according to Jenkinson and Anderson (28). Pairs of thymus lobes from individual embryos were cultured separately. To identify the phenotype of each mouse/thymus, crude liver cell preparations from each embryo were analyzed by FACS® for surface CD45 expression, and the genotype was routinely confirmed by Southern blotting of DNA from embryonic tissue. After 7 d in FTOC, thymocytes were released from the thymus by gentle chopping with fine knives. Quantitation of subpopulations was performed using FACS® as described above. As thymocytes from $CD45^{+/+}$ and $CD45^{+/-}$ mice were always equivalent, these were pooled (referred to as $CD45^{+}$), and directly compared with CD45-null ($CD45^{-/-}$) littermates.

Measurement of Apoptosis. Total DNA was prepared from 10^6 thymocytes immediately after release from the thymus, as in Smith et al. (17), and subjected to agarose gel electrophoresis.

Proliferation Assays. Splenocytes were incubated at 10^5 cells per well in RPMI supplemented with 5% FCS. Mitogens were added as follows: PMA (a gift of D. Wraith, Bristol University, Bristol, UK) at 50 ng/ml and ionomycin (Molecular Probes, Inc., Eugene, OR) at 400 ng/ml; LPS (*Escherichia coli*–derived, a gift of D. Wraith) at 3 μg/ml; $F(ab')_2$ fragments of goat anti–mouse IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 2–10 μg/ml; anti-CD40 (3/23, Serotec) at 1–10 μg/ml and anti-CD38 (NIM-R5, endotoxin–low ascites preparation, a gift of R.M.E. Parkhouse) at a 1/500–1/2,000 dilution. In the anti-IgD proliferation assay, splenocytes were incubated at 2×10^5 cells per well in RPMI supplemented with 10% FCS and anti-IgD (purified 1.19, a gift of R.M.E. Parkhouse) was added at 1.25–10 μg/ml. Controls consisted of medium plus serum only. All conditions were prepared in triplicate and incubated at 37°C in 5% CO_2. Unless otherwise stated, cultures were pulsed after 72 h with ^3H[TdR] at 1 μCi/well for 6 h. Results are expressed as the mean of each triplicate.

Results

The Targeted Mutation in Exon 9 of the CD45 Gene Produces a CD45-null Phenotype in Homozygous Mutant Animals. The technique of gene targeting by homologous recombination (29) was employed to insert the MC1NeopA cassette in inverse transcriptional orientation into a unique site in exon 9 of the CD45 gene in ES cells. This mutation introduces a number of stop codons into the CD45 reading frame downstream of the insertion point. A single targeted ES cell clone was used to generate chimeric animals which gave germline transmission of the mutated allele. The complete lack of expression of CD45 in homozygous mutant animals was determined in several ways. FACS® analysis of several different lymphoid compartments was performed using a panel of antibodies specific for CD45 and its isoforms, an example of the results obtained is shown in Fig. 1 *A*. CD45-null splenocytes stained with the pan-CD45 mAb YBM42.2 are indistinguishable from cells stained with an isotype-matched negative control (Fig. 1 *A*). No expression of CD45 was seen with seven other CD45 mAbs on thymocytes, splenocytes, lymph node or bone marrow cells (data not shown). Confirmation of these results was achieved by immunoprecipitation (data not shown) and by Western blotting of thymus lysates using both a pan-CD45 antibody (data not shown) and an antiserum specific for the cytoplasmic domain of CD45 (Fig. 1 *B*).

CD45 Is Not Required for the Development of B Lymphocytes. The percentage and numbers of mature lymphocytes found within the peripheral lymphoid tissue of CD45-null mice were determined by enumerating the total numbers of cells recovered from the spleens of homozygous mutant CD45-null transgenic mice and by analyzing cells stained for flow cytometry with a variety of T and B cell markers. In all cases, wild-type littermates were used as controls. The total numbers of lymphoid cells residing in the spleen of CD45-null mice (mean $178 \pm 54 \times 10^6$, $n = 6$) was consistently significantly greater than for wild-type control mice (mean $89 \pm 17 \times 10^6$, $n = 6$). However, despite some intersample variation, the percentage of B cells in the CD45-null mice fell within the normal range. As a result, CD45-null mice had approximately twice as many splenic B cells ($108 \pm 12 \times 10^6$, $n = 6$) as wild-type control mice ($49 \pm 12 \times 10^6$, $n = 6$). These results indicate that the development of mature B cells does not require CD45 expression.

CD45-null Mice Have Increased Numbers of Splenic B Cells Expressing High Levels of IgM. Analysis of surface Ig expression on CD45-null B cells revealed an altered profile of IgM and IgD staining. In wild-type mice, splenic B cells can be divided into three subpopulations on the basis of IgM versus IgD staining (30). Population I (IgM low, IgD intermediate-high) includes the predominant recirculating resting B cell population in spleen and lymph node. Population III (IgM high, IgD low) includes newly formed immature B cells and B-1 B cells and is not present in lymph nodes of normal mice (30, 31). A third population (II) expresses both IgM and IgD at high levels. In CD45-null mice, a large increase in the percentage of splenic B cells present in populations II and III is seen, whereas population I constitutes the smallest population of splenic B cells (Fig. 2). When total splenic cellularity is considered, population I is present at normal levels indicating that the in-

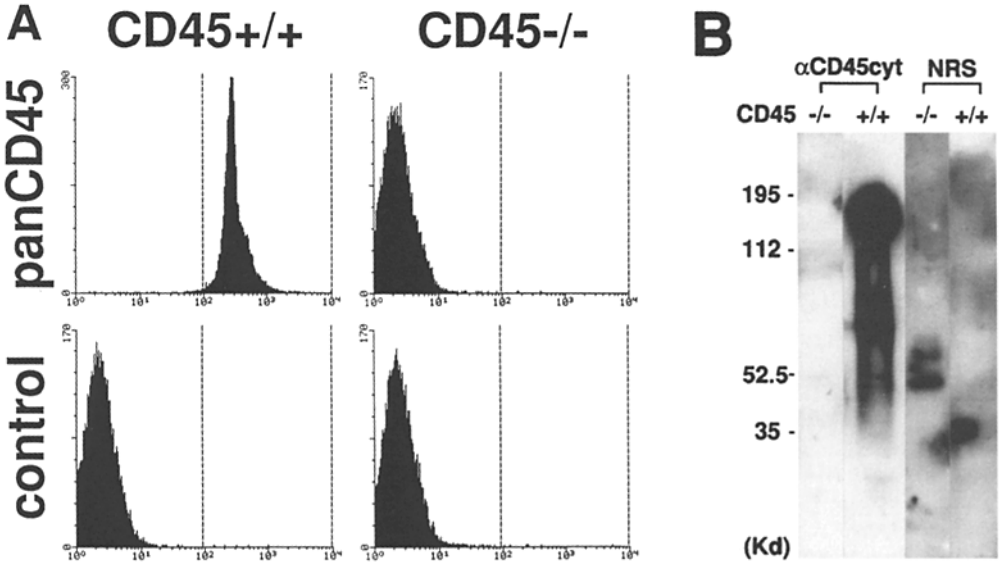

Figure 1. No expression of CD45 on lymphocytes from CD45-null transgenic mice. (*A*) Splenocytes were stained with the pan-CD45 antibody YBM42.2 (*top*) or an isotype-matched negative control YTH76.9 (*bottom*), detected with FITC-conjugated MARG2a and analyzed by flow cytometry. (*B*) Thymus lysates were prepared from CD45-null and wild-type mice and the equivalent of 4×10^6 cells per lane electrophoresed through a nonreducing SDS-PAGE gel and Western blotted. Duplicate samples were probed with anti-CD45 cytoplasmic domain antiserum (αCD45cyt, *left*) or heat-inactivated normal rabbit serum as a negative control (*NRS, right*).

creased splenic cellularity in CD45-null mice is partly due to a large increase in the total number of cells present in populations II and III (Table 1) and also to an increase in the total number of non-B, non-T cells (data not shown).

To investigate further the phenotype of CD45-null B cells, the expression profiles of several B cell surface markers on resting splenic B cells were analyzed (data not shown). Moderate increases were found in the mean fluorescence levels of several cell surface markers including class II MHC (increased by an average of 1.59 ± 0.06–fold over that of wild-type cells), CD40 (average 1.75 ± 0.17–fold increase), and CD19 (average 1.35 ± 0.02–fold). CD45-null and wild-type B cells upregulate both CD40 and class II MHC expression similarly after overnight culture with a combination of anti-CD40 mAb and recombinant IL-4, suggesting that these signals are not regulated by CD45 (data not shown). The mean fluorescence level of CD38 was unchanged.

CD45-null Mice Have Low Numbers of Mature T Cells. Fig. 3 shows the results of a flow cytometric analysis of splenocytes from CD45-null and control littermate mice for simultaneous staining for the CD3/CD4 (*top*) and CD3/CD8 (*bottom*) markers. The percentage of $CD3^+CD4^+$ T cells is very markedly reduced in CD45-null transgenic animals versus their littermate controls, typically by at least 10-fold. When assessed by setting a region around the $CD3^+CD8^+$ cell population in wild-type animals, the apparent percentage of such $CD3^+CD8^+$ cells in the CD45-null mutants is significantly reduced but to a lesser extent than for $CD4^+$ T cells, typically three- to fourfold. How-

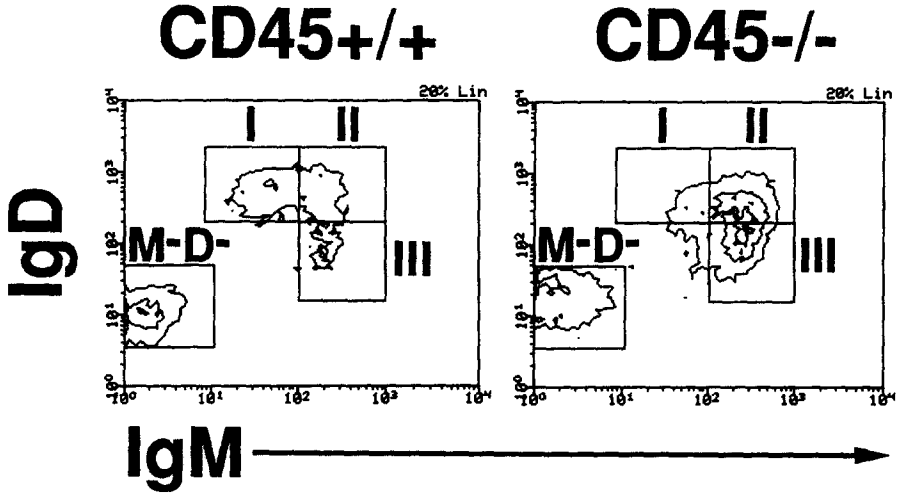

Figure 2. Profiles of IgM versus IgD staining for CD45-null and wild-type splenocytes. Splenocytes were stained with anti-IgM (B7.6, detected with a combination of biotinylated MARG1 and PE-coupled streptavidin) and anti-IgD (1.19, detected with FITC-conjugated MARG2a) and analyzed by two-color flow cytometry. Contour plots were generated for each sample showing IgM and IgD fluorescence on the x and y abscissa, respectively. Square gates show the distribution of the three major populations: I (IgD high-intermediate, IgM low), II (IgD high, IgM high), and III (IgD low, IgM high) and M-D- (DN cells). Both plots are representative of many similar profiles. Mice were 5-wk-old.

Table 1. *Percentage of B Cells in Subpopulations Defined by IgM and IgD*

	$CD45^{+/+}$		$CD45^{-/-}$	
	Percent B cells	Total B cells ($\times 10^{-6}$)	Percent B cells	Total B cells ($\times 10^{-6}$)
Population I	47.66 ± 7.59*	20.86 ± 5.70‡	22.14 ± 5.14*	21.72 ± 4.88‡
Population II	35.63 ± 8.35	17.32 ± 3.21	44.95 ± 7.63	47.22 ± 7.65
Population III	16.71 ± 4.81	8.50 ± 3.66	32.91 ± 11.00	29.40 ± 12.74

*Splenocytes were stained with anti-IgM (B7.6) and anti-IgD (1.19) and analyzed by FACS®. The percentage of splenic B cells in each population (corresponding to the regions in Fig. 2) is shown.
‡The total number of B cells in each population was calculated from the percentage of splenocytes in each population (using regions indicated in Fig. 2) and hemocytometer measurements of total lymphocytes. Mice were 5–10-wk-old. Data are expressed as an arithmetic mean ± SD. A minimum of six mice were included in each group.

ever careful inspection of the FACS® plots suggests the percentage of $CD8^+$ T cells may be overestimated in the CD45-null animals since most of the gated cells are only weakly stained for CD3 and CD8 and do not form a clearly distinct population as in the wild-type controls.

When the increased splenic cellularity of homozygous mutant animals is taken into account, the total numbers of T cells present in the spleen of CD45-null transgenic animals is ~20% of that of wild-type ($6 \pm 3 \times 10^6$, $n = 4$ for mutants compared with $27 \pm 6 \times 10^6$, $n = 4$ in wild-type animals).

Thymocyte Development Is Impaired at Two Distinct Points. To understand the basis for the reduced numbers of peripheral T cells in CD45-null transgenic mice, we have investigated the development of T cell precursors in the thymus. At a gross level, there is an ~50% reduction in the total numbers of thymocytes recovered from null mutant animals ($97 \pm 30 \times 10^6$, $n = 5$) versus their wild-type control littermates ($199 \pm 40 \times 10^6$, $n = 4$). No differences were seen in the numbers of thymocytes recovered from heterozygous animals ($191 \pm 46 \times 10^6$, $n = 4$) compared with wild-type controls.

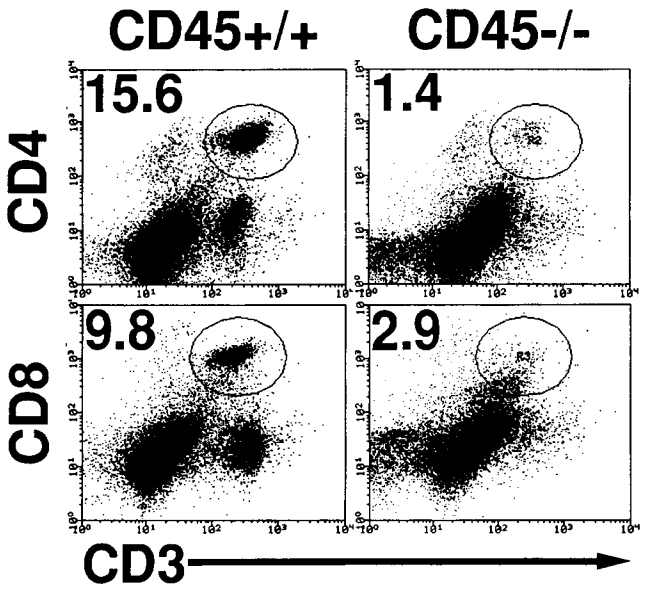

Figure 3. The frequency of splenic T cells is greatly reduced in CD45-null mice The percentages of $CD4^+$ and $CD8^+$ T cells in the spleen of a CD45-null homozygous mutant mouse are compared with a control wild-type littermate. Splenocytes from 5-wk-old mice were stained with PE-CD4, FITC-CD8, and biotin-CD3 (+Tricolor™-streptavidin) and 20,000 lymphocytes analyzed by FACScan® (Becton Dickinson & Co.). The percentages of total lymphocytes in the indicated region are shown in each panel. The fluorescence levels are displayed on a four-decade logarithmic scale.

The phenotype of the thymocytes recovered from CD45-null animals was clearly different from control mice in two ways. Fig. 4 *A* shows the results of an analysis for CD4/CD8 expression. CD45-null animals have a distinctly lower percentage of $CD4^+$ $CD8^-$ single positive (SP), mature thymocytes (2.8 versus 16.7%, Fig. 4 *A*). In addition, the mutant animals have an elevated percentage (32 versus 10%, Fig. 4 *A*) of $CD4^-CD8^-$ double negative (DN) precursors. The percentage of mature $CD8^+$ SP cells is also reduced in mutant animals. In Fig. 4 *A* this is obscured by the presence of an increased number of $CD4^{lo}$ $CD8^+$ intermediate thymocytes, but on close inspection it is clear that the $CD4^-CD8^+$ SP population is greatly reduced; this can also be elucidated by gating for $CD3^{hi}$ cells (data not shown). In addition, the level of both CD4 and CD8 expressed by the immature $CD4^+CD8^+$ double positive (DP) cells is elevated in CD45-null mutants versus controls.

To further investigate the DN precursors present in mutant animals, we analyzed cells stained simultaneously for CD4+CD8+CD3 (single color) and independently for CD25 and for CD44. When the CD25 and CD44 expression of the DN precursors (or triple negative in this case) was examined, only the subset of cells with the $CD25^+$ $CD44^-$ phenotype was found to be present at increased frequency (Fig. 4 *B*). Concomitantly, the percentage of $CD25^-$ $CD44^-$cells was reduced (Fig. 4 *B*).

The actual numbers of thymocytes at different stages of development that were recovered from CD45-null transgenic animals and control littermates are shown in Table 2. These data clearly indicate that the number, as well as the percentage, of DN precursors is higher in CD45-null transgenic mice. In addition, the absolute number of intermedi-

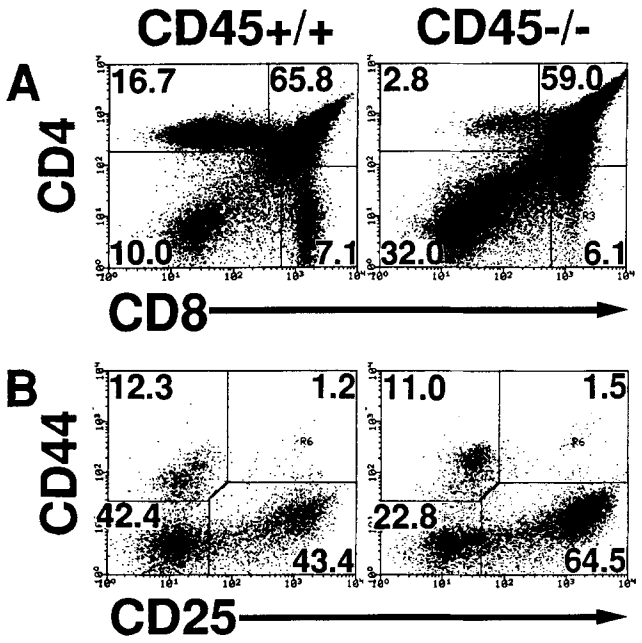

Figure 4. Thymic development is inhibited at both early and late selection checkpoints. Thymocytes from a 5-wk-old CD45-null mutant and a wild-type control littermate were stained as below and 50,000 cells analyzed by FACScan®. The percentages of the relevant gated cells in the regions marked on each panel are shown. (*A*) Cells were stained with PE-CD4 and FITC-CD8. (*B*) Cells were stained with a cocktail containing biotinylated antibodies to CD4, CD8, and CD3 and simultaneously with FITC-CD25 and PE-CD44. A gate was set to collect data for CD4, CD8, CD3 triple negative cells. The profile of CD25 versus CD44 staining is shown.

ate DP cells is reduced by more than twofold. Thus, absence of CD45 inhibits the development of DN cells into DP cells. This is most clearly indicated by the ratio of DN/DP cells in the mice. CD45-null transgenic mice have an average DN/DP ratio of 1:2.0, whereas their wild-type littermates have a ratio of 1:7.7. The absence of CD45 also inhibits the development of DP cells into mature SP cells. In absolute numbers, CD45-null mice have ~10% of the $CD4^+$ SP cells found in wild-type mice (Table 2). The ratio of DP/$CD4^+$ SP cells is 17.7:1 in CD45-null mice versus 4.2:1 in wild-type littermates. These data demonstrate that there is a second point at which CD45 exerts a positive effect on thymocyte differentiation, namely at the DP to SP transition which requires positive selection by MHC plus peptide (16).

In addition to the results described above, an ontogenetic analysis of thymocyte development in CD45-null transgenic mice was performed by determining CD4/CD8 profiles at various stages of development (data not shown). The results of this study confirm and expand the results obtained with adult mice. Before the appearance of DP cells, no differences were seen between mutant and wild-type thymuses. The first block in development or partial inhibition of the differentiation of $CD4^-CD8^-$ DN cells was already apparent by day 18 of gestation; the second point of inhibition at the DP to SP transition was apparent on the

Table 2. *CD4 and CD8 Thymocyte Subsets in CD45-deficient Mice*

	Total number of positive thymocytes ($\times 10^{-6}$)		
T cell subsets	$CD45^{+/+}$	$CD45^{+/-}$	$CD45^{-/-}$
$CD4^-CD8^-$	17.40 ± 3.85*	13.39 ± 2.79	29.17 ± 10.36
$CD4^+CD8^+$	134.03 ± 29.16	124.95 ± 33.59	59.41 ± 25.96
$CD4^+CD8^-$	32.25 ± 4.70	35.56 ± 6.87	3.35 ± 1.02
$CD4^-CD8^+$	12.12 ± 1.70	13.57 ± 2.94	2.59 ± 1.65
n	4	4	5

*Thymocytes from 5-wk-old mice were stained with anti-CD4 (PE) and anti-CD8 (FITC) and analysed by FACS®. Total number of cells in each subset were calculated from the percentage of positive cells in two-color dot plots and hemocytometer measurements of total thymocytes. Data are expressed as an arithmetic mean ± SD.

day after birth, and the stable adult phenotype was established by 1 wk after birth (data not shown). These observations reinforce the conclusion that the absence of CD45 does not affect the development of thymic stem cells or the entry of prothymocytes into the thymic rudiment since no differences are apparent at day 16 of gestation. By contrast, in the complete absence of CD45 there is a distinct inhibition of thymic development both at the DN to DP and at the later DP to SP transitions.

CD45 Is Required for Negative Selection in FTOC. The FTOC technique (28) was used to study further the development of thymocytes from CD45-null mice. As a first step we confirmed that the development of CD45-null thymocytes in FTOC reproduced the pattern seen in vivo. Thymuses from 14-d embryos were cultured for 7 d in vitro and the number and phenotype of the thymocytes recovered were determined. The data demonstrate that CD45-null thymuses contain an elevated percentage of DN precursors and very few cells of $CD4^+$ SP mature phenotype after 7 d of culture compared with control thymuses; this can be seen by comparing the ratios of the relevant cell populations in unmanipulated cultures shown in Fig. 5 *C*. No differences were noted between $CD45^{+/-}$ heterozygotes and $CD45^{+/+}$ homozygotes (data not shown). The total yield of thymocytes from CD45-null ($^{-/-}$) thymuses was 30–40% lower than from wild-type or heterozygote littermates (compare untreated control cultures in Fig. 5 *B*). This is also consistent with the in vivo data. The lower yield of total thymocytes is essentially accounted for by a reduction in the yield of $CD4^+CD8^+$ DP thymocytes, which in unmanipulated CD45-null thymuses is approximately half that from the $CD45^+$ controls (Fig. 5 *C*, *solid bars*). In addition, the data in Fig. 5 *C* show that more than twice the number of $CD4^-CD8^-$ DN precursors are recovered from CD45-null thymuses than from their $CD45^+$ littermates.

The results of the analyses of thymocyte development

both in vivo and in vitro are consistent with the hypothesis that the CD45-null mutation significantly diminishes the efficiency of positive selection. Further evidence that positive selection is inhibited in CD45-null thymocytes is provided by an increase in the basal level of apoptosis in CD45-null FTOC. Fig. 5 *A* shows the measurement of apoptosis by analysis of the integrity of DNA obtained from FTOC using agarose gel electrophoresis. A comparison of the DNA from unmanipulated thymic lobes shows that there is a clear increase in the degree of fragmentation, characteristic of apoptosis, in CD45-null versus $CD45^+$ control cultures (compare control lanes [*C*] in Fig. 5 *A*).

Negative selection of immature DP thymocytes may be achieved in FTOC by delivering a strong signal via the TCR complex, for example by adding anti-CD3ε mAb (17). Treatment of $CD45^+$ FTOC with anti-CD3 induced a significant level of apoptosis as indicated by DNA fragmentation; in contrast, no increase over the basal level of apoptosis was observed in similarly treated CD45-null thymocytes (Fig. 5 *A*). Furthermore, the yield of total thymocytes from $CD45^+$ thymuses was reduced on average by 38% when anti-CD3 mAb was included in the culture, whereas the yield of CD45-null thymocytes was not affected by anti-CD3 treatment (Fig. 5 *B*). Fig. 5 *C* shows that this reduction was almost entirely accounted for by a loss of $CD4^+CD8^+$ DP thymocytes in wild-type thymuses.

Apoptosis May Be Induced in CD45-null Thymocytes by Non-TCR Stimuli. Although the higher basal level of apoptosis

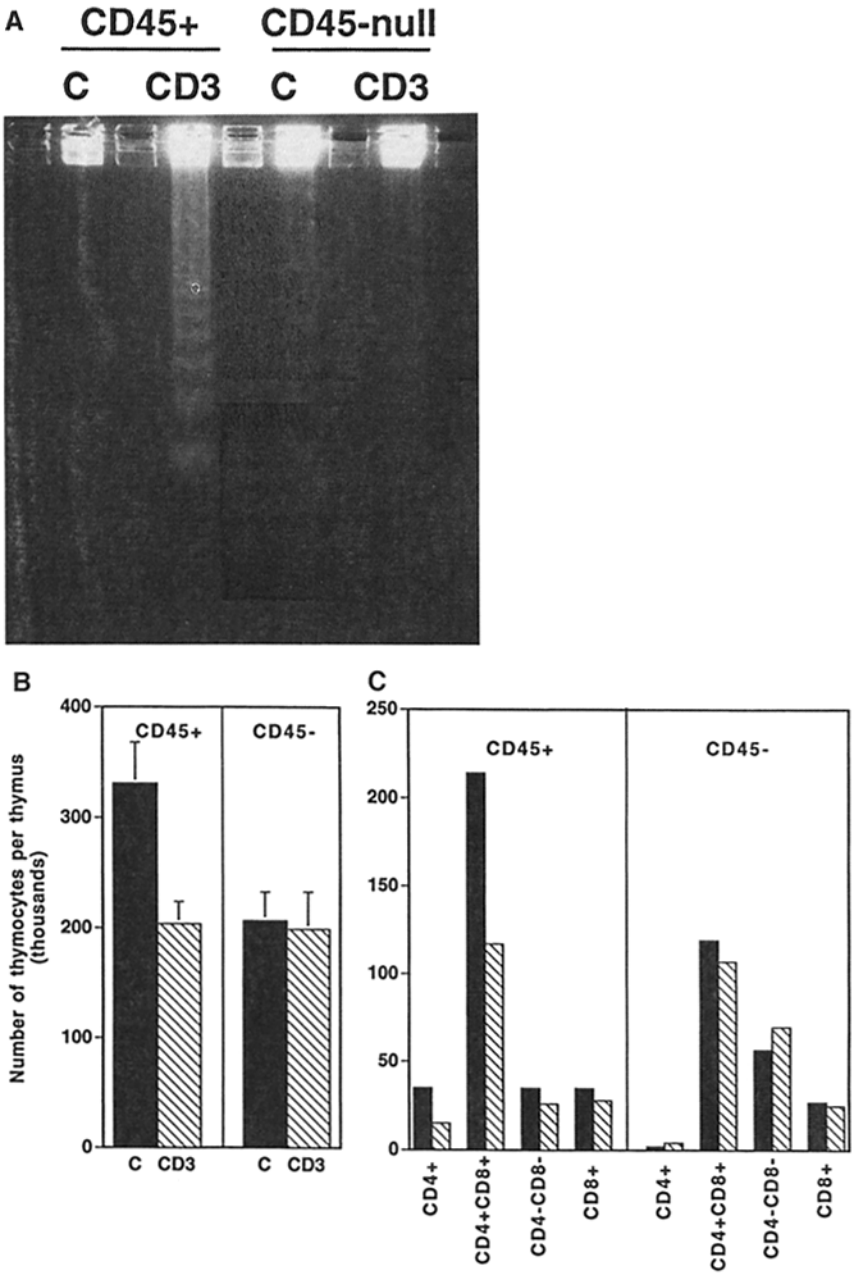

Figure 5. CD3 antibody does not induce apoptosis in CD45-null thymocytes. (*A*) Total DNA from 10^6 thymocytes from $CD45^+$ or CD45-null thymuses with or without anti-CD3 mAb (2 μg/ml) treatment for 15 h was analyzed by gel electrophoresis, as labeled. The presence of small molecular weight DNA fragments indicates the presence of apoptotic cells. (*B*) 7-d FTOC were treated with anti-CD3 mAb (2 μg/ml) for 15–20 h (*hatched bars*) or cultured in medium alone (*solid bars*) after which thymocytes were released and yields of thymocytes determined. The values shown are the means ± SD obtained from five independent experiments. (*C*) Thymocyte subpopulations were analysed by FACS® after surface staining with fluorescent CD4 and CD8 antibodies and the numbers of the four subpopulations determined with reference to total yields. The data shown are from one representative experiment of five separate experiments.

seen in CD45-null thymuses suggested that the apoptotic machinery per se was intact in CD45-null thymocytes, we wished to confirm that stimulation of apoptosis by signals other than via the TCR complex was intact. Similar FTOC experiments were performed in which apoptosis was induced by the calcium ionophore, ionomycin, the steroid dexamethasone, or via the Fas antigen. The results show that these three stimuli were equally effective at producing apoptosis in CD45-null thymocytes as in $CD45^+$ thymocytes (Fig. 6). All three stimuli reduced the number of viable thymocytes recovered by a similar degree in both CD45-null and $CD45^+$ thymuses (Fig. 6). The reduction in thymocyte yields is a direct result of apoptotic cell death induced by the three treatments. This was demonstrated by measuring the percentage of cells with subdiploid DNA content by flow cytometry using 7-amino actinomycin D. In each case, the percentage of apoptotic (subdiploid) cells is similar in $CD45^+$ and $CD45^-$ null thymocytes (data not shown). These data demonstrate that the apoptotic pathway is fully functional in CD45-null thymocytes.

CD45 Is Absolutely Required for B Cell Proliferation in Response to Antigen Receptor Cross-linking and Differentially for CD40 and CD38 Responses. A number of studies have implicated CD45 in signaling through the BCR. Proliferation assays were performed in order to analyze the response of the CD45-null B cells to a number of mitogenic agents. Splenocytes from homozygous mutant animals responded normally to LPS and at reduced levels to PMA plus ionomycin when compared with wild-type cells (data not shown). A mitogenic preparation of polyclonal $F(ab')_2$ anti-IgM produced a strong proliferative response in wild-type splenic preparations, whereas splenocytes from homozygous mutant mice failed to proliferate in response to anti-IgM at concentrations of ≤ 20 μg/ml (Fig. 7 *A*). Similarly, the CD45-null B cells were unable to respond to a mitogenic anti-IgD antibody (Fig. 7 *B*). These results confirm previous observations that in CD45-null B cells the antigen receptor is uncoupled from signaling pathways leading to proliferation and further demonstrate that signaling through IgD is also CD45 dependent.

Since the major function of CD45 appears to be the regulation of tyrosine phosphorylation in antigen receptor complexes, we considered whether it might also regulate tyrosine phosphorylation events triggered by other types of receptor. Several studies have described antibodies against CD45 that significantly inhibit anti-CD40–induced proliferation of human PBL and B cell lines and murine splenic B cells, suggesting a role for CD45 in the regulation of CD40 signaling (19–21). To investigate this further, we performed proliferation assays to assess the ability of CD45-null B cells to respond to a mitogenic anti-CD40 mAb (32). As shown in Fig. 7 *C*, splenocytes from homozygous mutant mice consistently proliferated as well and frequently better than their wild-type counterparts. This result clearly indicates that CD45 is not essential to the CD40 signaling pathway.

We also tested the requirement for CD45 in signaling through the CD38 receptor, which has been shown to trig-

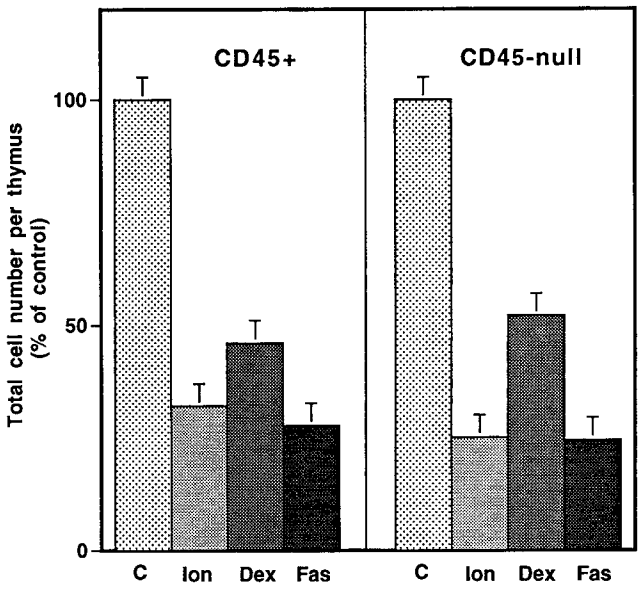

Figure 6. Normal induction of apoptosis by non-TCR pathways in thymocytes. $CD45^+$ and CD45-null 7-d FTOC were treated with ionomycin (1 μM), dexamethasone (0.01 mM), and anti-Fas mAb (0.5 μg/ml) for 15 h. Total thymocyte yields were determined and the results are expressed as a percentage of untreated control cultures in each case.

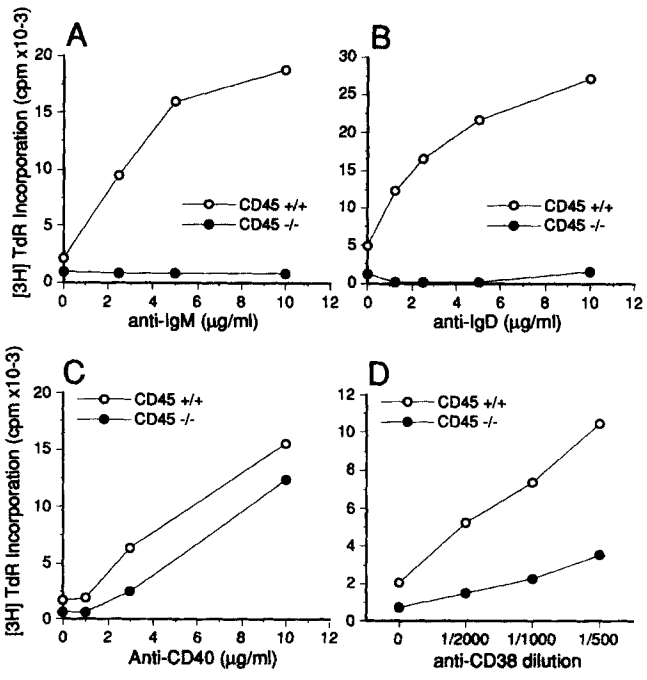

Figure 7. Proliferative responses of CD45-null splenocytes to stimulation of the B cell antigen receptor, CD40, and CD38. Splenocytes were incubated in RPMI supplemented with 5% FCS or with the addition of anti-IgM as a preparation of polyclonal $F(ab')_2$ fragments of goat anti-mouse IgM at 2.5–10.0 μg/ml (*A*); purified anti-IgD (1.19) at 1.25–10.0 μg/ml (*B*); anti-CD40 (3/23) at 1.0–10.0 μg/ml (*C*); or anti-CD38 ascites (NIM-R5) at a 1/2,000–1/500 dilution (*D*). Cultures were pulsed after 72 h. Graphs are plotted from the mean $[^3H]$TdR incorporation (*cpm*) of triplicate cultures of CD45-null splenocytes (●) or wild-type splenocytes (○).

ger tyrosine phosphorylation (24). The mAb NIM-R5 recognizes an epitope of CD38 (33); this mAb induced a dose-dependent proliferative response in wild-type B cells (Fig. 7 *D*). CD45-null B cells did proliferate in response to anti-CD38 but at significantly reduced levels compared with wild-type cells (Fig. 7 *D*). This result suggests that, although CD45 is not absolutely required for signalling via CD38, it may act to enhance the response through this receptor.

Discussion

The different isoforms of the CD45 family are encoded by multiple mRNA transcripts, generated by alternative splicing from a single gene. To achieve a complete knockout of the CD45 gene we had to adopt a strategy that would target an exon common to all isoforms. The insertion of a neomycin resistance cassette in inverse orientation within exon 9 is predicted to affect all transcripts by the introduction of a series of stop codons downstream of the site of disruption. Cell surface expression of CD45 in homozygous mutant animals was undetectable by FACS® analysis, however the possibility remained that an aberrant splicing event could remove the disrupted exon and the antigenic extracellular domain of CD45 while leaving the cytoplasmic PTPase domain intact. We were unable to demonstrate the presence of any form of CD45 in homozygous mutant animals by using an antiserum against the cytoplasmic domain of CD45 to probe Western blots of thymus lysates (Fig. 1 *B*), or by immunoprecipitation using the same antiserum (data not shown). We therefore conclude that the CD45-null mice described in this study completely lack expression of all isoforms of the CD45 gene. This contrasts with a previous report by Kishihara et al. (34) in which exon 6 of the CD45 gene was deleted by homologous recombination and replaced with a *neo* expression cassette. The CD45-exon $6^{-/-}$ mice lack expression of those isoforms of CD45 that require exon 6 and, less predictably, had variable but reduced expression of other isoforms. B cells, which in normal mice express the full-length product (B220) as their major isoform, appeared to lack detectable surface CD45 expression in the CD45-exon $6^{-/-}$ mice. However some peripheral T cells and mature thymocytes still expressed surface CD45. No analysis of cytoplasmic CD45 domains was reported (34).

The frequency of splenic T cells is reduced in CD45-null mice by a factor of 10 compared with wild-type animals, although the absolute number of T cells per spleen is reduced by a factor of only 5 because of a twofold increase in the total number of splenocytes. We also observed a similarly significant reduction in T cell numbers in lymph nodes (data not shown). The exon 6 mutant mice previously described also had a reduced frequency of T cells in the spleen and peripheral lymphoid organs, although the reduction (2.5-fold in spleen) was less than we observed in CD45-null mice. However, in contrast to the CD45-null transgenic mice, no reduction in absolute numbers of T cells was seen in the spleens of CD45-exon 6–deleted mice since such mice also showed an ~2.5-fold increase in total lymphocytes in the spleen compared with wild-type (34). The loss of CD45 appeared to have no adverse effect on B cell development since B cells were present in CD45-null mice in increased numbers. Kishihara et al. (34) reported no reduction in the percentage or absolute numbers of B cells in CD45-exon $6^{-/-}$ mice, and although mean numbers or percentages of B cells were not reported the available data on the CD45-exon $6^{-/-}$ mice are consistent with a similar expansion of total B cell numbers. The data reported here permit the conclusion that CD45 is not required at any stage in B lymphocyte development since we are confident that our CD45-null mice do not express any CD45 isoforms even in B cell progenitors or totipotential stem cells. Previously it has been reported that anti-IgM–induced growth inhibition and DNA fragmentation were enhanced in CD45-null mutants of an immature B cell line compared with the CD45-positive parental cells (35). We have not been able to measure directly the rate of loss of B cells due to this tolerance mechanism in CD45-null mice. However, the increased cellularity within the B cell compartment of CD45-null mice would argue against a significant enhancement of apoptotic signals in the absence of CD45. To test this conclusion it would be necessary to introduce a rearranged IgM transgene into the CD45-null background.

Analysis of the IgM versus IgD expression of CD45-null B cells revealed a large increase in the number of cells contained within populations II (IgM high, IgD high) and III (IgM high, IgD low), whereas the number of B cells in population I was normal. The relationship between these B cell subpopulations is not certain. Two alternative hypotheses have been proposed (31, 36). Population I may be derived from II and III by a peripheral maturation event (36). If so, the altered ratios in CD45-null spleen represent a late block in B cell maturation. Alternatively, populations I and II correspond to separate lineages (31). In this case our data would represent an expansion of a specific B cell lineage in CD45-null mice rather than a block in the maturation of B cells. The altered B cell phenotype might be a result of the lack of peripheral T cells in CD45-null mice rather than an intrinsic effect of the CD45 mutation in B cells. However, the IgM versus IgD profile of athymic (CBA *nu/nu*) mice does not resemble that of CD45-null mice and in fact such animals displayed a two- to threefold reduction in the percentage of splenic B cells within a population (BLA-1^-2^+), roughly corresponding to population II (IgM high, IgD high) (36), which suggests that the altered B cell profile in CD45-null mice cannot be solely attributed to a reduction in the number of peripheral T cells.

Our studies suggest that the reduced number of mature T cells in the spleen and peripheral lymphoid organs of CD45-null mutant mice can be explained by an ~10-fold reduction in the number of mature thymocytes produced during thymic development. Several lines of evidence point to an influence of CD45 at two distinct checkpoints in thymic development. The earliest effect of the CD45-null phenotype is an inhibition of the transition of $CD4^-CD8^-$

DN cells into the $CD4^+CD8^+$ DP compartment. This is seen in three ways. Firstly the "steady-state" yield of DP cells from CD45-null thymuses is approximately half that of normal thymuses. Second, the production of DP cells is delayed during fetal thymic ontogeny (data not shown). Third, in FTOC, where only a single cohort of developing thymocytes is present, the yield of DP cells from CD45-null mutant lobes is half that of normal thymic lobes (Fig. 5 *B*).

These data consistently show that the absence of CD45 reduces the number of DP cells by half. This reduction can be explained either by a reduction in the efficiency with which cells pass through the developmental checkpoint, or alternatively, by a reduced ability of successful cells to proliferate, since this transition is known to be accompanied by several rounds of cell division (37). The evidence suggests that the former explanation accounts for most if not all of this early developmental effect. Both in steady-state and kinetic (ontogenetic and FTOC) studies, the percentages and real numbers of DN precursor cells are increased. When the DN compartment is further divided by analyzing DN cells for CD25 and CD44 expression, it is evident that the partial block causes an accumulation of $CD25^+$ $CD44^-$ DN cells. Inhibition at this stage of differentiation is also seen in other transgenic mice mutated for TCR-β, pTα, CD3 components or the Lck tyrosine kinase (14, 15). These mutants, and other data, support the hypothesis that selection for successful rearrangement of TCR-β and formation of the pre-TCR complex takes place at this same developmental checkpoint (14). Although it has yet to be formally demonstrated that a signal via the pre-TCR is essential for the DN to DP transition, a role for CD45 in regulating this transition is consistent with this hypothesis.

The second point at which CD45 exerts a positive influence on thymic development is the maturation of immature DP thymocytes into mature SP cells. The dependence on CD45 expression of this second selection step required for maturation is greater than at the early checkpoint. The numbers and percentages of mature SP cells, developed either in vitro or in vivo, and the ratio of DP/SP cells demonstrate that the maturation of DP to SP cells is inhibited by about fivefold as compared with wild-type cells. In addition, the increased basal level of apoptosis observed in untreated CD45-null thymic lobes is consistent with the inhibition of positive selection since DP cells that fail to be positively (or negatively) selected die after about 3 d by apoptosis (38).

The exon 6 mutant mice described by Kishihara et al. (34) also showed an inhibition of thymocyte development, although in that case the early inhibition was less marked. This report significantly extends those observations since the partial nature of the inhibition seen in exon 6 mutant mice could have been explained by the residual level of CD45 expression, albeit much reduced. This study now demonstrates conclusively that CD45 is not absolutely required for thymocyte development, although it is clearly an important regulator of thymocyte maturation. Previous in vitro data are consistent with the hypothesis that the influence of CD45 on thymocyte development is exerted directly on developing thymocytes via involvement in TCR-coupled signal transduction (for a review see reference 2). One caveat, however, which should be borne in mind, is that CD45 is also expressed by thymic dendritic cells and macrophages and that FTOC experiments have shown that dendritic cells are essential for thymocyte development (39, 40). Neither the present nor previous experiments have formally excluded the possibility that the CD45 might be required by accessory cells for effective interactions with developing T cells critical in selective events. Although there are no data to support this possibility at present, cell transfer experiments, preferably in vivo as well as in organ culture, would be required to exclude this hypothesis.

Our data clearly show that CD45 is important in TCR complex–transduced signals leading to apoptosis in FTOC and are suggestive that CD45 regulates TCR-transduced signals in general. These data apparently conflict with the results of Kishihara et al. (34) who found that CD45 exon 6 mutant mice deleted their TCR $V\beta6^+$ and TCR $V\beta8.1^+$ thymocytes in Mls-1^a, H-2^d mice. This apparent discrepancy may be explained by a low level of CD45 expression in the exon 6–deleted mice. Alternatively, the difference between the in vivo and in vitro systems, perhaps in the strength of the TCR signals or the combination of TCR signals with accessory signals, may account for the apparently contradictory results. A clearer resolution of this issue could be provided by crossing the CD45-null mutation onto a TCR transgenic background. We note, however, that the avidity–affinity model of thymocyte selection would predict that early acting regulators of positive selection would also affect negative selection (16). Indeed, our data showing a more profound inhibition of negative than positive selection, is entirely consistent with the affinity–avidity model if, as we propose, CD45 acts as a positive regulator of TCR complex signaling.

Evidence from in vitro B cell lines and from in vivo–targeted mutagenesis demonstrates that CD45 is required for signal transduction after cross-linking of sIgM (6, 34). Consistent with these previous studies, splenocytes from CD45-null mice were completely unable to proliferate in response to anti-IgM. In addition, we have shown that CD45 is also absolutely required for sIgD signaling. The latter result is not surprising since despite the ability of sIgD and sIgM to trigger different physiological outcomes and some evidence for IgD-receptor–specific associated proteins (41), only subtle temporal differences have been observed in their signal transduction pathways (42).

Our finding that CD45-null B cells were not impaired in their ability to respond to a mitogenic anti-CD40 mAb contrasts with previous reports in which anti-CD45 mAbs inhibited CD40-mediated B cell proliferation (19–21). Antibody inhibition studies of this kind require careful interpretation and may be explained in terms of stochiometric mechanisms such as sequestration of CD45 or inappropriate interaction of CD45 with substrates (43). It remains possible that, under certain circumstances, CD45 may influence some aspect of the CD40 signaling pathway, but the physiological relevance of this interaction is unknown.

CD38 is a receptor that triggers tyrosine phosphorylation events but does not possess intrinsic PTK activity (24). The identity of the PTK(s) activated by CD38 is at present unknown, however our finding that CD38-mediated proliferation is reduced in CD45-null mice suggests that at least one of these PTKs is a src family kinase that is activated by CD45. Recently it was shown that in physiological states of B cells in which anti-IgM activation is blocked, anti-CD38 activation is also inhibited (44). Lund et al. (44) suggested that the data indicated that a critical signaling component was common to both pathways. Our data are consistent with an overlap between the pathways but do not identify the shared component(s).

In summary, CD45 acts as a positive regulator of signals delivered via the TCR and BCR complexes, so that the signaling threshold of these receptors is markedly increased in the absence of CD45. The extent to which functional consequences of antigen receptor triggering are inhibited in the absence of CD45 varies. Previous studies analyzing the defect in TCR signaling using in vitro T cell lines that lack CD45 expression have suggested that CD45 exerts its effect by regulating the phosphorylation state of the COOH-terminal tyrosine of the *lck* and *fyn* kinases (45–47). Analysis of the biochemical basis for the signal reduction in CD45-null lymphocytes is in progress.

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References

1. Thomas, M.L. 1989. The leukocyte common antigen family. *Annu. Rev. Immunol.* 7:339–369.
2. Trowbridge, I.S., and M.L. Thomas. 1994. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu. Rev. Immunol.* 12:85–116.
3. Weaver, C.T., J.T. Pingel, J.O. Nelson, and M.L. Thomas. 1991. $CD8^+$ T-cell clones deficient in the expression of CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli. *Mol. Cell. Biol.* 11:4415–4422.
4. Koretzky, G.A., J. Picus, T. Schultz, and A. Weiss. 1991. Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Acad. Sci. USA.* 88:2037–2041.
5. Shiroo, M., L. Goff, M. Biffen, E. Shivnan, and D. Alexander. 1992. CD45 tyrosine phosphatase-activated $p59^{fyn}$ couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4887–4897.
6. Justement, L.B., K.S. Campbell, N.C. Chein, and J.C. Cambier. 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science (Wash. DC).* 252:1839–1842.
7. Koretzky, G.A., J. Picus, M.L. Thomas, and A. Weiss. 1990. Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. *Nature (Lond.).* 346:66–68.
8. Koretzky, G.A., M.A. Kohmetscher, T. Kadleck, and A. Weiss. 1992. Restoration of T cell receptor-mediated signal transduction by transfection of CD45 cDNA into a CD45-deficient variant of the Jurkat T cell line. *J. Immunol.* 149: 1138–1142.
9. Hovis, R.R., J.A. Donavon, M.A. Musci, D.G. Motto, F.D. Goldman, S.E. Ross, and G.A. Koretzky. 1993. Rescue of signaling by a chimeric protein containing the cytoplasmic domain of CD45. *Science (Wash. DC).* 260:544–546.
10. Volarevic, S., B.B. Niklinska, C.M. Burns, C.H. June, A.M. Weissman, and J.D. Ashwell. 1993. Regulation of TCR signalling by CD45 lacking transmembrane and extracellular domains. *Science (Wash. DC).* 260:541–544.
11. Desai, D.M., J. Sap, J. Schlessinger, and A. Weiss. 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell.* 73:541–554.
12. Desai, D.M., J. Sap, O. Silvennoinen, J. Schlessinger, and A. Weiss. 1994. The catalytic activity of the CD45 membrane-proximal phosphatase domain is required for TCR signalling and regulation. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4002–4010.
13. Robey, E., and B.J. Fowlkes. 1994. Selective events in T cell development. *Annu. Rev. Immunol.* 12:675–705.
14. Anderson, S.J., and R.M. Perlmutter. 1995. A signalling pathway governing early thymocyte maturation. *Immunol. Today.* 16:99–105.
15. Fehling, H.J., A. Krotkova, C. Saint-Ruf, and H. von Boehmer. 1995. Crucial role of the pre-T-cell receptor α genes in development of $\alpha\beta$ but not $\gamma\delta$ T cells. *Nature (Lond.).* 375: 795–798.
16. Jameson, S.C., K.A. Hogquist, and M.J. Bevan. 1995. Positive selection of thymocytes. *Annu. Rev. Immunol.* 13:93–126.
17. Smith, C.A., G.T. Williams, R. Kingston, E.J. Jenkinson, and J.J.T. Owen. 1989. Antibodies to CD3/TCR receptor complex induce death by apoptosis in immature T-cells in thymic cultures. *Nature (Lond.).* 337:181–184.

18. Ren, C.L., T. Morio, S.M. Fu, and R.S. Geha. 1994. Signal transduction via CD40 involves activation of *lyn* kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase Cγ2. *J. Exp. Med.* 179:673–680.
19. Faris, M., F. Gaskin, J.T. Parsons, and S.M. Fu. 1994. CD40 signaling pathway: anti-CD40 monoclonal antibody induces rapid dephosphorylation and phosphorylation of tyrosine phosphorylated proteins including protein tyrosine kinase *lyn*, *fyn*, and *syk* and the appearance of a 28-kD tyrosine phosphorylated protein. *J. Exp. Med.* 179:1923–1931.
20. Gruber, M.F., J.M. Bjorndahl, S. Nakamura, and S.M. Fu. 1989. Anti-CD45 inhibition of human B cell proliferation depends on the nature of activation signals and the state of B cell activation. A study with anti-IgM and anti-CDw40 antibodies. *J. Immunol.* 142:4144–4152.
21. Kato, T., T. Kokuho, T. Tamura, and H. Nariuchi. 1994. Mechanisms of T cell contact-dependent B cell activation. *J. Immunol.* 152:2130–2138.
22. Santos-Argumedo, L., C. Teixeira, G. Preece, P.A. Kirkham, and R.M.E. Parkhouse. 1993. A B lymphocyte surface molecule mediating activation and protection from apoptosis via calcium channels. *J. Immunol.* 151:3119–3130.
23. Howard, M., J.C. Grimaldi, J.F. Bazan, F.E. Lund, L. Santos-Argumedo, R.M.E. Parkhouse, T.F. Walseth, and H.C. Lee. 1993. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science (Wash. DC).* 262: 1056–1059.
24. Kirkham, P.A., L. Santos-Argumedo, M.M. Harnett, and R.M.E. Parkhouse. 1994. Murine B cell activation via CD38 and protein tyrosine phosphorylation. *Immunology.* 83:513–516.
25. Yenofsky, R.L., M. Fine, and J.W. Pellow. 1990. A mutant neomycin phosphotransferase II gene reduces the resistance of transformants to antibiotic selection pressure. *Proc. Natl. Acad. Sci. USA.* 87:3435–3439.
26. Qin, S., S. Cobbold, R. Benjamin, and H. Waldmann. 1989. Induction of classical transplantation tolerance in the adult. *J. Exp. Med.* 169:779–794.
27. Watt, S.M., D.J. Gilmore, J.M. Davis, M.R. Clark, and H. Waldmann. 1987. Cell surface markers on hematopoietic precursors. Reagents for the isolation and analysis of progenitor cell subpopulations. *Mol. Cell. Probes.* 1:297–326.
28. Jenkinson, E.J., and G. Anderson. 1994. Fetal thymic organ cultures. *Curr. Opin. Immunology.* 6:293–297.
29. Mansour, S.L., K.R. Thomas, and M.R. Capecchi. 1988. Disruption of the proto-oncogene int-2 in mouse embryo–derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature (Lond.).* 336:348–352.
30. Hardy, R.R., K. Hayakawa, J. Haaijman, and L.A. Herzenberg. 1982. B-cell subpopulations identified by two-colour fluorescence analysis. *Nature (Lond.).* 297:589–591.
31. Hardy, R.R., K. Hayakawa, D.R. Parks, L.A. Herzenberg, and L.A. Herzenberg. 1984. Murine B cell differentiation lineages. *J. Exp. Med.* 159:1169–1188.
32. Hasbold, J., C. Johnson-Léger, C.J. Atkins, E.A. Clark, and G.B. Klaus. 1994. Properties of mouse CD40: cellular distribution of CD40 and B cell activation by monoclonal anti-mouse CD40 antibodies. *Eur. J. Immunol.* 24:1835–1842.
33. Harada, N., L. Santos-Argumedo, R. Chang, J.C. Grimaldi, F.E. Lund, C.I. Brannan, N.G. Copeland, N.A. Jenkins, A.W. Heath, R.M.E. Parkhouse, and M. Howard. 1993. Expression cloning of a cDNA encoding a novel murine B cell activation marker. Homology to human CD38. *J. Immunol.* 151:3111–3118.
34. Kishihara, K., J. Penninger, V.A. Wallace, T.M. Kundig, K. Kawai, P.S. Ohashi, M.L. Thomas, C. Furlonger, C.J. Paige, and T.W. Mak. 1993. Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase–deficient mice. *Cell.* 74:143–156.
35. Ogimoto, M., T. Katagiri, K. Mashima, K. Hasegawa, K. Mizuno, and H. Yakura. 1994. Negative regulation of apoptotic death in immature B cells by CD45. *Int. Immunol.* 6: 647–654.
36. Herzenberg, L.A., K. Hayakawa, R.R. Hardy, T. Tokuhisa, V.T. Oi, and L.A. Herzenberg. 1982. Molecular, cellular and systemic mechanisms for regulating IgCH expression. *Immunol. Rev.* 67:5–31.
37. Penit, C., and F. Vasseur. 1988. Sequential events in thymocyte differentiation and thymus regeneration revealed by a combination of bromodeoxyuridine DNA labeling and antimitotic drug treatment. *J. Immunol.* 140:3315–3323.
38. Shortman, K., D. Vremec, and M. Egerton. 1991. The kinetics of T cell antigen receptor expression by subgroups of $CD4^+CD8^+$ thymocytes: delineation of $CD4^+8^+3^+$ thymocytes as post-selection intermediates leading to mature T cells. *J. Exp. Med.* 173:323–332.
39. Anderson, G., E.J. Jenkinson, N.C. Moore, and J.J.T. Owen. 1993. MHC class II positive epithelium and mesenchyme cells are both required for T-cell development in the thymus. *Nature (Lond.).* 362:70–73.
40. Anderson, G., J.J.T. Owen, N.C. Moore, and E.J. Jenkinson. 1994. Thymic epithelial cells provide unique signals for positive selection of $CD4^+CD8^+$ thymocytes in vitro. *J. Exp. Med.* 179:2027–2031.
41. Terashima, M., K.-M. Kim, T. Adachi, P.J. Nielsen, M. Reth, G. Köhler, and M.C. Lamers. 1994. The IgM antigen receptor of B lymphocytes is associated with prohibin and a prohibin-related protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3782–3792.
42. Kim, K.-M., and M. Reth. 1995. The B cell antigen receptor of class IgD induces a stronger and more prolonged protein tyrosine phosphorylation than that of class IgM. *J. Exp. Med.* 181:1005–1014.
43. Shivnan, E., M. Biffen, M. Shiroo, E. Pratt, M. Glennie, and D. Alexander. 1992. Does co-aggregation of the CD45 and CD3 antigens inhibit T cell antigen receptor complex–mediated activation of phospholipase C and protein kinase C? *Eur. J. Immunol.* 22:1055–1062.
44. Lund, F.E., N.W. Solvason, M.P. Cooke, A.W. Heath, J.C. Grimaldi, R.M.E. Parkhouse, C.C. Goodnow, and M.C. Howard. 1995. Signaling through murine CD38 is impaired in antigen receptor–unresponsive B cells. *Eur. J. Immunol.* 25: 1338–1345.
45. Ostergaard, H.L., D.A. Shackelford, T.R. Hurley, P. Johnson, R. Hyman, B.M. Sefton, and I.S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the *lck*-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. USA.* 86:8959–8963.
46. Cahir McFarland, E.D., T.R. Hurley, J.T. Pingel, B.M. Sefton, A. Shaw, and M.L. Thomas. 1993. Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. *Proc. Natl. Acad. Sci. USA.* 90:1402–1406.
47. Hurley, T.R., R. Hyman, and B.M. Sefton. 1993. Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the lck, fyn, and c-src tyrosine protein kinases. *Mol. Cell. Biol.* 13:1651–1656.