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# The Differential Regulation of Lck Kinase Phosphorylation Sites by CD45 Is Critical for T Cell Receptor Signaling Responses

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## SUMMARY

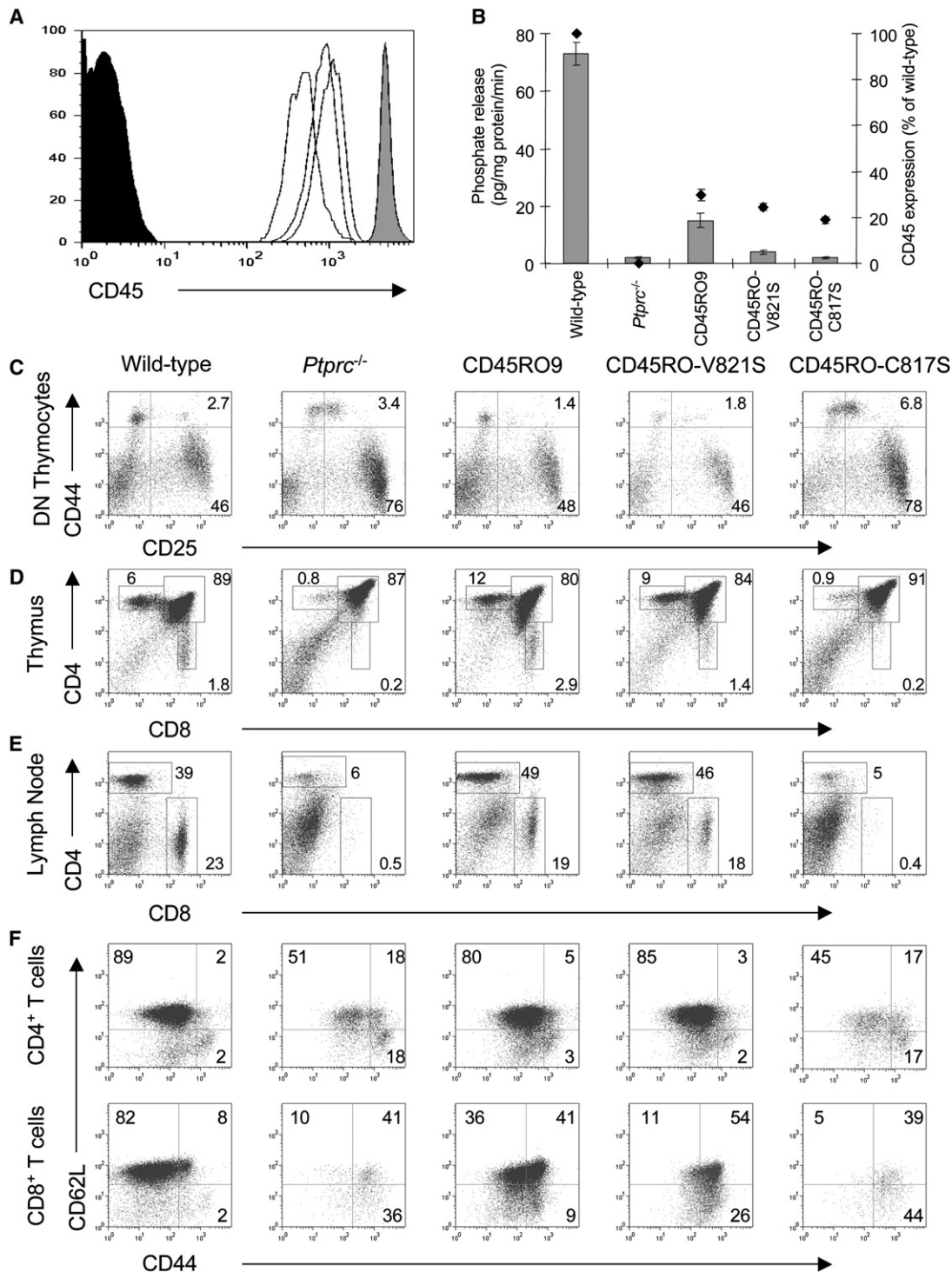
The molecular mechanisms whereby the CD45 tyrosine phosphatase (PTPase) regulates T cell receptor (TCR) signaling responses remain to be elucidated. To investigate this question, we have reconstituted CD45 (encoded by *Ptprc*)-deficient mice, which display severe defects in thymic development, with five different expression levels of transgenic CD45RO, or with mutant PTPase null or PTPase-low CD45R0. Whereas CD45 PTPase activity was absolutely required for the reconstitution of thymic development, only 3% of wild-type CD45 activity restored T cell numbers and normal cytotoxic T cell responses. Lowering the CD45 expression increased CD4 lineage commitment. Peripheral T cells with very low activity of CD45 phosphatase displayed reduced TCR signaling, whereas intermediate activity caused hyperactivation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. These results are explained by a rheostat mechanism whereby CD45 differentially regulates the negatively acting pTyr-505 and positively acting pTyr-394 p56<sup>lck</sup> tyrosine kinase phosphorylation sites. We propose that high wild-type CD45 expression is necessary to dephosphorylate p56<sup>lck</sup> pTyr-394, suppressing CD4 T<sup>+</sup> cell lineage commitment and hyperactivity.

## INTRODUCTION

The transmembrane and heavily glycosylated CD45 tyrosine phosphatase (PTPase) has attracted attention because of its high expression on the surface of T and B lymphocytes (Alexander, 2000; Hermiston et al., 2003; Holmes, 2006). Why does an enzyme need to be so abundant at the T cell surface? No physiologically relevant exogenous CD45 ligands have yet been identified, although various lectins have been suggested (van Vliet

et al., 2006). Considerable attention has been given to the alternative splicing of CD45 in T cells, generating up to eight different CD45 isoforms, differing only in their ectodomains (Beverley et al., 1992). These isoforms appear to differentially associate in *cis* with the CD4 and CD8 coreceptors, thereby providing a feasible mechanism whereby the CD45 phosphatase can act on CD4 and CD8-associated p56<sup>lck</sup> tyrosine kinase, which is critical for the initiation of T cell receptor (TCR)-mediated signals (Dorman et al., 2002; Leitenberg et al., 1996). Furthermore, CD45 isoforms can differentially homodimerize, particularly at higher expression, possibly leading to inhibition of the phosphatase activity, a finding with relevance to autoimmunity (Majeti et al., 1998, 2000; Xu and Weiss, 2002). A further possible isoform-dependent mechanism involves the differential targeting of CD45 to p56<sup>lck</sup> in lipid rafts (Irls et al., 2003). Several reports have also highlighted the importance of CD45 expression in its differential regulation of TCR signaling amplitude. For example, it is well established that CD45 expression increases during T cell development, becoming 2-fold higher in the periphery than in the thymus (McNeill et al., 2004). This correlates with a change in the sensitivity of TCR signaling responses from high in the thymus to much lower in the periphery (Grossman and Singer, 1996; Sebzda et al., 1996). The threshold of TCR engagement required for activation is reduced even further in *Ptprc*<sup>+/-</sup> (encoding CD45) mice thymocytes in which CD45 PTPase activity is lower and positive selection is amplified (Wallace et al., 1997). The varying concentration of CD45 in specific subcellular localizations has also received attention in the context of signaling studies on the immune synapse and on microclusters (Campi et al., 2005; Douglass and Vale, 2005; Varma et al., 2006; Yokosuka et al., 2005). Such findings have stimulated the focus of our present study, which is on the differential expression of CD45 and the way in which the consequent titration of its phosphatase activity is critical in dictating TCR signaling responses.

The most important substrate for CD45 in T cells is the p56<sup>lck</sup> tyrosine kinase (Ostergaard et al., 1989), p59<sup>lyn</sup> also being a substrate (Mustelin et al., 1992). CD45 is the only known phosphatase that dephosphorylates the



**Figure 1. Effects of Altered PTPase Activity on T Cell Differentiation in Mutant CD45RO-PTPase Mice**

(A) Cell-surface expression of CD45 on thymocytes from wild-type (gray histogram), *Ptpnc1*<sup>-/-</sup> (black histogram), and CD45RO-C817S, CD45RO-V821S, and CD45RO9 as unfilled histograms from left to right, respectively.

(B) Thymic CD45 expression (diamond) and membrane PTPase activity (bar) presented as average percentage of wild-type (mean ± SEM, minimum of 5 mice for each group).

(C) Flow cytometry of DN thymocytes (gated CD4<sup>-</sup>CD8<sup>-</sup> cells) showing CD44 and CD25 expression. Numbers in quadrants indicate the frequency of DN2 (CD44<sup>+</sup>CD25<sup>+</sup>) and DN3 (CD44<sup>-</sup>CD25<sup>+</sup>) thymocytes.

(D) Flow cytometry analysis of CD4 and CD8 expression on thymocytes with CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>SP, and CD8<sup>+</sup>SP thymocyte populations highlighted by gating. Numbers indicate the frequency of cells in each gated population.

negative regulatory p56<sup>lck</sup> pTyr-505 site, counteracting an intramolecular association that downregulates kinase function (Veillette et al., 1992). In addition, CD45 dephosphorylates the activatory autophosphorylation p56<sup>lck</sup> pTyr-394 site, thereby suppressing kinase activity (Doro and Ashwell, 1999; Zhao et al., 2004), although in this case other phosphatases are thought to act at this site, such as PEP (Hasegawa et al., 2004) and SHP-1 (Chiang and Sefton, 2001). In *Ptpnc<sup>-/-</sup>* mice, the threshold for TCR signaling becomes very high, in a way that cannot be compensated for by PEP or SHP-1, leading to severe thymic developmental defects and the accumulation of only about 5% of the normal numbers of peripheral T cells, rendering studies on mature murine *Ptpnc<sup>-/-</sup>* T cells problematic (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999). To circumvent this problem, we have in the present work reconstituted *Ptpnc<sup>-/-</sup>* mice with a broad range of expression of a single isoform, CD45RO, in both wild-type and mutant forms. Our results show that whereas CD45 function is absolutely dependent on its phosphatase activity in the whole animal context, only 3% of normal phosphatase functionality is sufficient to reconstitute thymic development, whereas levels in the range of 10%–20% are required for full peripheral function. We present data suggesting that the main reason for high CD45 expression in the wild-type context is to suppress the hyperactivation of peripheral T cells by dephosphorylating p56<sup>lck</sup> pTyr-394.

## RESULTS

### Generation of Transgenic Mice Expressing Mutant CD45RO Isoforms with Altered PTPase Activity

CD45 has two phosphatase homology domains located in the cytoplasmic tail, with only domain I being catalytically active, whereas domain II is thought to have a regulatory role. Point mutations in either of these domains can result in a reduction in PTPase activity (Johnson et al., 1992). We selected two of these previously published point mutations in domain I: C817S, which has been shown to ablate PTPase activity, and V821S, which has been shown to reduce wild-type PTPase activity to an estimated 25%, an amount we hypothesized might be sufficient to reconstitute thymic development in *Ptpnc<sup>-/-</sup>* mice. By using rCD45 cytoplasmic tail protein, we found that both these mutations significantly reduced PTPase activity (t test,  $p < 0.01$ ; Figure S1 in the Supplemental Data available online), whereas the so-called wedge mutant E613R was found to have normal PTPase activity (t test,  $p = 0.18$ ).

We have previously described transgenic mouse lines expressing varying amounts of CD45RO under the control of the *vav* promoter (Ogilvy et al., 2003). To complement

these, CD45RO-V821S and CD45RO-C817S transgenic lines were generated to give expression of the individual CD45RO isoform with low or null PTPase activity. To evaluate expression of the transgene, thymocytes were analyzed for surface CD45 expression by flow cytometry (Figure 1A): CD45RO-C817S thymocytes were found to express CD45 protein at  $19.0\% \pm 1.5\%$  and CD45RO-V821S at  $24.7\% \pm 0.5\%$  of total CD45 wild-type amounts (compared to a value including all isoforms). The CD45RO9 transgenic line, described previously (Ogilvy et al., 2003), provided a relevant control expressing the CD45RO isoform at a closely matched ( $29.8\% \pm 2.5\%$ ) amount to CD45RO-V821S. This allowed the effect of reduced CD45 PTPase activity to be compared directly between these two transgenic lines.

To determine the CD45 PTPase activity in primary transgenic cells in vivo, we prepared membrane fractions without detergent to prevent disruption of physiologically relevant associations and measured PTPase activities by using a phosphopeptide substrate. PTPase activity in *Ptpnc<sup>-/-</sup>* thymic membrane samples was reduced to 3.6% of the wild-type amount, showing that nearly all the PTPase measured by the assay was attributable to CD45 (Figure 1B). As expected, CD45 PTPase activity in CD45RO-C817S membranes was ablated. CD45RO-V821S values were 17% of control CD45RO9 membranes, and this reduction, in combination with the lower protein expression compared with C57BL/6, gave this transgenic line an equivalent CD45 PTPase function of just 3.0% of wild-type.

### Phenotypic Analysis of CD45RO-V821S and CD45RO-C817S Transgenic Mice

It is possible that the CD45 ectodomain has a function that is independent of CD45 PTPase activity. The generation of the PTPase-dead CD45RO-C817S line enabled us to address this question. For clarity, phenotypic data are presented below as two types of comparison: either comparing the CD45 mutant PTPase CD45RO-V821S line with its CD45RO9 expression-matched control, in order to assess the effect of reducing CD45 PTPase activity by 80%, or comparing mice varying in unmutated transgenic CD45RO expression. Comparison of CD45RO-C817S and *Ptpnc<sup>-/-</sup>* mice revealed equivalent blocks in thymic development at the DN and DP stages (Figures 1C and 1D), resulting in greatly reduced thymic CD4SP and CD8SP cell numbers (Figure 1D) and few mature peripheral T cells (Figure 1E; Table S1). These results show that the CD45RO ectodomain is unable to function independently of CD45 PTPase activity during T cell development.

However, expression of PTPase-low CD45RO-V821S restored thymic differentiation (Figures 1C and 1D) and

(E) Flow cytometry analysis of CD4 and CD8 expression on peripheral lymph node cells with CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells highlighted by gating. Numbers indicate the frequency of cells in each gated population.

(F) Flow cytometry analysis of CD44 and CD62L expression on gated CD4<sup>+</sup> and CD8<sup>+</sup> T cells from peripheral lymph node. Numbers indicate the frequency of cells in each quadrant. All flow cytometric data are representative of four independent experiments.

a peripheral T cell repertoire (Figure 1E) in which total T cell numbers were comparable with age-matched wild-type mice (Table S1). The absolute numbers of CD4SP and CD8SP thymocytes in CD45RO9 mice were significantly higher than wild-type (t test,  $p < 0.01$ ), by 82% and 53%, respectively, and this was reflected in the periphery by a 42% increase (t test,  $p < 0.01$ ) in CD4<sup>+</sup> T cell, but not CD8<sup>+</sup> T cell, numbers. CD45RO-V821S mice also showed a 78% increase (t test,  $p < 0.01$ ) in the number of CD4SP thymocytes, but this was not reflected in the periphery, where CD4<sup>+</sup> T cells numbers were comparable to wild-type. CD8SP numbers in CD45RO-V821S mice were not altered, but peripheral CD8<sup>+</sup> T cells showed a 38% decrease in absolute numbers (t test,  $p < 0.01$ ). Interestingly, an increased proportion of peripheral CD8<sup>+</sup> T cells from both CD45RO9 and CD45RO-V821S mice fell into the CD44<sup>hi</sup> population (Figure 1F), the majority of these cells also being CD62L<sup>hi</sup>, indicative of a memory-type phenotype.

Therefore, only 3% of the normal amount of CD45 PTPase is sufficient to reconstitute *Ptprc*<sup>-/-</sup> mice with normal numbers of mature T cells, albeit biased toward CD4<sup>+</sup> T cell lineage commitment and with changes within the peripheral CD8<sup>+</sup> T cell population.

#### Reduction in CD45 Expression Enhances CD4 Lineage Commitment

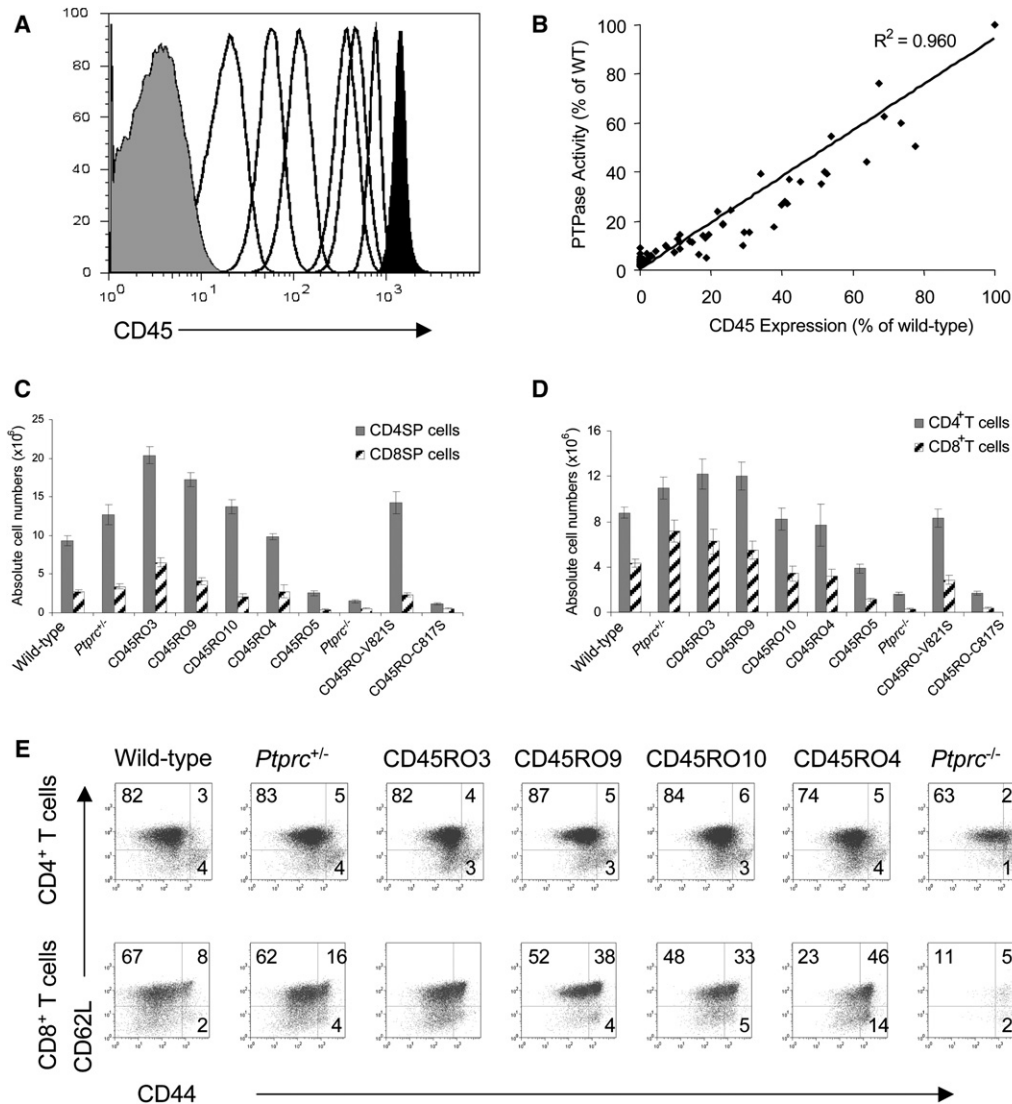
To further investigate the impact of CD45 on T cell development, we examined thymocytes from wild-type, *Ptprc*<sup>+/-</sup>, *Ptprc*<sup>-/-</sup>, and five independent CD45RO transgenic lines. The relative thymic CD45 expression on these lines varied from 1.3% ± 0.5% (CD45RO5) to 59.9% ± 2.4% (*Ptprc*<sup>+/-</sup>) of wild-type (Figure 2A; complete data are in Table S1). Measurement of thymic membrane CD45 PTPase activities revealed a linear relationship ( $R^2 = 0.969$ ) between CD45 expression and activity (Figure 2B), and comparable results were found in peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cell membranes with no difference in total CD45 expression or PTPase activity noted between these two T cell subsets (Figures S2 and S3). As the expression, and so CD45 PTPase activity, decreases, a bias toward the positive selection of CD4<sup>+</sup> T cells was apparent (Figure 2C); this resulted in a higher number of CD4<sup>+</sup> T cells in the periphery (Figure 2D). Only when the CD45 expression fell below 2% (as in the CD45RO5 line) was there a marked failure in the positive selection of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figures 2C and 2D). As noted for the CD45RO-V821S mice, the CD8<sup>+</sup> population also showed alterations in the frequency of CD44<sup>hi</sup> CD62L<sup>hi</sup> cells (Figure 2E), with the proportion of CD8<sup>+</sup> T cells in this population increasing as CD45 expression was reduced. In the following studies, CD44<sup>lo</sup> CD8<sup>+</sup> T cells from all mice were either gated or purified to ensure that analysis was carried out on comparable populations. Overall, these results show that wild-type CD45 expression restrains commitment to the CD4<sup>+</sup> T cell lineage, a restraint much reduced when CD45 PTPase activity is lowered to intermediate amounts (10%–60% of wild-type).

#### Reduced CD45 Expression Results in Altered p56<sup>lck</sup> Phosphorylation and Increased Thymic Signaling

Because the CD45-mediated restraint on the positive selection of CD4<sup>+</sup> T cells was reduced at lower CD45 expression, we investigated the signaling events that might explain this finding. Phosphorylation of activatory and inhibitory p56<sup>lck</sup> Tyr residues were assessed by FACS analysis via commercially available antibodies against active src family kinase and pTyr-505, respectively. Similar results were achieved with specific anti-Lck pTyr-394 sera (Holdorf et al., 2002; data not shown). In gated DP thymocytes, both p56<sup>lck</sup> pTyr-505 and pTyr-394 amounts increased as the expression of CD45 was reduced (Figure 3A). The increase in the phosphorylation of the two pTyr residues was comparable at intermediate CD45 expression, but in CD45RO5 and *Ptprc*<sup>-/-</sup> cells, the phosphorylation of pTyr-505 became very high. Stimulation with CD3 mAb made little obvious difference to the amount of pTyr-505 (data not shown). To demonstrate directly increased signaling capacity in cells with intermediate CD45 expression, we measured CD3-stimulated signals in DP thymocytes, showing that the proportion of DP thymocytes with enhanced staining of phospho-PKB (Figure 3B) was increased above wild-type at intermediate CD45 levels. Of particular importance was the finding that signaling via phospho-ERK was also markedly increased (Figure 3C) because a clear correlation between CD4 lineage commitment and activation of the Ras-MAPK pathway has previously been established (Sharp and Hedrick, 1999; Sharp et al., 1997; Sugawara et al., 1998). The kinetics of ERK and PKB activation were found to be similar in wild-type and CD45RO transgenic mice irrespective of the CD45 expression (data not shown). In the lowest expressing CD45RO5 line, in which the amounts of p56<sup>lck</sup> pTyr-505 were very high, both phospho-PKB and phospho-Erk signals were reduced compared to wild-type, whereas in *Ptprc*<sup>-/-</sup> thymocytes, no CD3-stimulated signaling responses were noted. Overall these results show that in the thymus, the sensitivity of cells to TCR stimulation increases as CD45 expression decreases, associated with increased pTyr-394 and amplified signals, until in CD45RO5 mice the inhibitory effects of pTyr-505 hyperphosphorylation become dominant, and then finally in *Ptprc*<sup>-/-</sup> cells, p56<sup>lck</sup> becomes dysfunctional (Stone et al., 1997).

#### Amplified TCR Signals in Peripheral T Cells with Intermediate CD45 PTPase Activity

Wild-type thymocytes generally display lower TCR signaling thresholds compared to mature peripheral T cells (Grossman and Singer, 1996; Sebzda et al., 1996). Given our finding that the sensitivity of thymocytes to TCR stimulation was affected by decreased CD45 expression, it was therefore of interest to determine whether comparable results were demonstrable in mature CD4 and CD8 T cells. The reconstitution of mature T cell repertoires on a *Ptprc*<sup>-/-</sup> background in a wide range of CD45RO, as well as mutant CD45RO-V821S, transgenic lines provided



**Figure 2. T Cell Differentiation in Transgenic Mice Varying in CD45RO Expression**

(A) Flow cytometry of cell-surface expression of CD45 on thymocytes from wild-type (gray histogram), *Ptprc*<sup>-/-</sup> (black histogram), and CD45RO5, CD45RO4, CD45RO10, CD45RO9, CD45RO3, and *Ptprc*<sup>+/-</sup> as unfilled histograms from left to right, respectively.

(B) Thymic CD45 expression and membrane PTPase activity from the transgenic CD45RO lines. Each data point represents an individual mouse and the linear regression analysis is shown.

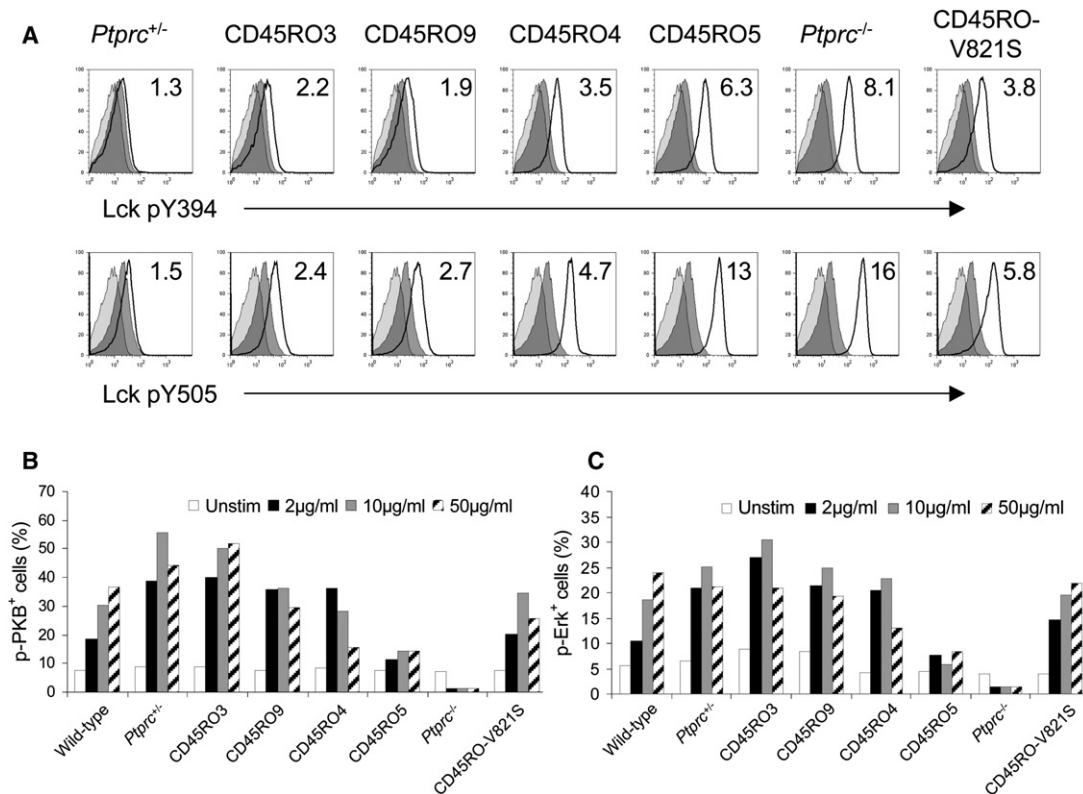
(C and D) Absolute cell numbers of CD4<sup>+</sup> (gray) and CD8<sup>+</sup> (striped) thymocytes (C) and CD4<sup>+</sup> (gray) and CD8<sup>+</sup> (striped) T cells in the spleen (D) determined by flow cytometry. Data are mean  $\pm$  SEM with a minimum of 3 mice per line; full data can be found in Table S1.

(E) Flow cytometry analysis of CD44 and CD62L expression on gated CD4<sup>+</sup> and CD8<sup>+</sup> T cells from peripheral lymph node. Numbers indicate the frequency of cells in each quadrant. All flow cytometric data are representative of four independent experiments.

a unique set of reagents for addressing this question. A striking increase in CD3-stimulated splenic CD4<sup>+</sup> T cell proliferation was observed with decreasing CD45 expression, up to a 5-fold increase when expression was reduced, up to a 5-fold increase when expression was reduced to 30%–40% of wild-type, and even CD45RO-V821S T cells displayed proliferation comparable with wild-type (Figure 4A). Costimulation with CD28 enabled CD4<sup>+</sup> CD45RO-V821S cells to proliferate 3-fold higher than wild-type (Figure 4B). An increase in CD3-stimulated CD44<sup>lo</sup> CD8<sup>+</sup> T cell proliferation (Figure 4C) was also noted

at intermediate CD45 expression, but in this case CD45RO-V821S proliferation was reduced compared with wild-type. CD28 costimulation (Figure 4D) was unable to enhance proliferation of CD8<sup>+</sup> T cells from the lower CD45-expressing lines.

The proliferation data suggest that at intermediate CD45 expression (10%–60% of wild-type), peripheral T cells are more responsive to TCR stimulation. We explored this question further by measuring TCR-induced signals. Flow cytometric analysis revealed a dose-response curve in



**Figure 3. CD45RO Transgenic Thymocytes Display Enhanced TCR Signaling**

(A) Flow cytometry of p56<sup>lck</sup> pTyr-505 and pTyr-394 staining (unfilled histogram) in gated DP thymocytes compared with wild-type (dark gray histogram) and isotype control (light gray histogram). Numbers indicate the n-fold increase in phosphorylation compared with wild-type. Data are representative of three independent experiments.

(B) Number of DP thymocytes becoming phospho-PKB +ve after 3 min TCR stimulation with various concentrations of CD3 antibody (2C11: white, none; black, 2 µg/ml; gray, 10 µg/ml; striped, 50 µg/ml). Phosphorylation was measured by flow cytometry and is representative of at least three independent experiments.

(C) Number of DP thymocytes becoming phospho-PKB positive after 3 min TCR stimulation with various concentrations of CD3 antibody (2C11: white, none; black, 2 µg/ml; gray, 10 µg/ml; striped, 50 µg/ml). Phosphorylation was measured by flow cytometry and is representative of at least three independent experiments.

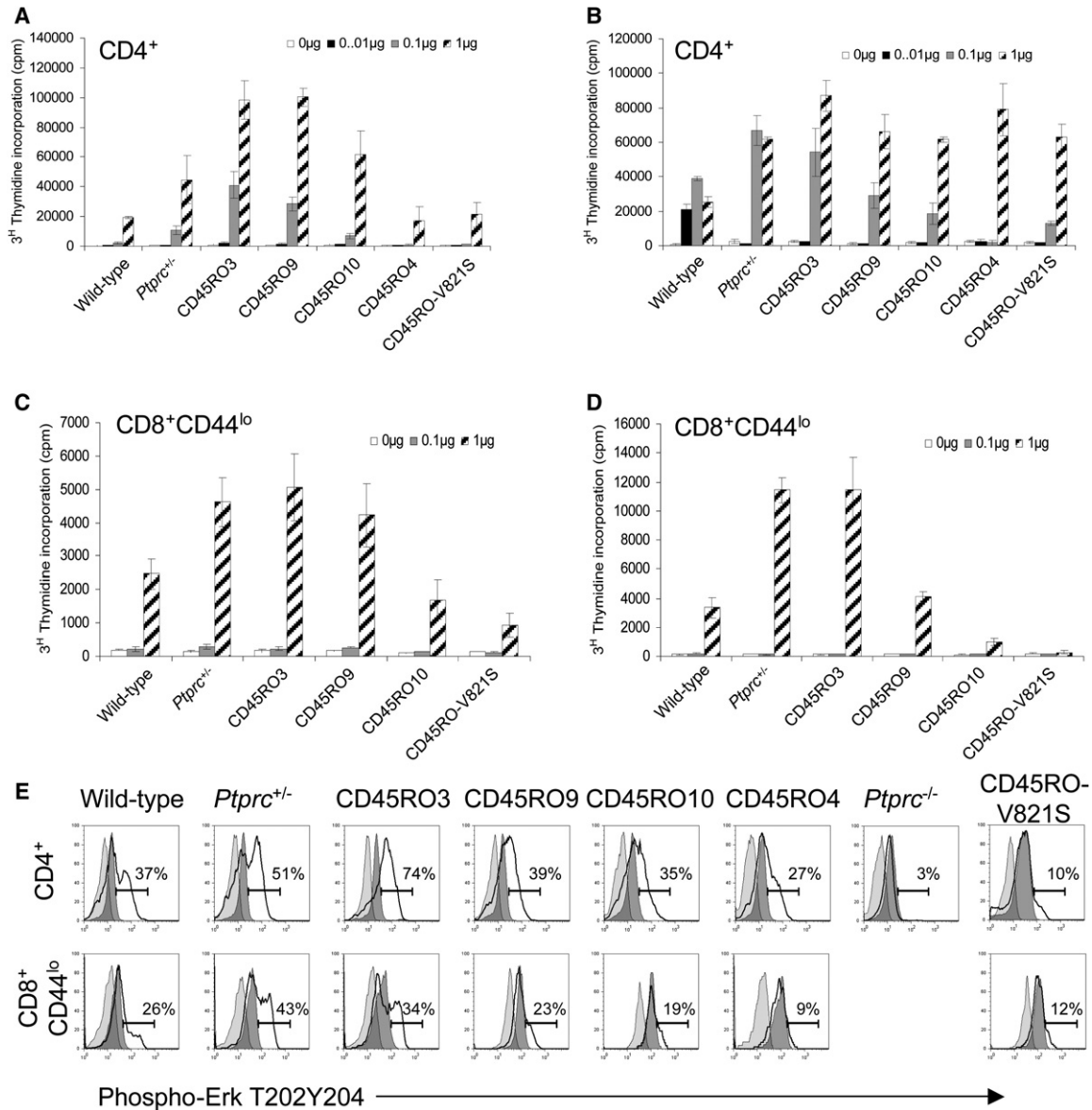
which the proportion of both CD4<sup>+</sup> and CD44<sup>lo</sup> CD8<sup>+</sup> T cells with high amounts of phospho-ERK (Figure 4E) and phospho-PKB staining (Figure S4D) was much increased above wild-type at intermediate CD45 expression, but the proportion was reduced in CD45RO-V821S and ablated in *Ptprc*<sup>-/-</sup> cells. Furthermore, TCR-induced calcium release and upregulation of the activation markers CD25 and CD69 by CD4<sup>+</sup> T cells followed the same pattern (Figure S4). Together, these data suggest that reducing amounts of CD45 in peripheral T cells initially enhance responses, whereas at low amounts of CD45 expression, TCR signaling is reduced.

In summary, two distinct conclusions can be drawn from these results: first, decreased CD45 expression increases TCR signaling responses with respect to proliferation for both CD4<sup>+</sup> and CD8<sup>+</sup> CD44<sup>lo</sup> T cells; and, second, when direct comparisons are made between CD45RO-V821S and its CD45RO9 expression-matched control, it is clear that the 80% reduction in CD45 PTPase activity in the former compared to the latter renders both

CD4<sup>+</sup> and CD8<sup>+</sup> T cells much less responsive to CD3 stimulation.

**Reduced Antibody Responses in CD45RO-V821S PTPase-Low Mice, but Normal CD8<sup>+</sup> CTL Responses**

To investigate further the impact of expressing only 3% of normal CD45 PTPase activity in peripheral T cells, we carried out two functional assays. First, wild-type, CD45RO9, and CD45RO-V821S mice were immunized with the T-dependent Ag TNP-KLH. Anti-hapten serum IgG<sub>1</sub> from individual mice was determined at 7, 14, and 77 days after primary immunization and then 7 and 14 days after rechallenge (Figure 5A). The EC<sub>50</sub> of anti-hapten IgG produced by the transgenic mice after the primary immunization was significantly lower (t test, p < 0.01) than wild-type, with CD45RO9 producing 30% and CD45RO-V821S only 15% of wild-type antibody amounts at day 14. By day 77, the primary response had declined in both the CD45RO9 and CD45RO-V821S lines to low levels,



**Figure 4. CD45RO Peripheral T Cells Display Enhanced Proliferation and TCR Signaling**

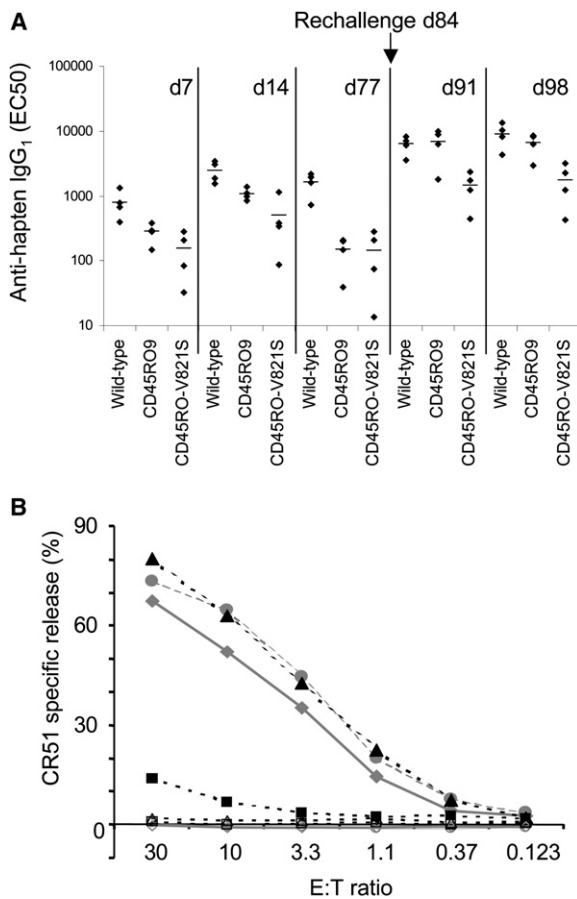
(A–D) Proliferation of purified splenic CD4 T cells (A, B) or purified CD44<sup>lo</sup> CD8 T cells (C, D) of wild-type, *Ptprc*<sup>+/-</sup>, *Ptprc*<sup>-/-</sup>, and CD45 transgenic mice after CD3 stimulation (A, C) or CD3-CD28 ligation (B, D) for 72 hr. For all assays, cells were stimulated with immobilized CD3 mAb at 0 μg/ml (clear bar), 0.01 μg/ml (black bar), 0.1 μg/ml (gray bar), and 1 μg/ml (black striped bar) with or without coimmobilized CD28 mAb at 5 μg/ml. Data are mean ± SD of triplicate samples and are representative of at least three independent experiments.

(E) Flow cytometry of phospho-Erk pThr-202pTyr-204 staining in gated splenic CD4<sup>+</sup> or CD44<sup>lo</sup> CD8<sup>+</sup> T cells either basally (dark gray histogram) or after TCR ligation for 3 min (black line) compared with isotype control (light gray histogram). In all cases, data are representative of three independent experiments.

whereas wild-type mice retained higher amounts of IgG. After rechallenge, CD45RO9 mice generated memory IgG responses comparable to wild-type, despite a lower initial primary response, whereas CD45RO-V821S mice still produced significantly less Ab ( $p < 0.01$ ) than either the wild-type or CD45RO9 mice. An 80% reduction in CD45 PTPase activity therefore leads to an 85%–90% reduction in the ability of mice to mount T-dependent Ab responses, suggesting defective T cell help.

In contrast, CD8<sup>+</sup> CTL responses were found to be comparable with wild-type in all three lines (Figure 5B). Comparison of wild-type, CD45RO transgenic, and CD45RO-V821S allospecific CTL cells showed equivalent killing of chromium-loaded target cells at a variety of effector cell ratios, with only *Ptprc*<sup>-/-</sup> cells unable to produce an effective CTL response (Figure 5B). So, despite lower proliferation, CD45RO-V821S CD8<sup>+</sup> T cells could still produce effective CTL responses.





**Figure 5. T-Dependent Ab Production Is Reduced in CD45RO-V821S Mice but CTL Responses Are Normal**

(A) EC<sub>50</sub> of total anti-hapten IgG from wild-type, CD45RO9, and CD45RO-V821S mice at 7, 14, and 77 days after primary TNP-KLH immunization and at 7 and 14 days (d91 and d98, respectively) after rechallenge. Data points represent results from individual mice.

(B) CTL assays of target cell killing, measured by chromium release, of effector T cells from wild-type (gray diamond), CD45RO9 (gray circle), CD45RO-V821S (black triangle), and *Ptpnc<sup>-/-</sup>* (black square) at various effector-to-target ratios incubated with allogeneic (filled symbols) or syngeneic (empty symbols) targets. Effector CTL were primed by culture with allogeneic (BALB/c) target cells for 5 days prior to killing assay. Data are representative of three independent experiments.

**Differential Regulation by CD45 of p56<sup>lck</sup> pTyr-394 and pTyr-505 in Peripheral T Cells**

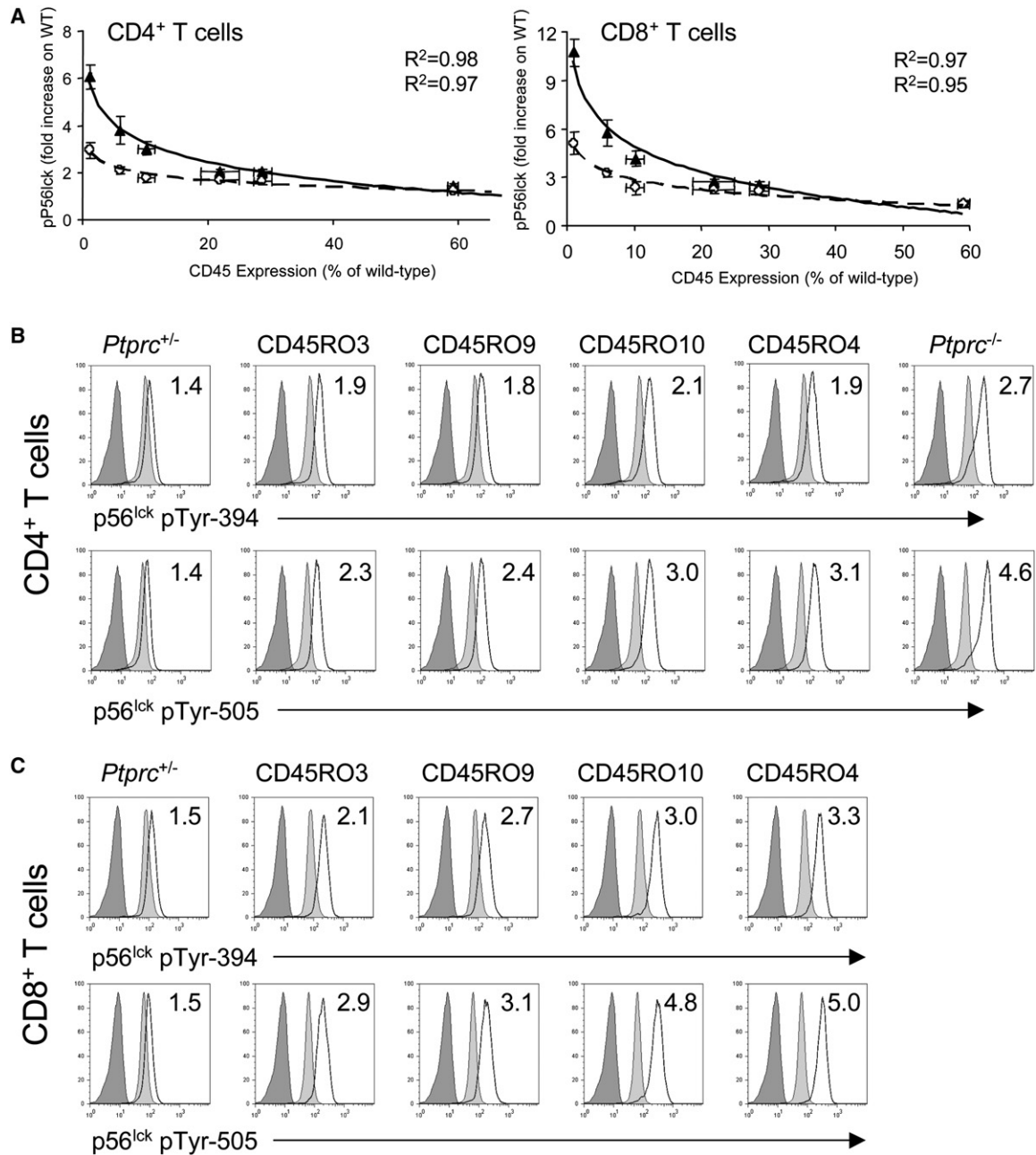
It is known that CD45 has access to p56<sup>lck</sup> Tyr phosphorylation sites under basal conditions because p56<sup>lck</sup> Tyr-505 becomes hyperphosphorylated in the absence of CD45 (Stone et al., 1997). In the present work, we used in situ proximity ligation to demonstrate both CD45-CD3 and CD45-CD4 or CD8-p56<sup>lck</sup> associations in primary resting CD4 and CD8 T cells, confirming previous observations (Figure S5). To elucidate the enzymatic consequences of these associations on TCR signaling responses at different CD45 PTPase activity, we carried out a detailed examination of the phosphorylation status of the activatory pTyr-394 and inhibitory pTyr-505 p56<sup>lck</sup>

phosphorylation sites, assaying phosphorylation separately in CD4 and CD8 T cells by means of flow cytometry. Interestingly, wild-type cells have very low basal p56<sup>lck</sup> phosphorylation, with about 10% of molecules phosphorylated at each tyrosine (Figure S6). Figure 6A illustrates dose-response curves, and Figures 6B and 6C are examples of primary flow cytometric data, in which the increases relative to wild-type in either basal pTyr-505 or pTyr-394 were assessed in relation to CD45 expression in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Increased basal phosphorylation in *Ptpnc<sup>-/-</sup>* cells was greater for the pTyr-505 residue than for the pTyr-394 residue, in the range of 5- to 10-fold and 3- to 5-fold, respectively, with greater increases apparent in CD8 than in CD4 cells. A precipitous drop in pTyr-505 from 5- to 10-fold increase over wild-type to 3- to 4-fold was noted over the range 0%–10% CD45 expression (Figure 6A), a reduction clearly detectable even when CD45 PTPase activity was only 3% of wild-type, as in CD45RO-V821S cells (data not shown), followed by a much gentler decline to 100% of wild-type amount in both CD4 and CD8 cells. In contrast, the dephosphorylation of pTyr-394 was less marked over the 0%–10% CD45 expression range, and whereas CD45 expression at 30% of wild-type, as in the CD45RO9 mice, caused a striking 80% reduction in pTyr-505 compared to *Ptpnc<sup>-/-</sup>*, only a 40% reduction in pTyr-394 was noted. p56<sup>lck</sup> pTyr-394 remained elevated over the intermediate CD45 expression range at which T cells display hyperactivity.

Taken together, these results show that a very low CD45 expression dephosphorylates sufficient pTyr-505 to maintain p56<sup>lck</sup> functionality for TCR signal transduction, whereas much higher amounts of CD45 are necessary to suppress the pTyr-394 site that is critical for p56<sup>lck</sup> activity. Elucidating the differential effects of CD45 at these two sites provides the key to understanding how CD45 regulates TCR signaling responses.

**DISCUSSION**

We have reconstituted *Ptpnc<sup>-/-</sup>* mice with five different fixed amounts of CD45RO and two mutants of CD45R0, and we have shown that T cell membrane CD45 PTPase activity increased in parallel with CD45 expression. Comparison of the transgenic lines showed that CD45 acted as a rheostat to modulate the sensitivity of cells to TCR signals and differentially regulate two critical p56<sup>lck</sup> kinase phosphorylation sites. Thus, in CD45 null mice, TCR signaling is very weakly transduced, resulting in nonresponsive peripheral T cells. However, the incorporation of only 3% of wild-type CD45 PTPase, as in the CD45RO-V821S mice, can restore peripheral T cell signaling and function, albeit at a reduced level compared to wild-type. As the CD45 PTPase activity titrates upwards, TCR signaling responses increase until they are clearly higher than wild-type in the CD45RO expression range 10%–60%, typically generating bell-shaped responses with respect to CD45 expression. The sensitivity of thymocytes to TCR signals also conform to the same



**Figure 6. Changes in CD45 Expression and PTPase Activity Cause Differential Changes in p56<sup>lck</sup> pTyr-394 and pTyr-505 Phosphorylation in Peripheral T Cells**

(A) Peripheral CD45 expression and phosphorylation of p56<sup>lck</sup> pTyr-505 (solid line) and pTyr-394 (dashed line) from CD4<sup>+</sup> and CD8<sup>+</sup> peripheral T cells. CD45 expression is presented as percent of wild-type and phosphorylation of p56<sup>lck</sup> presented as n-fold increase compared to wild-type. Data show mean  $\pm$  SEM of a minimum of three individual mice per line.

(B and C) Flow cytometry of p56<sup>lck</sup> pTyr-505 and pTyr-394 staining (unfilled histogram) in gated splenic T cells compared with wild-type (gray histogram) and isotype control (dark gray histogram). Numbers indicate the n-fold increase in phosphorylation compared with wild-type. Data are representative of three independent experiments. Note that no differences in p56<sup>lck</sup> pTyr-505 and pTyr-394 levels were noted between CD8<sup>+</sup> CD44<sup>hi</sup> and CD44<sup>lo</sup> populations, so these cells were pooled for the purposes of the data shown. Insufficient CD8<sup>+</sup> *Ptprc*<sup>-/-</sup> cells were available to generate histograms for this subset in the series shown in (C) (cf. Table S1), but prolonged data collection in further experiments enabled pTyr-505 and pTyr-394 values to be generated for this subset for (A).

general pattern, although the fall in responsiveness over the range 0%–10% CD45 PTPase activity appears to be even sharper than for peripheral T cells, with

thymocytes in the 10%–14% CD45 expression range displaying reduced TCR-induced signals as compared to wild-type.

One explanation for the decreased sensitivity of TCR signaling as CD45 expression increases from 40% to 100% (of wild-type) is based on the possibility that CD45 homodimerization, which is likely to increase as CD45 expression increases (Dornan et al., 2002), causes inhibition of CD45 PTPase activity (Majeti et al., 1998). However, two observations in particular make this explanation unlikely. First, both thymic and peripheral T cell membrane CD45 PTPase activity increased with CD45 expression in a linear fashion, whereas PTPase inhibition at higher concentrations would predict a different result. Second, if PTPase activity had been inhibited at the highest CD45RO concentrations, then our results suggest that TCR signaling responses would have increased, not decreased, as we in fact observed.

Our experiments focused on anti-CD3 ± anti-CD28 stimulation of T cells, which did not result in major changes in the phosphorylation status of Lck (data not shown). Thus, the effect of CD45 expression on signaling sensitivity in our experiments is likely to arise from the changes in basal state Lck phosphorylation observed, consistent with the associations between CD45, CD3, and CD4/8 whereby CD45 PTPase is brought into proximity with its Lck substrate. Interestingly, in preliminary experiments, CD4-coligation very effectively costimulated wild-type CD4<sup>+</sup> T cell proliferation, whereas anti-CD3 alone was sufficient to drive maximal proliferation in CD45RO3 cells (data not shown). Therefore, it is likely that the interaction of CD45 with multiple, different pools of intracellular p56<sup>lck</sup> are relevant for full activation of TCR-coreceptor signaling pathways. It is important to note that previous reports have indicated that crosslinking of TCR-CD4 initiates 4-fold greater p56<sup>lck</sup> kinase activity and increased tyrosine phosphorylation of intracellular substrates compared with TCR-CD8 crosslinking (Ravichandran and Burakoff, 1994). Furthermore, it has been reported that 10-fold more p56<sup>lck</sup> binds to CD4 compared to the CD8 coreceptor (Maroun and Julius, 1994) and that this interaction is 18-fold stronger (Campbell et al., 1995), a fact likely to affect the dynamics of the interaction between CD45 and its kinase substrate. These observations may, in part, explain the different CD45 expression required for maximal CD4<sup>+</sup> versus CD8<sup>+</sup> T cell function described in the present work. These reports are also relevant to the bias of thymocytes expressing intermediate amounts of CD45 to develop along the CD4 rather than CD8 lineage, an observation consistent with previous results showing that thymocytes are exquisitely sensitive to changes in p56<sup>lck</sup> activity during CD4 and CD8 lineage commitment (Sharp and Hedrick, 1999; Sohn et al., 2001). The p56<sup>lck</sup> kinase has been shown to play a direct role in activating the MAP kinase pathway (Denny et al., 1999; Lin and Abraham, 1997), one of the main downstream signaling pathways implicated in thymic differentiation (Sebzda et al., 1999), with a clear correlation between Ras-MAPK pathway activation and CD4 lineage commitment, consistent with the observed hyperactivity of this pathway.

Our analysis of p56<sup>lck</sup> Tyr phosphorylation sites provides a molecular explanation for the proposed rheostat

function of CD45. Although phosphorylation at Tyr-505 and Tyr-394 is known to exert negative and positive actions, respectively, on p56<sup>lck</sup> function, these effects are not equivalent. Phosphorylation at Tyr-505 causes an intramolecular association with the p56<sup>lck</sup> SH2 domain, thereby perturbing the interaction(s) of this domain with potentially important targets in the TCR signaling complex, but exerting relatively little effect on p56<sup>lck</sup> kinase activity (Chong et al., 2005; Pelosi et al., 1999; Sicheri and Kuriyan, 1997). In contrast, phosphorylation at Tyr-394 has a major effect on p56<sup>lck</sup> kinase activity (Doro et al., 1996). Our results showed that in mature T cells, intermediate amounts of CD45 PTPase (10%–60% of wild-type) were highly effective at dephosphorylating pTyr-505, but relatively less effective in this range at dephosphorylating pTyr-394. Therefore, the 1.4- to 2.1-fold increases in pTyr-394 levels in CD4 cells over this CD45 expression range appeared to dominate p56<sup>lck</sup> function, causing TCR signaling responses greater than wild-type. In contrast, CD45 at low expression was relatively less efficient at dephosphorylating pTyr-505, especially in CD8<sup>+</sup> cells, so the hyperactivatory influence of increased pTyr-394 in this context may be counteracted by the inhibitory consequences of high pTyr-505. But in peripheral T cells, at least, the dominant effect on p56<sup>lck</sup> function appears to be the increased pTyr-394 observed over the 10%–60% CD45 expression range. We therefore propose that the main reason why T cells express so much CD45 is to ensure effective pTyr-394 dephosphorylation under basal conditions, thereby damping T cell activation and preventing autoimmunity. However, a low amount of CD45 is absolutely required to effectively dephosphorylate pTyr-505 and prevent p56<sup>lck</sup> dysfunction, as is apparent in *Ptpnc<sup>-/-</sup>* mice.

After TCR stimulation, additional phosphatases are likely to be involved in regulation of p56<sup>lck</sup> function. Indeed, recent data from Germain and colleagues indicate a critical role for SHP-1 in inhibition of p56<sup>lck</sup> function and TCR signaling in response to partial agonist or antagonist peptides (Stefanova et al., 2003). Our results are pertinent to the role of CD45 in the immune synapse and in the recently described microclusters that sustain TCR signaling (Campi et al., 2005; Yokosuka et al., 2005). Whereas exclusion of CD45 from microclusters has been associated with prolongation of TCR signaling, CD45 was present in cSMACs in which signaling stops (Varma et al., 2006). CD45-primed p56<sup>lck</sup> may diffuse into clusters from surrounding CD45-rich domains (Douglass and Vale, 2005). In the context of these models, our results suggest that very small amounts of CD45 would be sufficient to prime p56<sup>lck</sup> for cluster signaling, whereas much higher amounts are needed to stop signaling, conditions that appear to be generally consistent with the reported imaging data.

Our results showed that a higher proportion of CD8<sup>+</sup> T cells from CD45RO transgenic mice express high amounts of CD44, indicative of an activated or “memory” phenotype. Given that numbers of thymic CD8<sup>+</sup> SP cells were similar in all lines of mice with the exception of the

very low CD45 expressors, this was unlikely to be the result of lymphopenia-induced proliferation and gain of memory-like attributes. We have assessed the phenotype of thymic CD8<sup>+</sup> SP cells in CD45RO mice: these are mature (TCR<sup>hi</sup>) and express low amounts of CD44, indicating that the acquisition of a memory phenotype occurs when these cells leave the thymus (data not shown). The underlying reason for this phenotype is not yet fully understood and requires further investigation. In contrast, CD44 expression on CD45RO transgenic CD4<sup>+</sup> T cells was not altered.

When *in vivo* antibody responses to the T-dependent antigen TNP-KLH were measured, the intermediate line RO9 (29.8% wild-type CD45 level) was found to make a lower primary IgG<sub>1</sub> than the wild-type strain despite being mildly hyper-responsive to anti-CD3 ( $\pm$  anti-CD28) *in vitro*. This may reflect the greater influence of costimulation in wild-type cells in the *in vivo* situation as well as possible impairment of B cell function and survival in the RO9 mice (Huntington et al., 2006); the secondary response of RO9 was not different to wild-type. By contrast, the ability of effector allospecific CTL from the CD45 transgenic lines to kill fully allogeneic targets was not different even for the V821S line (3% activity). The sensitivity of CTL is believed to be very high with less than 10 complexes required to elicit effector function for CD45 wild-type cells (Purbhoo et al., 2004); given the relatively high density of allo-MHC on targets, such considerations may well permit effective activation even with inefficient signal transduction capabilities.

Comparison of CD45RO-V821S with expression-matched CD45RO9 transgenic mice enabled direct investigation of the consequences for primary T cell activation of reducing the CD45 PTPase activity by 80% in mice with comparable amounts of CD45 protein. This reduction was associated with a major reduction in proliferation, primary and secondary responses to antigen, and TCR-mediated signaling, associated with a 2-fold increase in pTyr-505 levels. Yet assessment of CD45 as a potential pharmaceutical target to induce immunosuppression must consider the wider picture (Alexander, 2005). Unless 90% or more of CD45 PTPase activity is inhibited, our results suggest that partial inhibition of CD45 would run a serious risk of rendering T cells hyperactive, a considerable challenge to selective pharmaceutical intervention.

## EXPERIMENTAL PROCEDURES

### Mice

All mice were bred and maintained in specific pathogen-free conditions in the animal facilities at The Babraham Institute (Cambridge, UK), and all procedures were conducted under United Kingdom Home Office guidelines. Point mutations Cys<sup>817</sup>-Ser (C817S) and Val<sup>821</sup>-Ser (V821S) were introduced into cDNA encoding the CD45RO isoform by the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The cDNA was inserted into the NotI site of the HS321/45 *vav-hCD4* plasmid (Ogilvy et al., 1999), and transgenics were created as previously described (Ogilvy et al., 2003). Transgenic pups were identified by PCR on DNA extracted from tail biopsies with primers specific to the transgenic SV40 poly sequence (Ogilvy et al., 1999). Transgenic founder mice were mated to previously described

B6.129-Ptprc<sup>tm1holm</sup> *CD45 exon 9*<sup>-/-</sup> mice (Byth et al., 1996) to generate transgene-bearing animals that lacked expression of all endogenous CD45 isoforms. Mice were backcrossed to the *Ptprc*<sup>-/-</sup>, C57BL/6 background for a minimum of 7 generations. Mice expressing the *vav*-CD45RO transgene at a variety of levels have been previously described (Ogilvy et al., 2003) and *Ptprc*<sup>-/-</sup>, *Ptprc*<sup>+/-</sup>, and wild-type C57BL/6 were used as control mice. Experiments used age- and sex-matched mice.

### T Cell Stimulation, Flow Cytometry, and Antibodies

Single-cell suspensions were stained for 30 min with antibodies obtained from BD PharMingen, unless otherwise stated, to the following cell-surface markers: CD3 (Clone 145-2C11; in-house hybridoma), CD4 (Clone CT-CD4, Caltag; Clone YTA3.1, in-house hybridoma), CD8 (Clone YTS169, in-house hybridoma), CD25 (Clone PC61), CD44 (Clone IM7), CD45 (Clone 30-F11), CD62L (Clone MEL-14; Caltag), and CD69 (Clone H1-2F3) in flow cytometry buffer (PBS with 0.5% FCS and 0.05% sodium azide). Cells were washed and analyzed on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo FACS Analysis software (Tree Star). For intracellular staining, T cells were resuspended in RPMI medium and prewarmed to 37°C for 30 min at a concentration of 10<sup>7</sup>/ml. T cells were stimulated by the addition of anti-CD3 (Clone 2C11) at a concentration of 2  $\mu$ g/ml, 10  $\mu$ g/ml, or 50  $\mu$ g/ml for 1, 3, or 10 min. Stimulation was stopped by fixing with 2% formaldehyde for 10 min at 37°C prior to permeabilization with ice-cold 90% methanol for a minimum 30 min. Cells were stained with signaling protein antibodies (Cell Signaling Technology): anti-phospho-p44/42 Map Kinase (Thr-202/204), anti-phospho-AKT (Ser-473), anti-phospho-Lck (Tyr-505), and anti-phospho-Src family (Tyr-416) and detected with a Cy-5-labeled minimal crossreactive AffiniPure Donkey anti-rabbit IgG (Jackson ImmunoResearch Labs).

### T Cell Purification

Spleen and lymph node cells were prepared from 8- to 10-week-old mice and T cells purified with the Spinsep mouse T cell enrichment kit (Stem Cell Technologies), with the addition of anti-CD19 (Clone 1D3; PharMingen) to remove transgenic B cells that did not express B220. For purification of T cell subsets, further addition of anti-CD8 (Clone 53.8.7; PharMingen) for CD4 T cell enrichment, or anti-CD4 (Clone GK1.5; PharMingen) for CD8 T cell enrichment, was used. CD8 T cells were further purified into CD44<sup>lo</sup> and CD44<sup>hi</sup> populations by the addition of FITC-conjugated anti-CD44 (Clone IM7; PharMingen) and sorted with the FACSaria cell sorter (BD Biosciences). Final cell suspensions were greater than 90% CD3<sup>+</sup>, 90% CD4<sup>+</sup>, or 98% CD44<sup>lo</sup> CD8<sup>+</sup>, as determined by flow cytometry.

### Membrane PTPase Activity

Membrane fractions from thymocyte or purified CD4 or CD8 peripheral T cell suspensions were prepared by sonication and ultracentrifugation. Cells were suspended in 20 mM HEPES (pH 7.4), 5 mM EDTA, 1 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, and were disrupted by sonication. Nuclei were pelleted by centrifugation, and the remaining homogenate was resolved into cytoplasmic (soluble) and membranous (particulate) components by ultracentrifugation (95,000  $\times$  g for 90 min at 4°C). Membrane pellets were resuspended in 20 mM HEPES with 10 mM 2-mercaptoethanol and analyzed with the PTP Assay Kit 1 (Upstate Biotechnology) according to the manufacturer's instructions. PTPase activity was standardized between samples to protein concentration as measured with the BioRad Protein Assay (BioRad).

### Immunization

Samples of preimmune sera were taken 7 days before the initial immunization. Groups of mice were immunized *i.p.* with 100  $\mu$ g trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH; Biosearch Technologies) emulsified in alum (Alu-Gel-S; Serva Electrophoresis). Samples of blood from tail veins were taken on days 7, 14, and 77 postimmunization. For secondary responses, mice received a further 100  $\mu$ g of TNP-KLH/alum on day 84, with tail bleeds taken on days 91 and 98.

For analysis of serum anti-TNP Abs, Nunc Maxisorp plates were coated with 5 µg/ml TNP-OVA (Biosearch Technologies) overnight. Plates were blocked by addition of PBS/1% BSA for 1 hr. Serial dilutions of sera were added and specific Ab isotype detected with Clonotyping System-HRP (Southern Biotechnology Associates). EC<sub>50</sub> values for anti-TNP titers were calculated by nonlinear regression analysis with Prism software (GraphPad software).

#### CTL Assay

Cells were obtained from disaggregated spleens of transgenic or control C57BL/6 mice (responder) and from Balb/c mice (stimulator). Allogeneic CTL were stimulated by culture in Falcon 3013 flasks containing 10<sup>7</sup> irradiated (30Gy) stimulator cells and an equal number of responder cells, except where the individual transgenic animal had significantly (<66%) fewer CD8<sup>+</sup> T cells, in which case the number of responder cells was adjusted to give a similar ratio of CD8 responders to stimulators for all cultures. Cells were cultured in RPMI 1640 supplemented with 10% FCS, 5 × 10<sup>-5</sup> M 2-mercaptoethanol, and penicillin, streptomycin for 5 days. Effector cells were recovered from cultures and pooled, and viable counts were determined. Cytotoxicity assays were performed by a 6 hr Cr<sup>51</sup> release method in Falcon 3072 96-well plates. M12C3 cells (H-2<sup>d</sup>) and control RMA cells (H-2<sup>b</sup>) were used as targets (10<sup>4</sup>/well) and effectors titrated as shown.

#### Proliferation Assay

Proliferation was assessed by [<sup>3</sup>H]-thymidine incorporation. 1 × 10<sup>5</sup> enriched CD4 and CD44<sup>lo</sup> CD8 T cells were stimulated for 72 hr (proliferation) with plate-bound anti-CD3 (Clone 2C11, 0.01–1 µg; PharMingen) with or without anti-CD28 (Clone 37.52, 5 µg; PharMingen). Cells were pulsed for the final 16 hr of culture with 1 µCi [<sup>3</sup>H]-thymidine (Amersham) per well, and incorporated label was quantified by scintillation counting.

#### Statistical Methods

An unpaired two-tailed Student's *t* test was used to determine significant differences to wild-type, with *p* values less than 0.01 considered significant.

#### Supplemental Data

Six figures, one table, and Experimental Procedures are available at <http://www.immunity.com/cgi/content/full/27/3/425/DC1/>.

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