Combinations of CD45 Isoforms Are Crucial for Immune Function and Disease

Ritu Dawes, Svetla Petrova, Zhe Liu, David Wraith, Peter C. L. Beverley and Elma Z. Tchilian

J Immunol 2006; 176:3417-3425; doi: 10.4049/jimmunol.176.6.3417
http://www.jimmunol.org/content/176/6/3417

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

References This article cites 55 articles, 21 of which you can access for free at: http://www.jimmunol.org/content/176/6/3417.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Combinations of CD45 Isoforms Are Crucial for Immune Function and Disease

Ritu Dawes,* Svetla Petrova,* Zhe Liu,* David Wraith,** Peter C. L. Beverley,* and Elma Z. Tchilian1*

Expression of the CD45 Ag in hemopoietic cells is essential for normal development and function of lymphocytes, and both mice and humans lacking expression exhibit SCID. Human genetic variants of CD45, the exon 4 C77G and exon 6 A138G alleles, which alter the pattern of CD45 isoform expression, are associated with autoimmune and infectious diseases. We constructed transgenic mice expressing either an altered level or combination of CD45 isoforms. We show that the total level of CD45 expressed is crucial for normal TCR signaling, lymphocyte proliferation, and cytokine production. Most importantly, transgenic lines with a normal level, but altered combinations of CD45 isoforms, CD45RABC+/+ and CD45RO+/+ mice, which mimic variant CD45 expression in C77G and A138G humans, show more rapid onset and increased severity of experimental autoimmune encephalomyelitis. CD45RO+/+ cells produce more TNF-α and IFN-γ. Thus, for the first time, we have shown experimentally that it is the combination of CD45 isoforms that affects immune function and disease. The Journal of Immunology, 2006, 176: 3417–3425.

The CD45 (leukocyte common) Ag is a hemopoietic cell-specific tyrosine phosphatase, essential for efficient T and B cell Ag receptor signal transduction (1, 2). Multiple CD45 isoforms can be generated by complex alternative splicing of exons 4(A), 5(B), and 6(C) in the extracellular domain of the molecule. The expression of different CD45 isoforms is cell type specific and depends on the state of activation and differentiation of hemopoietic cells (3, 4). In humans, naïve T lymphocytes express high molecular mass isoforms containing the A exon (CD45RA cells), but, following activation, the low molecular mass 180-kDa isoform is expressed (CD45RO cells), and CD45RO expression is maintained on the majority of primed (memory) cells (3, 5). In both rats and mice, low molecular mass isoform expression is also associated with T cell memory and more differentiated T cell function (4, 6).

Although CD45 alternative splicing is highly regulated and conserved among vertebrates, the function of different CD45 isoforms is not clear. No specific ligand has been found for CD45, although interactions with lectin-like molecules have been reported (7–10). Nevertheless, it is clear that a large CD45 extracellular domain is required for normal TCR signaling, at least in transfected cell lines (11). The formation of homo- and heterodimers by CD45 isoforms has been proposed as another mechanism that may regulate CD45 phosphatase activity (12); however, recent structural data indicate that this is unlikely (13). Spatiotemporal mechanisms for regulating CD45 activity have been suggested involving partitioning of CD45 into lipid rafts or the immunological synapse (14–17).

However, although the function of CD45 isoforms remains unknown, what is clear is that CD45 is crucial for normal immune function and altered CD45 expression has major effects. Both mice and humans lacking CD45 expression are severely immunodeficient (18, 19), and point mutations of CD45 that cause altered splicing have been associated with autoimmune and infectious diseases (20–22). The two best described human CD45 variant alleles have contrasting phenotypes, geographical distribution, and disease associations. The exon 4 C77G variant is found in 1–3% of Europeans and North Americans and is present at a higher frequency in multiple sclerosis (MS),2 HIV, autoimmune hepatitis, systemic sclerosis, and other diseases (20, 22–25). C77G carriers cannot splice out exon A, and their memory/effector lymphocytes continue to express both CD45RA and CD45RO isoforms. In contrast, exon 6 A138G is present in nearly 40% of Far Eastern Orientals (26). The allele promotes splicing toward the CD45RO isoform, resulting in the presence of more memory/effector CD45RO-positive T cells and more T cells producing IFN-γ. A138G is associated with protection against Graves’ disease and hepatitis B (21). The high frequency of A138G indicates that it may also affect susceptibility and pathogenesis in other autoimmune or infectious diseases, and therefore, has an important effect on disease burden in a large part of the human population.

At least two mechanisms may underlie CD45 regulation of immune function. The first is by control of the threshold of signaling through the TCR. The critical role of CD45 phosphatase activity for TCR signal transduction is well established, and the Src family of kinases has been identified as its primary targets (1, 2). The second mechanism is by regulation of cytokine production and responses. CD45−/− mice have increased phosphorylation of Jaks, and their hemopoietic cells show increased responses to cytokines (27), while recent studies have shown that CD45 is required for chemokine and cytokine production in NK cells after stimulation via Fc or MHC-binding receptors (28).

To investigate the role of distinct CD45 isoforms, we constructed transgenic (Tg) mice expressing single high (CD45RABC) or low (CD45RO) molecular mass isoforms at different levels on a CD45−/− background. Because in humans expressing CD45 variant alleles it is the combinations of isoforms

---

2 Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; Tg, transgenic.
expressed on cells that are altered, we also generated mice expressing defined combinations of isoforms mimicking the expression of C77G or A138G human variants. In these CD45 Tg models, we asked how CD45 expression patterns affect proliferative responses and cytokine production. We show first that a threshold of CD45 expression is required for adequate TCR signaling and cytokine production. Second, we show that changes in combinations of CD45 isoforms alter the threshold for TCR signaling and cytokine production, resulting in altered susceptibility and severity of disease in a model autoimmune disease, experimental autoimmunencecephalomyelitis (EAE). Collectively, our results show that both the levels of expression and combinations of CD45 isoforms are crucial for lymphocyte function.

Materials and Methods

CD45 Tg mice

Tg mice expressing single CD45RABC or CD45RO isoforms under the control of the human CD2 promoter have been described previously (29). The Tg mice were bred onto CD45 homozygous knockout mice. Two lines with each isoform were constructed with high and low levels of transgene expression. These are referred to as CD45RABC<sup>high</sup>, CD45RABC<sup>low</sup>, CD45RO<sup>high</sup>, and CD45RO<sup>low</sup>. We also generated mice with one normally splicing CD45 allele and a fixed nonsplicing single CD45 transgene (CD45RABC/H11032 and CD45RO<sup>high</sup> mice) by breeding single isoform CD45RABC<sup>high</sup> or CD45RO<sup>high</sup> Tg mice with C57BL/6 mice. The presence of the CD45 transgenes in the F<sub>1</sub> crosses was detected by PCR on tail genomic DNA, using forward, 5'-GAACCTCAAGATCCAAAGAGAGG-3' and reverse, 5'-TAATTCACAGTAATGTTCCCAAACATGGC-3' primers, generating 1000- and 710-bp products for the CD45RABC and CD45RO transgenes, respectively. All mice were bred in the specific pathogen-free facilities of the Institute for Animal Health and used for experiments at 6–8 wk of age, unless otherwise stated. All experiments fully complied with relevant Home Office guidelines and were approved by the animal ethical committee of the Institute for Animal Health.

Flow cytometric analysis

The following reagents and Abs were also used to stain cell suspensions: CD4 FITC (GK1.5), CD8 PE (53-6.7), pan CD45 CyC (30-F11), CD45RA biotin (14.8), and CD45RB biotin (16A), all from BD Biosciences. Apoptosis was visualized by annexin V staining. Cells were first surface stained using CD4 and CD8 Abs, followed by annexin staining, according to the manufacturer’s instruction (BD Pharmingen).

T cell separation and activation

CD4 and CD8 lymph node T cells were purified by negative selection by first incubating the cells with anti-MHC class II (clone TIB120), B220 (clone RA36B2), CD11b (clone M1/70), and either CD4 (GK1.5) or CD8 (3168) Abs (all gifts from D. Tough, The Edward Jenner Institute for Vaccine Research, Compton, U.K.), followed by Dynabead separation. The purity of the resulting subsets was checked by flow cytometry and was better than 90%.

For T cell stimulation, 2 x 10<sup>5</sup> lymph node cells were placed into round-bottom 96-well plates. PMA (50 ng/ml) and calcium ionophore (ionomycin; 200 ng/ml) were added to the wells. For TCR-CD3 cross-linking, the plates were coated overnight at 4°C with varying concentrations (from 0 to 10 μg/well) of anti-mouse CD3ε (clone 145-2C11; BD Biosciences) in the presence or absence of 1 μg/well CD28 (clone 37.51; BD Biosciences) and washed three times before incubation with the T cells. For MLR, 2 x 10<sup>5</sup> lymph node cells of CD45 Tg H-2<sup>d</sup> mice were placed into round-bottom 96-well plates in the presence of 2 x 10<sup>5</sup> (10:1), 1 x 10<sup>6</sup> (5:1), and 2 x 10<sup>6</sup> (1:1) T cell-depleted BALB/c H-2<sup>d</sup> stimulator cells. These were prepared by treating the splenocytes with anti-Thy-1.2 mAb, followed by addition of guinea pig complement (gifts from D. Tough) and irradiating with 2500 rad. T cells were harvested at various intervals (24–96 h) after a 12-h pulse with 1 μCi of [<sup>3</sup>H]thymidine per well.

Cytokine production

Cytokine production was measured using BD Biosciences mouse Th1/Th2 cytokine cytometric bead array kit, following the manufacturer’s protocol. Briefly, for each test sample, 10 μl of each cytokine capture bead suspension was mixed, and 50 μl of the mixed beads was transferred to each assay tube, followed by 50 μl of PE detection reagent and 50 μl of diluted standard or neat sample. The samples were incubated for 2 h at room temperature in the dark, washed, and resuspended in 300 μl of wash buffer. The standards and test samples were analyzed on a FACSCalibur (BD Biosciences), using CellQuest and BD cytometric bead array software, in accordance with the manufacturer’s instructions.

Western blot analysis

For total protein extraction, cell lysis (2 x 10<sup>6</sup> cells/ml) was performed in radioimmunoprecipitation assay buffer (PBS (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, and 0.5 mM PMSF) buffer, containing protease and phosphatase inhibitors (protease inhibitor mixture and phosphatase inhibitor mixture 2; Sigma-Aldrich), on ice at 4°C for 30 min. Insoluble materials were removed by centrifugation (15,000 x g for 10 min at 4°C), and the total protein concentration of each sample was quantified by bicinchoninic acid protein assay (Pierce Biotechnology). Cell lysates were analyzed by 10% SDS-PAGE (10 μg of total protein per lane), transferred onto a nitrocellulose membrane, and immunoblotted using Abs specific for STAT1, pY701STAT1 (Upstate Biotechnology), and T-bet (Santa Cruz Biotechnology). The basal and phosphorylated status of Lck was determined using Abs against Lck (Upstate Biotechnology), pY505Lck, or pY416Src (Cell Signaling Technology). pY416Src Ab cross-reacts with pY539Lck. Blots were developed using ECL donkey anti-rabbit HRP-linked F(ab)'<sub>2</sub> and ECL Western blotting detection reagents (Amer sham Biosciences).

Induction of EAE

EAE was induced by s.c. injection of myelin oligodendrocyte glycoprotein peptide 35-55, MEVGYR5FSFRVHLYRNKGQ (29) of 200 μg of peptide Ag in a final volume of 100 μl of CFA (Difco Laboratories) containing Mycobacterium tuberculosis (4 mg/ml) (30). On the same day and 2 days later, all mice received two i.p. doses of pertussis toxin (200 ng injection; Sigma-Aldrich). Mice were scored twice daily for symptoms of EAE, as follows: 0; no signs; 1, flaccid tail; 2, partial hind limb paralysis and/or impaired righting reflex; 3, full hind paralysis; 4, hind limb plus fore limbs paralysis; and 5, moribund and dead. The two scores for each day were added together and divided by the number of surviving mice to give the mean EAE grade for each day.

Statistical analysis

Student’s t test assuming equal variance was used.

Results

Proliferative responses in CD45 Tg mice expressing single CD45RABC or CD45RO isoforms

To investigate the role of different CD45 isoforms in T cell function, we constructed Tg mice expressing single high (CD45RABC) or low (CD45RO) molecular mass isoforms on a CD45<sup>−/−</sup> background (29). Two CD45RABC and two CD45RO lines were generated with high and low levels of expression of the respective transgenes (Fig. 1). These are referred to as CD45RABC<sup>high</sup>, CD45RABC<sup>low</sup>, CD45RO<sup>high</sup>, and CD45RO<sup>low</sup>. We compared the responses to titrated amounts of plate-bound CD3/CD28 over time, of Tg or control CD45<sup>−/−</sup> cells throughout the experiment, and/or impaired righting reflex; 3, full hind paralysis; 4, hind limb plus fore limbs paralysis; and 5, moribund and dead. The two scores for each day were added together and divided by the number of surviving mice to give the mean EAE grade for each day.

Student’s t test assuming equal variance was used.

Results

Proliferative responses in CD45 Tg mice expressing single CD45RABC or CD45RO isoforms

To investigate the role of different CD45 isoforms in T cell function, we constructed Tg mice expressing single high (CD45RABC) or low (CD45RO) molecular mass isoforms on a CD45<sup>−/−</sup> background (29). Two CD45RABC and two CD45RO lines were generated with high and low levels of expression of the respective transgenes (Fig. 1). These are referred to as CD45RABC<sup>high</sup>, CD45RABC<sup>low</sup>, CD45RO<sup>high</sup>, and CD45RO<sup>low</sup>. We compared the responses to titrated amounts of plate-bound CD3/CD28 over time, of Tg or control CD45<sup>−/−</sup> lymph node T cells. Proliferation of both CD45RABC<sup>high</sup> and CD45RO<sup>high</sup> cells was greatly reduced compared with CD45<sup>−/−</sup> cells throughout the response (Fig. 2A). Even in the presence of IL-2, although the response was increased, it was still no more than 50% of that of CD45<sup>−/−</sup> mice (data not shown). The number of T cells in lymph nodes in CD45RABC<sup>high</sup> and CD45RO<sup>high</sup> was the same as in CD45<sup>−/−</sup> mice, as detected by CD3, CD4, and CD8 FACS analysis, and did not account for the reduced response. Both CD4 and CD8 T cells showed similarly reduced responses (Fig. 2A). Neither CD45RABC<sup>high</sup> nor CD45RO<sup>high</sup> cells were able to respond to
there is reduced phosphatase activity in cells expressing CD45RABChigh and CD45ROhigh cells. These results show that CD45RABClow, CD45R0high, and CD45R0low mice, as well as wild-type CD45.

pY505Lck is reduced at 2 min in CD45Balloantigens in MLR (Fig. 2A), while both CD45RABChigh and CD45ROhigh lymph node T cells responded to PMA-ionomycin as well as wild-type CD45+/+ cells (Fig. 2C). No difference between the proliferative responses of CD45RABChigh or CD45ROhigh cells to any of the stimuli was detected.

In contrast with CD45RABChigh and CD45ROhigh, mice of the low expressing lines (CD45RABC
ew and CD45RO
ew) gave minimal responses to CD3/CD28, while the response to PMA and ionomycin was not affected (Fig. 2D), suggesting that the level of CD45 expressed influences the ability of T cells to respond to TCR stimulation and that a threshold level of CD45 single isoform expression is required, below which no response can be generated.

To understand the basis for the reduced response of CD45RABChigh and CD45ROhigh, we next examined one of the early phosphorylation events downstream of the TCR. Lck is a substrate for CD45 phosphatase activity, and the dominant role of CD45 is to dephosphorylate the inhibitory tyrosine Y505Lck. Therefore, we measured the basal level of Lck and its pY505Lck phosphorylation status following activation via CD3/CD28. As shown in Fig. 2E, the level of Lck protein is the same in the control CD45+/+, CD45RABC
ew, and CD45RO
ew mice. However, pY505Lck is reduced at 2 min in CD45+/+, but not in the CD45RABChigh and CD45ROhigh cells. These results show that there is reduced phosphatase activity in cells expressing CD45RABChigh and CD45ROhigh single isoforms, which explains the decreased proliferative response to CD3/CD28 and suggests that a higher level of expression or a combination of isoforms is required to restore this defect. Because CD45 can also dephosphorylate the activating tyrosine Y394Lck, we also measured its phosphorylation using Ab against Src phosphorylated at tyrosine 416. No differences in phosphorylation of Y394Lck were detected between the three mice strains (Fig. 2E).

Taken together, these results support two important conclusions. First, a threshold level of single CD45 isoform expression is required for TCR signaling. Second, cells expressing single CD45RABC or CD45RO isoforms at similar levels show identical patterns of response.

**Cytokine production in CD45 Tg mice expressing single CD45RABC or CD45RO isoforms**

We next studied the role of single CD45RABC and CD45RO isoforms in cytokine production. There were reduced amounts of IFN-γ in the supernatants of CD45 Tg cells compared with controls (Fig. 3A), while no consistent changes in TNF production were seen. IL-5 and IL-2 were not detected in supernatants. When separated CD4 and CD8 cells were analyzed, IFN-γ was reduced in both subsets.

We also measured cytokine production after stimulation of lymph node cells with PMA and ionomycin. PMA-ionomycin bypasses CD3 signaling, causing Ca2+ influx directly and activation of PKC. Increased production of TNF-α, IL-5, and IFN-γ and decreased IL-2 were detected in both CD45 Tg mice at 72 h (Fig. 3B) as well as at 24 and 48 h (data not shown). Similar trends were observed in supernatants of separated CD4 and CD8 cells. Increased cytokine production was detected also in the low expressing CD45RABC
ew and CD45RO
ew mice (data not shown). No significant differences were observed between the CD45 Tg mice irrespective of the level or isoform expressed, suggesting that these cells do have the capacity both to proliferate and produce cytokines if TCR signaling is bypassed.

At first sight, the differences in cytokine production of single isoform Tg cells following stimulation with CD3/CD28 or PMA-ionomycin are puzzling. However, single isoform mice have mostly activated/effector type T cells (29). When stimulated with CD3/CD28, the threshold of TCR signaling is not reached so that less cytokines are produced than by CD45+/+ control cells. In contrast, when PMA-ionomycin bypasses TCR signaling, abundant effector cytokines are produced by the activated effector T cells in the Tg mice. Less IL-2 is detected because this cytokine is produced by naive T cells.

These data suggest that, as with the proliferative response, a higher level of CD45 expression or more than one CD45 isoform is required for normal cytokine production.

**Responses in mice expressing two CD45 isoforms**

Mice expressing single CD45RABChigh or CD45ROhigh at levels five times less than the total CD45 in wild-type CD45+/+ mice give grossly abnormal in vitro proliferative and cytokine responses. These results, therefore, indicate that a higher level of single CD45 isoform expression or, alternatively, a combination of isoforms is required to reconstitute normal proliferative and cytokine responses. We next asked whether combinations of CD45 isoforms would restore these responses, first studying the response of T cells in mice expressing both CD45RABC and CD45RO isoforms (CD45RABChigh × CD45ROhigh). Neither the proliferative nor cytokine responses were reconstituted by the simultaneous expression of CD45RABC and CD45RO isoforms, and the responses were not different from single CD45RABChigh or CD45ROhigh cells (data not shown). However, although twice as much CD45 was expressed on the surface of CD45RABChigh × CD45ROhigh cells, the total level of CD45 is still less than in CD45+/+ mice. Therefore, it appears that the threshold level for normal responses was not reached or that more than two isoforms are required to restore the response.

**Proliferative and cytokine responses in CD45 Tg/+ mice**

Because in humans expressing C77G or A138G CD45 variant alleles it is the combinations of isoforms expressed on cells that are altered rather than the total amount of CD45, we generated mice expressing one normally splicing CD45 allele and a CD45RABC or CD45RO transgene. We constructed CD45RABC+/+ mice (F1 of CD45RABChigh × CD45+/+), which mimic the expression in CD457 variant humans, and CD45RO+ mice (F1 of CD45ROhigh × CD45+/+), which model the isoform expression of the A138G allele.

We analyzed the expression of CD45 isoforms and total CD45 expression in CD4 and CD8 cells (Fig. 4A). The level of total CD45 expression is very similar in both CD4 and CD8 cells in
CD45<sup>+/+</sup>, CD45<sup>RABC</sup><sup>+/+</sup>, and CD45<sup>RO</sup><sup>+/+</sup> mice. However, staining with CD45RA indicates that the balance of different isoforms is altered in these mice. As expected, more CD45RA is detected in CD45<sup>RABC</sup><sup>+/+</sup> cells because of the presence of the CD45RABC transgene. Both CD45<sup>RABC</sup><sup>+/+</sup> and CD45<sup>RO</sup><sup>+/+</sup> cells have a lower expression of CD45RB isoforms in CD4 and CD8 cells, suggesting more splicing of the normal CD45 allele toward lower molecular mass isoforms. Both Tg strains have up to 20% more activated T cells, as detected by CD44 and CD122 staining (data not shown), which may also explain the lower CD45RB expression seen in both lines.

Because the total level of CD45 expression is normal, we next determined whether the alterations in the balance of isoforms would affect CD3/CD28 responses. Lymphocytes from lymph nodes of CD45<sup>+/+</sup>, CD45<sup>RABC</sup><sup>+/+</sup>, or CD45<sup>RO</sup><sup>+/+</sup> mice were activated with plate-bound CD3 and 1 μg/ml CD28 Abs. Separated CD4 and CD8 lymph node cells were stimulated with 2 μg/ml CD3 and 1 μg/ml CD28 Abs or with PMA-ionomycin. Cells were pulsed with [¹H]thymidine at the indicated times and harvested 12 h later. Means and SDs of triplicate cultures from three mice are shown. Background counts were <500 cpm and have been subtracted. E. Western blot of Lck, pY055Lck, and pY394Lck in lymph node T cells stimulated with soluble CD3 at 2 μg/ml and CD28 at 1 μg/ml for the times indicated. Equal amount of cell lysate protein (10 μg) was loaded on each lane. Data are representative of six experiments.

FIGURE 2. Activation of lymph node T cells from CD45<sup>+/+</sup> and CD45 Tg mice expressing single isoforms. A, Total mesenteric and peripheral lymph node cells from CD45RABC<sub>high</sub> and CD45RO<sub>high</sub> mice were stimulated with varying amounts of plate-bound CD3 and 1 μg/ml CD28 Abs. Separated CD4 and CD8 lymph node cells were stimulated with 2 μg/ml CD3 and 1 μg/ml CD28 Abs. B, Lymph node cells were stimulated with T cell-depleted and irradiated allogeneic BALB/c splenocytes at ratios of 10:1, 5:1, and 1:1 (stimulator:target), or C, with PMA-ionomycin. D, CD45RABC<sub>low</sub> and CD45RO<sub>low</sub> lymph node cells were stimulated with 2 μg/ml plate-bound CD3 and 1 μg/ml CD28 Abs or with PMA-ionomycin. Cells were pulsed with [¹H]thymidine at the indicated times and harvested 12 h later. Means and SDs of triplicate cultures from three mice are shown. Background counts were subtracted. E, Western blot of Lck, pY055Lck, and pY394Lck in lymph node T cells stimulated with soluble CD3 at 2 μg/ml and CD28 at 1 μg/ml for the times indicated. Equal amount of cell lysate protein (10 μg) was loaded on each lane. Data are representative of six experiments.
We next analyzed cytokine production in CD45RABC/ and CD45 Tg/ mice expressing single CD45RABC or CD45RO isoforms. A. Amount of TNF-α, IFN-γ, IL-5, and IL-2 present in cell culture supernatants after 72-h stimulation with 2 μg/ml CD3 and 1 μg/ml CD28, or PMA-ionomycin. Histograms show the response and SD of three mice of each type. Data are representative of four experiments for total CD3-positive cells and two experiments for CD4- and CD8-separated cells. Statistically significant differences from wild-type CD4−/− controls are shown.

FIGURE 3. Cytokine production by total or CD4- and CD8-separated lymph node cells of CD45 Tg mice expressing single CD45RABC or CD45RO isoforms. A. Amount of TNF-α, IFN-γ, IL-5, and IL-2 present in cell culture supernatants after 72-h stimulation with 2 μg/ml CD3 and 1 μg/ml CD28, or PMA-ionomycin. Histograms show the response and SD of three mice of each type. Data are representative of four experiments for total CD3-positive cells and two experiments for CD4- and CD8-separated cells. Statistically significant differences from wild-type CD4−/− controls are shown.

Cytokine production in CD45 Tg/+ mice

We next analyzed cytokine production in CD45RABC/+ and CD45RO/+ mice after stimulation with CD3/CD28 or PMA-ionomycin. Both CD45RABC/+ and CD45RO/+ cells produced more TNF-α and IFN-γ compared with CD45−/− in response to CD3/CD28 stimulation, although there were no differences between the two Tg mice (Fig. 5A). Similar trends in cytokine production were seen in supernatants of separated CD4 and CD8 lymphocytes.

Interestingly, when stimulated with PMA-ionomycin, the CD45RO/+ mice consistently showed the highest production of TNF-α and IFN-γ at 72 h (Fig. 5B). Increased TNF and IFN-γ production was also observed in separated CD4 and CD8 subsets. There was less consistent trend to production of more IL-2 and IL-5 in CD45RABC/+ and CD45RO/+ mice. No IL-4 was detected in the supernatants. Despite finding differences in cytokine production, proliferative responses to PMA-ionomycin are the same in all strains of mice (Fig. 5C).

Because the change in IFN-γ production was particularly striking and because increased IFN-γ was detected in the human A138G variant individuals (21), for which the CD45RO/+ mice are a model, we concentrated on understanding the increased production of IFN-γ in CD45RO/+ cells. It has been shown that PMA-ionomycin-induced IFN-γ production is dependent on STAT1 and that the Th1-specific transcription factor T-bet, which increases IFN-γ production, is regulated by IFN-γ signaling through STAT1 (31). We therefore measured the basal and phosphorylated level of STAT1 and T-bet protein following PMA-ionomycin stimulation. Fig. 5D indicates that in CD45RO/+ mice more phosphorylated STAT1 and more T-bet are detected, providing an explanation for the increased IFN-γ production in these mice.

EAE in CD45 Tg mice

Human CD45 variant alleles have been associated with autoimmune diseases. The C77G variant has been found with increased frequency in MS (20, 25, 32), while the A138G allele has been shown to be protective in Graves’ disease (21). Because the CD45RABC/+ and CD45RO/+ mice have altered TCR signaling and cytokine production, we next tested whether this would lead to altered disease susceptibility or progression. EAE is a model for both MS in humans and the induction of autoimmune reactivity. We therefore used a myelin oligodendrocyte glycoprotein peptide to induce EAE in CD45−/−, CD45RABC/+, and CD45RO/+ mice (30).

Both CD45RABC/+ and CD45RO/+ developed more severe disease compared with CD45−/− mice with average total score of 2.2 ± 0.8 and 2.5 ± 1.1, respectively, vs 1.6 ± 1.1 in control CD45−/− mice (Table and Fig. 6). Disease onset was also earlier in CD45RABC/+ and CD45RO/+ mice (day 9.6 ± 1.1 and 10.3 ± 1.8 vs 12.6 ± 2.4 in CD45−/− mice). In all three experiments, the CD45RABC/+ mice developed the disease first (day 9.6 ± 1.1), while the signs of disease were more severe in CD45RO/+ mice (average maximal disease score 4.8 ± 1.3 vs 3.5 ± 0.9 and 3.1 ± 1.1).
These results indicate that altering the balance of isoforms is important for immune function, and that CD45RABC/ and CD45RO/ mice are more susceptible to induction of autoimmune disease in the EAE model.

**Discussion**

CD45 expression patterns can have profound effects on immune function and disease, and CD45 variant alleles that alter CD45 isoform expression are associated with infectious and autoimmune diseases. However, how CD45 isoform expression regulates lymphocyte function is not clear. To understand this, we studied how TCR signaling, cytokine production, and disease are altered in Tg mice expressing single, combinations of two, or more complex combinations of CD45 isoforms.

Our results with Tg mice expressing single CD45RABC/ and CD45RO/ mice are more informative for understanding the role of isoforms in immune function than the latter is likely due to the level of expression rather than the lack of a combination of isoforms for three reasons. First, mice with higher level of a single CD45 isoform than ours give normal proliferative responses (33). Second, the magnitude of proliferative response correlates with the level of expression in our single isoform high and low CD45RABC and CD45RO mice. Third, combinations of two isoforms at a subnormal level do not restore the response.

Interestingly, both CD45RABC/ and CD45RO/ mice are able to mount protective immune response during lymphocytic choriomeningitis virus and influenza virus infection (29), suggesting that in the context of a viral infection, when the innate system is strongly activated to produce cytokines and coreceptor signals (34), the activation threshold may be reached, allowing productive signaling to occur. However, when less innate activation occurs, a threshold level for an adequate response is not achieved. In addition to showing the importance of adequate levels of CD45 expression, both the in vitro and in vivo data indicate that there is no difference in the proliferative response and cytokine production between cells expressing either single CD45RABC or CD45RO isoforms at the same level or a combination of these two.
All hemopoietic cells express more than one CD45 isoform, and alternative splicing is conserved throughout vertebrate evolution, so that it seems certain that combinations of isoforms must have vital functions. Therefore, the most important new finding reported in this study is that combinations of CD45 isoforms do indeed affect immune function. Thus, even when a normal level of total CD45 expression was reached, but the balance of isoforms changed, as in CD45^{RABC^{+}} or CD45^{RO^{+}} mice, both strains showed altered kinetics and magnitude of proliferative response accompanied by more rapid and efficient dephosphorylation of pY505Lck. This leads to an altered TCR threshold reflected in the rapid proliferative responses of CD45^{RABC^{+}} or CD45^{RO^{+}} cells.

As a result of the more vigorous TCR response, more TNF-α and IFN-γ are produced by CD45^{RABC^{+}} or CD45^{RO^{+}} cells.

Convincing differences in the function of distinct CD45 isoforms have been difficult to identify. Many studies have been performed in which specific CD45 isoforms have been transfected into transformed cell lines. In some cases, a CD45RO transgene, while in others CD45ABC, was more efficient in restoring TCR signaling (35–38). These inconsistent observations are most likely due to inadequate levels of CD45 isoforms expressed, differences in CD45 negative cell lines, and in the stimuli or assays used (1).

In contrast, our studies and those of others (33) with CD45 Tg mice expressing identical levels of CD45 isoforms did not reveal

Table I. EAE induction and assessment in CD45^{+/+}, CD45^{RABC^{+}}, and CD45^{RO^{+}} mice

<table>
<thead>
<tr>
<th>Incidence of Disease</th>
<th>Mean Day of Onset (±SD)</th>
<th>Average Total Score (±SD)</th>
<th>Average Maximal Score (±SD)</th>
<th>Fatality</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45^{+/+}</td>
<td>21/22</td>
<td>12.6 ± 2.4</td>
<td>1.6 ± 1.1</td>
<td>3.1 ± 1.1</td>
</tr>
<tr>
<td>CD45^{RABC^{+}}</td>
<td>24/25</td>
<td>9.6 ± 1.1*</td>
<td>2.2 ± 0.8***</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td>CD45^{RO^{+}}</td>
<td>19/20</td>
<td>10.3 ± 1.5*</td>
<td>2.5 ± 1.1***</td>
<td>4.8 ± 1.3**</td>
</tr>
</tbody>
</table>

EAE was induced in groups of five to nine mice. The mean and SDs from three experiments are shown. The average total score was calculated by adding the scores from each day from the onset of disease and dividing by the number of days scored. The average maximal score was the maximal score in each experiment for each strain of mice divided by three. Statistically significant differences from wild-type CD45^{+/+} controls are indicated as *, p < 0.05; **, p < 0.01; and (**), p < 0.001.

**FIGURE 5.** Cytokine production and response of CD45^{+/+}, CD45^{RABC^{+}}, and CD45^{RO^{+}} mice. A, Total lymph node cells or separated CD4 and CD8 cells were stimulated for 72 h with CD3/CD28, or B, PMA-ionomycin. The histograms show means and SD of the amounts in supernatants of triplicate cultures from three mice. Results are representative of six experiments for total lymph node cells and three for separated CD4 or CD8 cells. C, Activation of lymph node T cells with PMA-ionomycin. Cells were pulsed with [³H]thymidine at 72 h and harvested 12 h later. Means and SDs of triplicate cultures from three mice are shown. D, Western blot of STAT1, pY701STAT1, and T-bet in lymph node T cells stimulated with PMA-ionomycin for the times indicated. Data are representative of three experiments.
FIGURE 6. EAE in CD45+/+, CD45RABC+, and CD45RO+/+ mice. EAE was induced in groups of five to nine mice of each strain, and disease was monitored, as described in Materials and Methods. The graph represents the mean score for each day from three experiments.

The authors have no financial conflict of interest.

Acknowledgments
We thank Dr. Agnes Le Bon for helpful discussions, and Jenny Piercy for technical assistance.

Disclosures
The authors have no financial conflict of interest.

References


ic to bone marrow-derived cells and is genetically controlled. J. Immunol. 166: 2944–2952.


