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Either of the CD45RB and CD45RO Isoforms Are Effective in Restoring T Cell, But Not B Cell, Development and Function in CD45-Null Mice

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The protein tyrosine phosphatase CD45 is expressed as a series of isoforms whose tissue and differentiation stage specificity is broadly conserved in evolution. CD45 has been shown to be an important regulator of a variety of functions in many different hemopoietic lineages. We have chosen an in vivo genetic complementation strategy to investigate the differential functions between isoforms. In this study, we report the characterization of transgenic mice which express the isoforms CD45RO or CD45RB as their only CD45 molecules, at a variety of expression levels and in the majority of hemopoietic lineages. Both CD45RO and CD45RB isoforms reconstitute thymocyte development in a CD45-null mouse background when expressed above a threshold level. The resulting mature T cells populate the peripheral lymphoid organs where they are found at normal frequency. Both CD45RO and CD45RB isoforms also permit T cell function in the periphery, although the threshold for normal function here appears to be set higher than in the thymus. In contrast, neither isoform is capable of fully restoring peripheral B cell maturation, even at levels approaching those in heterozygous CD45+/− mice in which maturation is normal. In vitro activation of B cells by Ag-receptor stimulation is only minimally complemented by these CD45RO and CD45RB transgenes. Our results suggest that CD45 isoforms play unique roles which differ between the T and B lineages.


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5 Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; Tg, transgene.
The mouse vav1 gene is widely expressed in hemopoietic cells but few other cell types. Thus, there is a substantial overlap in its expression pattern and that of CD45. The unusual lineage-restricted, yet compartment-wide, vav1 expression pattern and the regulation of its expression have been the subject of recent studies (23–25). These established the existence of DNaseI hypersensitive sites within the vav1 locus, marking putative transcriptional regulatory elements. The identification of such regulatory regions led to the development of var transgenic vectors which can be used to direct expression of heterologous coding regions expressly to hemopoietic cells (23–25). In this study, we take advantage of the availability of a var transgenic vector to restore CD45 protein, specifically the CD45RO and CD45RB isoforms, to mice deficient in endogenous CD45 and thereby investigate the ability of these individual isoforms to compensate functionally for endogenous CD45 protein in lymphocytes. Given their expression in nonlymphoid lineages, these mice should also facilitate the investigation of CD45 isoform function in other hemopoietic cells.

Materials and Methods

Generation of CD45 isoform transgenes and transgenic mice

The CD45RO isoform encoding cDNA (26) was kindly provided by Dr. Y. Saga (Sloan-Kettering Cancer Institute, New York, NY). A cDNA encoding CD45RB was generated by amplification from splenic cDNA, using the proof-reading Pfu DNA polymerase. The resulting 437-bp fragment was then inserted at the corresponding portion of the CD45RO sequence, hence producing a full-length CD45RB cDNA. The cDNAs were inserted into the NotI site of the HS231/45 var-hCD4 plasmid (described previously in Ref. 25) using NotI linkers. By this means, the cDNA encoding truncated human CD45 was replaced with one encoding either CD45RO or CD45RB.

Importantly, the normal order of the var1 DNasel hypersensitive sites and all other elements in the transgenic vector are maintained, with the CD45RO or CD45RB cDNAs replacing the coding portion of var1 exon 1. The integrity of the cDNAs was confirmed by sequencing. Before microinjection, the resulting var1-CD45RO and var1-CD45RB transgenes were excised from the plasmid vector. SacII digests were electrophoresed through low melting point agarose and the transgene fragments were purified using an elutrap-D column (Scherlieh & Schnuell, Dassel, Germany). The transgene was then ethanol precipitated and dissolved in sterile 10 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L EDTA. The DNA was micro-injected into fertilized (C57BL/6J × DBA/2J)F1 embryos which were transferred to pseudo-pregnant (CBA/H × C57BL/6J)F1 recipient females. Transgenic founder mice were mated to previously described B6.129-Ptprc<sup>iem9</sup>s1Jtm1Bmg/cdx1 exon 9<sup>−/−</sup> mice (5) to generate isoform transgene-bearing animals which simultaneously lacked expression of all endogenous CD45 isoforms (referred to as Tg<sup>−/−</sup>, CD45<sup>−/−</sup>). In the experiments described here, transgene-negative, CD45-deficient (CD45<sup>−/−</sup>) mice were tested simultaneously, along with CD45<sup>+</sup> (CD45<sup>+/+</sup>), or C57BL/6J control mice. Where possible, control mice were littermates, with the exception of those from the inbred C57BL/6 strain. The status of transgenic and control mice with respect to the CD45 locus was routinely tested using PCR primers located within a CD45 intron (5′-CGA TGA TGA TGA TAC TAA TTT CCT TTT TTT), exon 9 (5′-GTA ATC AGA GCT TTA AGG CAC ACC TC-3′ and 5′-GCT GGA GCA CAT GAG TCA TTA GAC AC-3′), and the neomycin resistance gene which formed part of the targeting construct (5′-TGG GCT ACC GGT GAT ATT GCT G-3′). In addition, flow cytometric analysis (see below) confirmed the lack of endogenous CD45 protein in the CD45<sup>−/−</sup> control mice.

Flow cytometric analysis

Peripheral blood and other hemopoietic tissues were analyzed for expression of CD45 protein by flow cytometry using pan-CD45-specific Abs to detect all CD45 isoforms. The mice analyzed were between 10 and 26 wk of age. Blood leukocytes, obtained after HEpCl lysis of erythrocytes, were incubated on ice with PE-labeled rat anti-mouse CD45 mAb (clone 30F11; BD PharMingen, San Diego, CA) plus anti-Fcγ receptor Ab (clone 2.4G2; BD PharMingen) to reduce nonspecific binding. Alternatively, the pan-CD45-specific mAb YB4M2.2 was used in conjunction with FITC anti-rat mAb (MARG2A; Serotec, Oxford, U.K.). Single cell suspensions of femoral bone marrow, thymus, spleen, mesenteric, and inguinal lymph nodes, as well as peritoneal cells, were all analyzed similarly. In some cases, mature RBCs were gated out by forward scatter rather than removed by lysis. To determine hemopoietic lineage, the cells were also incubated in saturating quantities of fluorochrome- or biotin-conjugated mAbs to anti-cell surface marker-specific Abs and, where necessary, streptavidin-allophycocyanin (Caltag Laboratories, Burlingame, CA). Cell surface marker-specific mAbs used included anti-CD3 (clone 14-5-2C11), anti-CD4 (H129.19), anti-CD8 (53-6.7), anti-CD25 (IL-2Rα chain, clone PC61-5.3), anti-CD44 (IMT.8.1), anti-CD45RB (16A or MB23G2), anti-Thy1 (T24.32.1), anti-Gr-1 (RB6-8C5), anti-Mac-1 (CD11b, clone M1/70-15), anti-CD19 (ID3), anti-IgM (anti-Ca 5.1 or 5.67), anti-IgG (11-26C or 1.19), and anti-Thy-1.19, all purchased from BD PharMingen or prepared in our laboratory. After staining, cells were washed and resuspended in PBS containing 2% FCS, 10 mmol/L Na3N, and 1 μg/ml propidium iodide or in PBS, 1% formaldehyde. At least 10,000 viable, nucleated cells were analyzed, using a FACSCalibur or FACSscan cytometer and CellQuest software (BD Biosciences, San Jose, CA).

Measurement of lymphocyte proliferative responses in vitro

T cell proliferation in response to anti-mouse IgD (clone 145-2.11) and CD45-decient (CD45<sup>−/−</sup>) mice were analyzed as described above. T lymphocytes were purified from spleen by Spin-Sep (StemCell Technologies, London, U.K.), and were plated at 2 × 10<sup>5</sup> cells/well into 96-well plates precoated with either 1 or 5 μg/ml CD3ε (BD PharMingen) or CD3 (1 μg/ml) plus CD28, 5 μg/ml (BD PharMingen) for 48 h at 37°C 5% CO<sub>2</sub> in air. Each well was pulsed with 1 μCi [H]thymidine 18 h before harvesting and incorporation into DNA was measured as above. Cell purities were checked before each proliferation by flow cytometry.

B cell proliferation in response to anti-mouse IgM (clone b7.6) mAb was measured as follows: B cells were purified by positive selection from the spleens of CD45RO, CD45RB, CD45<sup>−/−</sup> or C57BL/6 mice using FITC-labeled anti-MHC class II Ab (clone 145-2.11), anti-ITC-IFC MACs beads (Miltenyi Biotec, Bislewy, U.K.). Purity was assessed by staining with PE-labeled anti-CD19 and flow cytometry (as above). The selected populations were typically found to be comprised of ~85% CD19<sup>+</sup> cells. The purified B cells (10<sup>5</sup>/well) were cultured for 3 days in RPMI 1640 supplemented with 10% FCS and 1 × 10<sup>−4</sup> M 2-ME in 96-well plates (Falcon 353072; BD Biosciences). Six hours before harvesting, 1 μCi [H]thymidine (Amersham, Aylesbury, U.K.) was added to each culture. Thymidine incorporation into DNA was assessed using a Tomtec cell harvester (Hemden, CT) and a Wallac 1450 scintillation counter (PerkinElmer, Boston, MA).

Measurement of T-dependent Ag responses in vivo

Mice between 10 and 17 wk of age were immunized i.p. on day 1 and again on day 21 with 100 μg of alum-precipitated DNP-BSA (Calbiochem, Nottingham, U.K.). On days 10 and 28, serum was collected from tail vein blood samples. The presence of DNP-specific Abs (IgM and IgG1) in the serum was detected by ELISA using DNP-γ-globulin (Calbiochem) coated plates (Maxisorp F96; Nunc, Roskilde, Denmark). After blocking nonspecific binding sites with 1% gelatin in PBS, serial dilutions of serum were added to the wells and allowed to bind at 4°C overnight. After extensive washing, biotin-conjugated goat anti-mouse IgM or anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) were added. These were developed with streptavidin-HRP (Southern Biotechnology Associates) and o-phenylenediamine dihydrochloride substrate (Sigma-Aldrich, St. Louis, MO) before measuring the absorbance at 490 nm.

The CD45-deficient mice used to generate Tg<sup>−/−</sup>, CD45<sup>−/−</sup> mice were equivalent to six generations backcrossed to C57BL/6J mice. As the transgenic lines were of mixed genetic background, all mice used in the immunization protocols were selected for the H-2K<sup>b</sup> haplotype by PCR on genomic DNA from tail biopsies.

Results

The generation of transgenic mice expressing only single CD45 isoforms

A transgenic vector containing mouse vav1 gene sequences surrounding five hemopoietic cell-specific DNaseI hypersensitive sites has previously been shown to effectively direct expression of a eukaryotic cDNA (encoding a truncated human CD4 DNA molecule)
to cells of the hemopoietic compartment (25). In this study, we have used equivalent HS321/45 vav-CD45RO or HS321/45 vav-
CD45RB transgenes to target individual CD45 isoform expression, namely CD45RO or CD45RB, to the blood cells of mice which are
deficient for endogenous CD45 gene expression and therefore lack all endogenous CD45 protein.

We generated 11 vav-CD45RO and 3 vav-CD45RB isoform primary transgenic mice. All but one were crossed to CD45 exon
9-mutated mice (5) to give 10 CD45RO and 3 CD45RB independent transgenic lines on a CD45-deficient background. To evaluate the expression of the transgene, PBLs were analyzed for surface
CD45 protein by flow cytometry (Fig. 1). As with previous vav-driven transgenes (24, 25), both the vav-CD45RO and vav-
CD45RB transgenes were expressed in the majority of the independent transgenic lines (8 of 10 and 3 of 3, respectively). Analysis of the major hemopoietic cell populations in peripheral
blood samples by flow cytometry revealed three patterns of transgene expression among the independent transgenic lines. In 6 of 10
CD45RO (RO3, RO4, RO5, RO7, RO9, RO10) and 3 CD45RB (RB2, RB3, RB4) lines, transgene expression was observed in all
cells of a particular hemopoietic lineage, whereas in 2 CD45RO lines (RO8 and RO11) no transgene expression was observed in any
tissue or cell type tested (Fig. 1 and data not shown). Most striking was the observation that mice from lines in which the
transgene was active had substantial populations of T cells in their peripheral blood (Fig. 1). This gave an early indication that one of
the main functions of CD45, namely its role in T cell development, could be successfully compensated for by the introduction of either
single isoform transgene.

In two further CD45RO lines (RO6 and RO12), a “heterocellular” pattern of expression, similar to that seen in some vav-hCD4
primary transgensics, was noted (Fig. 1; see also Ref. 25). Unlike the vav-hCD4 transgensics, in this case the heterocellular pattern
observed cannot be attributed to genetic mosaicism because the mice analyzed were progeny animals rather than primary trans-
genics, and would therefore be expected to carry the transgene in all cells. Interestingly, although only a proportion of cells of the B
lymphoid (CD19+ cells) or myeloid (Mac-1+ or Gr-1+1) lineages displayed transgene expression (for the RO12 example shown in

![FIGURE 1. Flow cytometric analysis of surface CD45 protein on PBLs of vav-CD45RO transgenic, CD45-deficient mice. CD45 transgene expression in CD3+ T cells, CD19+ B cells, Mac-1-, and Gr-1+1 myeloid lineage cells from Tg+, CD45+/− mice of the indicated transgenic lines, compared with expression from C57BL6 (CD45+/+) and CD45+/− control mice (as indicated). The results shown are representative of multiple experiments using mice of multiple lines.](http://www.jimmunol.org/)

![FIGURE 2. CD45 isoform transgene expression in hemopoietic cells. Flow cytometric analysis of CD45 protein on (A and C) CD4+ CD8+ thymocytes, (B and D) CD19+ bone marrow B lymphocytes, splenic (E and G) CD3+ T cells, and (F and H) CD19+ B cells from Tg+, CD45+/− mice of the indicated vav-CD45RO or vav-CD45RB lines. Cell number is represented by histogram height. Results from C57BL6 (CD45+/+) control mice are shown as lightly shaded histograms and those from nontransgenic CD45+/− control mice as dark filled histograms. The results shown are representative of multiple experiments.](http://www.jimmunol.org/)
Expression of the vav-CD45RO or vav-CD45RB transgene rescues thymic development

Development of committed T cell precursors formed in the bone marrow takes place in the thymus by a tightly controlled series of steps (27). To proceed from one stage to the next, the cell must overcome a series of developmental hurdles, including adequate signaling through the pre-TCR (28). One consequence of the loss of endogenous CD45 expression has been shown to be a severe perturbation of T cell development (5–7). CD45-deficient mice display greatly decreased mature T cell numbers, a defect which has its origins in two substantial developmental blocks during their production in the thymus. Thus, the production of DP T cell precursors is reduced 2-fold and maturation of DP to SP cells an additional 5-fold (5).

Expression of the CD45RO and CD45RB transgenes occurred in the vast majority of thymocytes (Fig. 2, A and C; also data not shown). We investigated whether this expression could reverse the thymic defects seen in CD45-null (exon 9−/−) mice. Flow cytometric analysis of the four main thymic T cell subsets (DN, DP, CD4 SP, and CD8 SP; based on their expression of CD4 and CD8) revealed that, dependent on the level of expression within a given line, the more mature SP subsets (CD4+CD8− (CD4 SP) and CD4+CD8+ (CD8 SP)), which are almost totally absent in CD45−/− mice, could be substantially restored (Fig. 3 and Table I). Similarly, there was a concomitant decrease in the proportion of DP cells when compared with CD45−/− mice (Fig. 3 and Table I) as well as a reduction in the proportion of DN cells. The level of transgene activity in the individual line appeared to correlate with the degree of complementation of the CD45-null phenotype. Low levels of transgene expression (for example, RO5) gave little or no restoration of normal thymocyte development, the highest-expressing lines (for instance, RO3) gave essentially complete restoration, and those lines with intermediate CD45 expression showed partial restoration (Fig. 3 and Table I).

As we have observed previously (2, 5), DP thymocytes from CD45−/− mice have increased levels of CD4− and CD8-associated fluorescence (indicating increased amounts of protein expressed at the cell surface) when compared with those seen on cells from C57BL/6 (CD4+CD8−) mice. The significance of increased levels of CD4 and CD8 on CD45-deficient cells is unclear, however carefull examination of levels on the transgenic cells demonstrated an inverse correlation between the level of CD45 protein expressed and the surface CD4− and CD8-associated fluorescence.

**Table 1.** Composition of the thymus in CD45 isoform transgenic mice with respect to CD4 and CD8 expression

<table>
<thead>
<tr>
<th>Mouse Line (n)</th>
<th>Thymocyte Subset</th>
<th>CD45−/− (12)</th>
<th>CD45+/+ (4)</th>
<th>CD45+/+ (4)</th>
<th>RO3 (3)</th>
<th>RO4 (5)</th>
<th>RO5 (2)</th>
<th>RO7 (5)</th>
<th>RB2 (2)</th>
<th>RB4 (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DN</td>
<td>6.8 ± 1.5</td>
<td>4.8 ± 1.5**</td>
<td>3.3 ± 0.9**</td>
<td>4.3 ± 2.3</td>
<td>4.6 ± 1.7</td>
<td>5.2 ± 1.0</td>
<td>4.0 ± 1.6**</td>
<td>2.4 ± 0.16**</td>
<td>3.9 ± 2.2**</td>
</tr>
<tr>
<td></td>
<td>DP</td>
<td>89 ± 2.2</td>
<td>78 ± 4.8**</td>
<td>83 ± 2.6**</td>
<td>78 ± 2.2**</td>
<td>81 ± 3.4**</td>
<td>90 ± 1.4</td>
<td>80 ± 2.9**</td>
<td>88 ± 1.8</td>
<td>77 ± 5.0**</td>
</tr>
<tr>
<td></td>
<td>CD4 SP</td>
<td>0.89 ± 0.23</td>
<td>9.1 ± 1.1**</td>
<td>7.5 ± 1.5**</td>
<td>11 ± 2.0**</td>
<td>8.8 ± 1.7**</td>
<td>2.1 ± 0.19**</td>
<td>9.9 ± 1.9**</td>
<td>6.4 ± 1.1**</td>
<td>12 ± 1.9**</td>
</tr>
<tr>
<td></td>
<td>CD8 SP</td>
<td>1.2 ± 0.69</td>
<td>4.3 ± 1.2**</td>
<td>3.6 ± 0.88**</td>
<td>3.6 ± 1.3*</td>
<td>2.4 ± 0.67**</td>
<td>0.75 ± 0.13*</td>
<td>3.1 ± 0.74**</td>
<td>1.8 ± 0.40</td>
<td>3.5 ± 0.71**</td>
</tr>
</tbody>
</table>

*Shown are the mean percentage and SD of cells in each thymocyte subset as a proportion of total thymocytes analyzed. Thymocytes were simultaneously stained for CD4 and CD8. Results which were significantly different (in a t test) from those of the CD45−/− mice are shown in bold.

*, p < 0.05

**, p < 0.01

Importantly, all members of the same transgenic line displayed the same (or very similar) pattern and relative level of transgene expression. However, as indicated in Fig. 2, there was variation among the individual lines in the level of CD45 isoform-associated immunofluorescence, indicating varying levels of CD45 protein on any given cell type. RO3 line mice consistently displayed the highest levels of CD45RO and RO5 the lowest. Line RO4 mice were intermediate between the two; all other mice expressing RO exhibited levels between that of RO4 and RO3. Similarly, RB2 mice had high levels on par with RO3, whereas the expression in line RB4 was intermediate. No low-expressing RB line was obtained, so subsequent analysis was mainly confined to mice of lines RB2 and RB4.

Further, as expected from previous vav-driven transgenic mice (25), there was a hierarchy in levels of expression between the different hemopoietic lineages, with lymphoid cells displaying the highest levels of transgenic CD45, myeloid cells an intermediate level, and erythroid cells the lowest levels (Fig. 1 and data not shown).
CD45−/− DN thymocytes to signal appropriately through the pre-TCR, a requirement for further development to the DP stage. The precise point at which the block occurs is believed to be the DN3 to DN4 transition, resulting in an increase in DN3 cell numbers and a corresponding decrease in DN4 cells (5, 29). As previously reported, the CD45−/− mice displayed a higher proportion of DN3 cells than their CD45+/+ C57BL/6 counterparts (Fig. 4) indicative of a block at the DN3 to DN4 transition. The lowest-expressing line (RO5; Fig. 4) was comparable to that of the CD45−/− mice, whereas lines with higher expression of either CD45RO or CD45RB (Fig. 4) were similar to the CD45−/− mice, indicating that, at least in the higher-expressing lines, appropriate pre-TCR signaling was restored.

Interestingly, we observed a CD44−, CD25low intermediate DN2 population (see the region outlined in bold in Fig. 4), the relative presence or absence of which appeared to correlate with the degree of isoform transgene expression. For instance, whereas cells with these characteristics were infrequent among DN2 cells from a C57BL/6 thymus (Fig. 4) or those from the higher-expressing CD45RO and CD45RB lines, they appear to form quite a significant population among DN thymocytes from CD45−/− mice (Fig. 4) or low-expressing line RO5 (Fig. 4). The significance of this population is unclear, although it is possible that it reflects an additional stage at which CD45 is normally active during T cell development.

Peripheral T cell populations are restored in CD45RO and CD45RB transgenic mice

In the majority of the vav-CD45RO and vav-CD45RB lines generated, the level of transgenic isoform expression was sufficient to restore normal proportions of peripheral T cells (Fig. 5A). Although there was some variation apparent in individual mice, the percentage of CD3+ T cells in the spleen was within the normal range for all the single isoform lines examined, with two exceptions. First, those lines which failed to express the CD45 transgene (RO8 and RO11) were indistinguishable from CD45−/− mice (data not shown). Second, in the line which had the lowest level of transgene expression (RO5), we observed an intermediate frequency of splenic T cells which was consistently different from that of CD45−/− and CD45+/+ mice (Fig. 5A). Both the CD4 and CD8 splenic T cell subpopulations were restored to normal levels.
for the majority of CD45 isoform transgenic mice; these mice also 
had increased numbers of both CD4 and CD8 T cells (relative to 
CD45-null mice) in their peripheral lymph nodes (data not shown).

The expression of single CD45RO or CD45RB transgenes does not restore peripheral B cell maturation

The ability of the CD45RO and CD45RB isoform transgenes to substitute for endogenous CD45 isoform expression in B cells was markedly poorer than in T cells. Maturation of B cells from the immature to mature stages occurs in the spleen and has previously been shown to be defective in CD45<sup>−/−</sup> mice (Ref. 5; see also Fig. 5B). In particular, the transition from the IgM<sup>high</sup>IgD<sup>high</sup> intermediate stage cells (sometimes referred to as T2 B cells; Ref. 30) to the mature IgM<sup>low</sup>IgD<sup>high</sup> phenotype is markedly impaired. We examined the splenic B cells from single isoform transgenic lines 
and found that this B cell maturation step was variably complemented in the different lines (Fig. 5B). However, in no case did the 
percentage of mature B cells approach that found in CD45<sup>−/−</sup> mice (Fig. 5B). Thus, neither CD45RO nor CD45RB, when expressed as a single isoform, appeared to substitute effectively for endogenous CD45 expression during B cell maturation.

In vitro analysis of lymphocyte function

We have previously reported that neither B cells (5) nor T cells (2) 
from CD45 exon 9<sup>−/−</sup> mice proliferate in response to Ag-receptor cross-linking in vitro. Therefore, we characterized the ability of lymphocytes from the vav-CD45RO and vav-CD45RB transgenic mice to respond to such stimulation. Lymphocytes from transgene-expressing lines (RO3, RO4, RO5, RO7, RO9, RB2, RB4) proliferated in vitro to stimulation with anti-CD3 Ab. In contrast, cells from the nonexpressing lines (RO8) did not (data not shown). Fig. 6A shows a representative experiment in which the proliferation of purified T cells in response to immobilized CD3 mAb, or coimmobilized CD3 and CD28 mAbs, was investigated using isoform lines matched for their CD45 expression levels. No consistent differences in proliferative responses were noted between the CD45RO and CD45RB lines, and the small differences noted in Fig. 6A were not reproducible in multiple experiments (n = 5 for each matched pair). Interestingly, however, there was a clear trend for the T cells expressing single CD45 isoforms to proliferate better than the wild-type C57BL/6 T cells at low doses of CD3 (1 μg/ml; Fig. 6A).

Strikingly, transgenic B lymphocytes did not proliferate substan-
tially in response to anti-IgM stimulation regardless of which 
line was analyzed (Fig. 6B and data not shown). Either no re-


tion, or one at a substantially reduced level, was observed when 
unfractionated spleen or lymph node cells were stimulated in vitro.

Fig. 6B shows the response of purified splenic B cells from the 
highest-expressing lines, RO3 and RB2, compared with that of 
C57BL/6 mice. As can be seen, although both lines responded with 
proliferation above that engendered with medium alone, neither reached >~15% of the wild-type (CD45<sup>−/−</sup>) level. In contrast, in all cases, the B cell preparations responded equally well to LPS (as do CD45-null B cells; Ref. 5).

T-dependent Ab production and Ig class switching can occur in 
CD45RO and CD45RB isoform mice

We investigated the ability of vav-CD45RO and vav-CD45RB transgenic mice to respond to vivo to a T cell-dependent Ag, DNP-
BSA. In each case, the serum from individual mice was titrated 
over a wide range and the level of DNP-specific IgM and IgG1 was 
determined at both 10 days after primary immunization and 7 days 
after rechallenge. The data shown in Fig. 7 plot the level of DNP-
specific IgG1 Ab (in absorbance units) in individual animals at a 
particular dilution of serum taken from mice 10 days after the 
primary immunization. Comparisons between genetically matched 
groups of animals were made by one-way ANOVA and produced 
the same qualitative results regardless of whether single or multi-
ciple dilution points were compared. The data are presented as single 
dilution point values for simplicity.

Whereas IgM levels did not significantly differ between any 
groups of animals, even between wild-type and CD45-knockout 
mice, significant differences were seen in the ability of different 
lines of mice to produce hapten-specific IgG1 (Fig. 7). CD45-null 
mice produce essentially no DNP-specific IgG, the values found 
being within the range for unimmunized serum (data not shown). 
The level of specific IgG1 produced by other mice broadly corre-
lated with the level at which the CD45 transgene was expressed in
FIGURE 7. Production of DNP-specific IgG1 Abs by vav-CD45RO and vav-CD45RB transgenic mice in response to immunization with the T cell-dependent Ag DNP-BSA. Shown is the relative amount of DNP-specific IgG1 Ab (in absorbance units) in the serum of individual mice (genotype as indicated) immunized i.p. 10 days previously with 100 μg of DNP-BSA in alum. The results of two independent experiments have been pooled. The data for each group of mice was then compared by one-way ANOVA using the GENSTAT, 1993 package. Where the results for a particular group differed significantly from those of the C57BL/6 (CD45+/−) mice the level of significance (p value) is given.

the relevant line. The order of expression levels for the lines shown in Fig. 7 is RO7 ~ RB4 > RO10 > RO4. RO4 mice produced only low DNP-specific IgG1 levels, whereas those of the higher-expressing RB4 and RO7 lines were comparable to wild-type mice. The intermediate RO10 line, while producing a substantial response, made significantly less Ab than C57BL/6 wild-type control mice. Very similar results were obtained for secondary IgG1 Ab measurements. In summary, Ab responses correlated with the level of CD45 expression at the cell surface whereas either CD45RO or CD45RB appeared to be equally proficient at rescuing Ab responses in CD45-null mice.

Discussion

We have used a transgenic expression vector which uses mouse vav1 gene transcriptional regulatory elements to direct expression of two CD45 isoforms, namely CD45RO and CD45RB, specifically throughout the hemopoietic compartment. This vector has been previously shown to efficiently direct expression of human CD4 in a wide variety of hemopoietic cells (25). Nine of 13 resulting transgenic lines give consistent expression in all cells of the expected lineages. These data provide further evidence for the usefulness of the HS321/45-vav vector for transgenic expression within the hemopoietic compartment. Two lines (RO6 and RO12) demonstrated a variegated pattern of expression which may be attributable to integration site-specific effects as have been seen with other transgenes (23, 31). However, these “heterocellular” lines do reveal a strong bias in T cell development. For instance, despite the majority (>70%) of B cells and myeloid cells lacking expression of the transgene, almost all (>95%) of the CD3+ cells in peripheral blood were CD45+ (Fig. 1, RO12); this provides good evidence for a very significant advantage for CD45(RO)+ cells during thymic development. This result is entirely consistent with previous findings that CD45 is a key positive regulator of positive selection (2, 5–7). Indeed, the present findings show that both the CD45RO isoform and the CD45RB isoform can act effectively to permit efficient positive selection of thymocytes (Figs. 1, 3, 4, and 5A). A previous study has shown that CD45RO or CD45RABCC permitted efficient thymocyte development in an exon 6 CD45-targeted mouse, which is largely, although not completely, deficient in endogenous CD45 expression (32).

In the normal thymus, the majority of thymocytes express the CD45RO isoform as their predominant isoform, with expression of CD45RB and CD45RC isoforms appearing after positive selection (33). Thus, it appears that the identity of the extracellular domain of CD45 makes no gross difference to its ability to function during pre-TCR and TCR signaling in the thymus. However, a quantitative effect was observed. Although we have not directly quantified expression, the fluorescent staining was done in conditions of Ab excess and clearly distinguishes between the surface CD45 level on homoyzogotes, heterozygotes, and the various transgenic lines. Our lowest line, RO5, was observably very inefficient in selection of DP thymocytes into the SP pool (Fig. 3). This line demonstrates ~2–3% of the homoyzogous wild-type level of staining. By contrast, the RO4 line (6–8%) is almost equivalent to wild-type with regard to thymocyte development (Fig. 3, Table I). All lines with expression levels higher than RO4 are very similar to the wild-type thymic phenotype. Therefore, there appears to be a critical level of CD45RO expression (between ~2 and ~8% of wild-type) which is sufficient to restore thymic TCR signaling functions. We also observed the same quantitative effect with regard to the amount of CD45 required for normal differentiation within the CD4+CD8− DN thymocyte populations. The analysis of this subset revealed a novel population of CD44highCD25low cells. The significance of this population is unclear, but it may represent an additional checkpoint at which CD45 is normally acting to promote differentiation; alternatively, lack of CD45 may amplify a transient intermediate population by derepressing some proliferative signal(s), possibly via cytokines (10).

We also observed an inverse correlation between the level of staining for CD45 and the level of staining of CD4 and CD8 in DP thymocytes (Fig. 3). This may represent a genuine increase in CD4, CD8 expression, perhaps as compensation for reduced signal amplification. However, alternative explanations are possible including a reduction in masking/steric hindrance or fluorescence quenching at reduced CD45 levels.

With the exception of the lowest-expressing line (RO5), expression of either CD45RO or RB at levels considerably below that seen in wild-type mice (e.g., RO4, RB4) restores the presence of CD3+ T cells in the periphery to normal levels (Fig. 5A). This result is in marked contrast to the results obtained by Kozieradzki et al. (32). Their study reported that CD45RO and CD45RABCC cDNA transgenes, driven by the lck proximal promoter, restored T cell numbers to near normal in lymph nodes whereas no restoration of T cell populations in the spleen was found (32). They used only one line in each case. We have consistently observed reconstitution of T cells in the spleen in five CD45RO and three CD45RB lines. In the case of the CD45RABCC transgenic line analyzed by Kozieradzki et al. (32), the level of CD45 expression may have influenced their results, given that they found staining at ~2–4% of the wild-type level, similar to our RO5 line. However, they reported an ~15% wild-type-staining level for their RO line, more than we found for RO4 and comparable with our RO7 line, both of which give normal T cell numbers in the spleen (Fig. 5A and data not shown). It may be noted that the proximal lck promoter is more efficient at driving gene expression in the thymus than in the periphery, whereas the vav1 promoter is efficient in both contexts, possibly explaining these conflicting results.

The finding that T cells expressing single CD45 isoforms proliferated more efficiently in response to CD3 mAb was unexpected, particularly in light of the lower isoform expression on these cells in comparison with the higher total CD45 expression on the wild-type cells. It is possible that whereas low CD45 levels exert a dominantly positive role in TCR signaling, at higher expression levels the negative actions of CD45, particularly in dephosphorylating Lck pTyr-394, counteract the positive effects to some extent (4).
The successful reconstitution of thymocyte development, T cell migration, and TCR responsiveness by the majority of our novel transgenic lines is in significant contrast to the inability of the vav-CD45RO and vav-CD45RB transgenes to complement B cell maturation and in vitro responses. As has been shown by us and others (5, 34), CD45-deficient mice are impaired in the maturation of IgM\textsuperscript{low}IgD\textsuperscript{high} (T2) cells into the mature IgM\textsuperscript{low}IgD\textsuperscript{high} phenotype typical of follicular B cells. This maturation step is also known to involve Btk, Vav1/2 and other components of the B cell Ag receptor signaling cascade (35–39). The single isoform mice examined in this study did not properly restore the ability of B cells to mature from the T2 stage despite the fact that the level of CD45 on the highest-expressing lines was greater than 50% of the homozygous wild-type level on CD19.

CD45-deficient mice are impaired in the maturation and in vitro responses. As has been shown by us and others (5, 34), CD45-depleted exon 9 mice were restored in isotype switching in response to a different T cell-dependent Ag receptor signaling cascade (35–39). The single isoform mice examined in this study did not properly restore the ability of B cells to mature from the T2 stage despite the fact that the level of CD45 on the highest-expressing lines was greater than 50% of the homozygous wild-type level on CD19+ splenic cells and closely approached the level on heterozygous CD45+/− mice, in which maturation is normal. Comparison of pairs of mice from CD45RO and CD45RB lines, well-matched for expression, did suggest that CD45RO might function more effectively than CD45RB in this role (Fig. 5B).

It has long been known that peripheral B cells will proliferate in vitro when stimulated with anti-IgM or anti-IgD, a phenotype completely lacking in CD45-null exon 9−/− mice (5). We found that isoform transgenic B cells would proliferate above background levels when stimulated with anti-IgM but that the level of proliferation was greatly reduced compared with wild-type B cells. This was demonstrated with highly purified B cells as well as unfractionated spleen and lymph node cells to rule out the possibility that an inhibitory population of other cells (e.g., myeloid) might be responsible. It is most unlikely that this effect is caused by the partial block in B cell maturation. Firstly, T2 and mature B cells proliferate equally well to anti-IgM in vitro (30) and CD45-null mice possess a normal fraction of T2 cells (5). Secondly, some of the RO lines do contain significant numbers of mature B cells (e.g., RO3) and yet still proliferate very poorly (compare C57BL/6 and RO3 in Figs. 5B and 6).

Although, like almost all hemopoietic cells, B cells express more than one CD45 isoform, CD45RABC appears to be the predominant form on normal B cells (40). It remains to be seen whether the relatively poor functionality of CD45RO/RB in B cells is due to a requirement for a different extracellular domain or a particular combination of isoforms. It is tempting to speculate that the CD45RABC extracellular region is required for critical interactions with other molecules within the B cell membrane which cannot be mimicked by CD45RO or CD45RB (13). Although it is possible that our transgenic lines simply require a higher threshold level of CD45, if so the threshold must be extremely close to the heterozygous level because the RO3 and RB2 lines approach that level. Very recently, Virts and coworkers (41) reported the complementation of our CD45-null mice using a CD45 minigene expressed under control of the LFA-1 promoter. The normal pattern of isoform expression was observed and in this case B cell maturation and function was largely restored. Although the level of expression on B cells was not specifically quantified, it appears similar to their wild-type control. Therefore the data of Virts et al. (41) does not assist to answer the question as to which CD45 isoforms, and at what level, are needed for B cell development and function.

CD45-null (exon 9−/−) mice do not produce IgG1 against T-dependent Ags due to a lack of functional peripheral T cells. Indeed, B cells from these exon 9-targeted animals, as well as those bearing the exon 6 mutation, have been shown to be capable of isotype switching in response to a different T cell-dependent Ag (DNP-KLH) in the presence of T cells from CD45+/+ mice (N. Holmes, unpublished data; Ref. 34). Furthermore, both exon 9−/− mice (N. Holmes, unpublished data) and exon 6−/− mice (34) make T cell-independent antigenic responses which are somewhat greater than CD45+/+ wild-type mice. In light of these data, the ability of the single isoform transgenic mice to mount a T cell-dependent Ab response will depend on the helper function, and perhaps abundance, of their T cells. It is noteworthy, then, that RO4 mice make only a poor primary (Fig. 7) and secondary (data not shown) T-dependent Ab response despite having normal numbers of T cells in their peripheral lymphoid organs. Furthermore, the RO10 line (with an expression level intermediate between RO4 and RO7) also makes a demonstrably lower response, whereas RO7 and RB4 give quantitatively normal responses. We interpret these data as showing that CD45RO and CD45RO isoforms can supply all the functions a T cell needs for Ag responsiveness and B cell help, providing they are expressed in sufficient quantity. For this reason, the demonstration by Koziara et al. (32) that CD45RABC single isoform mice, expressing 2−4% of wild-type levels, did not produce T-dependent Ab needs to be interpreted cautiously. Xu and Weiss (14) have recently proposed a model in which CD45RO forms inactive dimers in a concentration-dependent manner. Our data, which demonstrate a positive correlation between CD45RO expression levels and lymphocyte function, do not seem consistent with this hypothesis.

In summary, we have compared the abilities of CD45RO and CD45RB isoforms to function in both T and B cells in an in vivo complementation model. Despite the broad conservation of the switching from CD45RO to larger isoforms (predominantly CD45RB in the mouse) upon T cell-positive selection and its reversal upon mature T cell activation, in general both isoforms studied here behaved similarly. The predominant finding is that there is a distinct quantitative correlation between expression level and function. The critical level required seems to vary depending on the context: less CD45 will apparently suffice for thymic development than is required for T cell function in the periphery. However, in the B cell lineage the CD45RO and CD45RB isoforms do not effectively substitute for the normal endogenous isoforms. These transgenic lines should also permit analysis of CD45 function in nonlymphoid lineages.

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