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Zuopeng Wu

Adele L. Yates

Gerard F. Hoyne
University of Notre Dame Australia, gerard.hoyne@nd.edu.au

Christopher C. Goodnow

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Consequences of Increased CD45RA and RC Isoforms for TCR Signaling and Peripheral T Cell Deficiency Resulting from Heterogeneous Nuclear Ribonucleoprotein L-Like Mutation

Zuopeng Wu, Adele L. Yates,[1] Gerard F. Hoyne,[2] and Christopher C. Goodnow[2]

CD45 is the most abundant protein tyrosine phosphatase in the plasma membrane of T cells and serves a critical role in TCR signaling. Different CD45 isoforms are made by alternative mRNA splicing depending on the stage of T cell development and activation, yet their role remains unclear. Expression of CD45RA and RC isoforms is increased 20- to 200-fold on T cells from *thunder* mice with a loss-of-function mutation in the RNA-binding protein, heterogeneous nuclear ribonucleoprotein L-like (hnRNPLL), although total CD45 expression is unaltered. In this study, we test the hypothesis that this shift in CD45 isoform expression alters TCR signaling, thymic selection, and accumulation of peripheral T cells. There was no discernable effect of the change in CD45 isoform expression upon Lck phosphorylation or T cell positive and negative selection, whereas these indices were strongly affected by a decrease in the overall amount of CD45 in *Ptprc* mutant animals. The one exception to this conclusion was in thymocytes from *Ptprc*$^{loc/loc}$ animals with 4% of normal CD45 protein levels, where Lck505 phosphorylation was increased 25% in *Hnrpll* mutant cells, suggesting that high m.w. CD45 isoforms had lower Lck505 phosphatase activity in this context. In T cells with no CD45 protein, hnRNPLL mutation still diminished peripheral T cell accumulation, demonstrating that hnRNPLL regulates T cell longevity independently from its effects on CD45 splicing. *The Journal of Immunology*, 2010, 185: 000–000.

The membrane protein tyrosine phosphatase CD45 is expressed on the surface of all leukocytes and plays a crucial role in regulating TCR signal strength and selection of the T cell repertoire in the thymus and periphery (1–6). CD45 has been shown both to promote and to inhibit TCR signaling by dephosphorylating two regulatory tyrosine residues on the Src kinase p56^{lck}, an inhibitory phosphate on Lck Y505, and an activating phosphate on Lck Y394 (1, 7, 8). CD45 is encoded by the *Ptprc* gene, which contains three variably spliced exons (exons 4, 5, and 6) that encode the CD45 RA, RB, and RC segments of the extracellular domain containing numerous O-glycosylation sites (1). *Ptprc* mRNA in B lymphocytes includes all three exons and encodes the highest molecular mass 220 kDa isoform, referred to as B220 or CD45RABC. Naive circulating T cells express intermediate m.w. isoforms including two segments (CD45RAB, BC) or one segment (CD45RB). Memory T cells exclude all three variable exons and express the low m.w. CD45RO isoform, so the presence of CD45RO or absence of CD45RB is widely used as a marker of memory or activated T cells in man and other animals. Despite extensive research, the functional significance of the regulated changes in CD45 isoform expression during T cell differentiation remains obscure.

The different CD45 isoforms can differentially associate in *cis* at the cell surface with the CD4$^+$ and CD8$^+$ T cell coreceptors to modulate access of the CD45 phosphatase domain to p56^{lck}, which is tightly associated with the coreceptors in the cytoplasm (9, 10). CD45 can also homodimerize at the cell surface when expressed at high levels, and dimerization is modulated by sialylation and O-glycosylation of the variable exons in the extracellular domain (1). The expression of the high m.w. isoforms shifts the balance toward the expression of CD45 monomers due to the repulsive activity exerted by the negative charge produced by the glycosylated variable exons (1). High m.w. CD45 isoforms have also been reported to bind more strongly to macrophage galactose-like lectin, which inhibits TCR signaling (2). Comparison of human T cells with CD45RO, RA or other isoforms has suggested a profound difference in TCR signaling, although other differences in the T cells may contribute to their different responses (11, 12). Inherited human *PTPRC* single-nucleotide variants that alter the splicing of protein tyrosine phosphatase, receptor type C exons 4 or 6 have been associated with differences in TCR signaling, activated T cell numbers, and susceptibility to autoimmune or infectious disease (3). Collectively, these results favor the view that TCR signal strength and quality are modulated by developmentally regulated CD45 splicing.

To address the function of CD45 isoforms in TCR signaling, transgenic mouse strains have been produced on a *CD45*$^{0/0}$ genetic background that expressed either a high (CD45RABC) or low (CD45R0) m.w. isoform (6, 13–16), yielding varying conclusions

John Curtin School of Medical Research, Australian National University, Canberra, Australia

[1]Current address: Food Standards Australia New Zealand, Barton, Australia.

[2]G.F.H. and C.C.G. contributed equally to this work.

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Address correspondence and reprint requests to Assoc. Prof. Gerard F. Hoyne or Prof. Christopher C. Goodnow at the current address: School of Health Sciences, University of Notre Dame Australia, Fremantle, Western Australia 6959, Australia (G.F.H.) or John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory 0200, Australia (C.C.G.). E-mail addresses: ghoyne@nd.edu.au (G.F.H.) or chris.goodnow@anu.edu.au (C.C.G.)

The online version of this article contains supplemental material.

Abbreviations used in this paper: DP, double-positive; ENU, N-ethyl-N-nitrosourea; HEL, hen egg lysozyme; hnRNPLL, heterogeneous nuclear ribonucleoprotein L-like; SP, single-positive.

about the extent to which the different isoforms are different or comparable (16–18). A complexity with these experiments is that the transgene-encoded proteins were expressed on the cell surface at 10–30% of the normal levels. Recently, it was shown that relatively small decreases in the amount of surface CD45 reduce dephosphosphorylation of the activating LckY394 site, whereas much larger decreases are needed to compromise dephosphorylation of the inhibitory LckY505 residue. Consequently, transgenic mice with intermediate levels of CD45 on T cells exhibit hyperresponsive TCR signaling (4). When comparisons have been made between transgenic mice expressing different isoforms at subnormal but relatively comparable levels, although the transgenic mice show consistent differences in TCR signaling or activation relative to that of wild-type controls, it has been difficult to ascribe any functional difference to specific isoforms (16–18). Hence, the question remains unresolved as to whether there is a change in TCR signaling and T cell selection due to altered splicing of CD45 isoforms in the context of normal levels of CD45 expression.

The heterogeneous nuclear ribonucleoprotein L-like protein (hnRNPLL; gene symbol *Hnrpll*) was recently discovered as a *trans*-acting factor that regulates alternative splicing of the three variable exons on *Ptprc* mRNA (5, 19, 20). The RNA recognition motif 1 (RRM1) domain of hnRNPLL binds specifically to RNA containing the activation-responsive motif sequence responsible for silencing *Ptprc* exons 4–6 (5). Folding of this domain is destabilized by a V136D missense mutation in the *thunder* mouse strain, disrupting *Ptprc* exon 4–6 silencing and resulting in 20–200 times higher expression of CD45RA-, RB-, and RC-containing CD45 isoforms on thymocytes and mature T cells but without any change in total amount of CD45 per cell (5). The hnRNPLL^{thunder} mutant mouse thus provides a way to test whether there is a change in TCR signaling and T cell selection due to altered splicing of CD45 isoforms in the context of normal levels of CD45 expression. The hnRNPLL^{thunder} mutation does not affect the number of single-positive (SP) T cells formed in the thymus, but there is a marked reduction in the numbers of peripheral naive CD4$^+$ and CD8$^+$ T cells due to a T cell-intrinsic decrease in persistence (5). Given the important function of CD45 in TCR signaling and the role of TCR signaling in persistence of peripheral T cells, we therefore wished to test whether the diminished accumulation of peripheral T cells caused by the hnRNPLL^{thunder} mutation was due to the large shift toward high m.w. CD45 isoforms. In this paper, we address these issues by comparing T cells from normal and hnRNPLL^{thunder} mutant mice, in the context of either a normal *Ptprc* gene, a targeted deletion in *Ptprc* exon 6 (6) that results in complete absence of CD45 (*CD45$^{0/0}$*), or a *Ptprc* point mutation that decreases CD45 surface protein to 4% of normal amounts. We find that the abundance of CD45 on the cell surface is critical for TCR signaling and selection, but there is no discernable effect of increasing the proportion of high m.w. CD45 isoforms, at either normal or limiting amounts of total CD45. Moreover, mutation of hnRNPLL disrupts peripheral T cell accumulation even in the absence of CD45 protein, indicating that hnRNPLL acts through an independent mechanism to promote peripheral T cell longevity.

Materials and Methods

Mice

The *thunder* mutation has been described previously and was maintained on a C57BL/6 background (5). The *lochy* mouse strain was derived from the same N-ethyl-N-nitrosourea (ENU) screen as the *thunder* strain and was maintained on a C57BL/6 background (21). *CD45$^{0/0}$* mice have been previously described (6) and were bred on a C57BL/6 background. The 3A9 *TCR* transgenic and *TCR* × *insHEL* mice have been previously described (22, 23) and were bred with *thunder* mice to introduce the *Hnrpll* mutation onto the B10.Br transgenic background. Diabetes incidence was measured using urine glucose testing with Diastix (Siemens Australia, Bayswater, Victoria, Australia) at weekly intervals or when a cage was wet. To be recorded as diabetic, mice had to be Diastix 4+ on at least two readings tested 1 wk apart. Nondiabetic mice were culled at 24 wk. All of the animals were housed in the Australian Phenomics Facility, and procedures were approved by the Australian National University Animal Experimentation Ethics Committee.

Flow cytometry and intracellular flow cytometry

Lymphoid tissues were prepared as single-cell suspensions in ice-cold PBS/10% FCS buffer. Ab conjugates were from BD Pharmingen (San Diego, CA), including anti-mouse pLckY505, or from Caltag Laboratories (Burlingame, CA). Phospho-Src family (Tyr^{416}) (100F9) rabbit Ab was from Cell Signaling Technology (Danvers, MA) with the sheep anti-rabbit IgG F(ab′)$_2$ FITC-conjugated secondary Ab from Chemicon International (North Ryde, New South Wales, Australia). Cell surface staining follows the standard protocol. Cell permeabilization/fixation buffer from eBioscience (San Diego, CA) was used for intracellular staining. Data were acquired on a FACSCalibur (BD Bioscience, North Ryde, New South Wales, Australia) and analyzed with FlowJo (Ashland, OR) software.

In vitro stimulation

A total of 10^6 lymphocytes were resuspended in 1 ml prewarmed RPMI/10% FCS medium with anti-CD3ε (2C11) (10 μg/ml for thymocytes) for 5 min and cross-linked with anti-hamster IgG (H+L) (50 μg/ml). The response was terminated by adding ice-cold media after 20 min of stimulation, and the cells were processed for surface and intracellular staining and analyzed by flow cytometry immediately after stimulation.

Sequencing

Total RNA were isolated from spleen of wild-type and mutant mice using TRIzol reagent (Invitrogen, Mulgrave, Victoria, Australia) and reverse-transcribed to cDNA using SuperScript First-Strand cDNA Synthesis Kit (Invitrogen). Transcripts or genomic sequence of candidate genes were amplified by PCR using Elongase DNA polymerase kit (Invitrogen) and were sequenced using BigDye terminator mix (Applied Biosystems, Mulgrave, Victoria, Australia) in the Biomedical Research Facility of John Curtin School of Medical Research.

Statistical analysis

Analysis was performed Student *t* test, paired *t* test, ratio *t* test, or log-rank test.

Results

Consequence of hnRNPLL *mutation for CD45 levels and isoforms in an allelic series of* Ptprc *mutant mice*

To examine the relationship among hnRNPLL-induced splicing of CD45 isoforms, TCR signaling, and T cell selection, we analyzed T cells from normal and hnRNPLL^{thu} mutant mice that also carried either a normal *Ptprc* gene, a targeted knockout of *Ptprc* exon 6 (6) (*Ptprc$^{0/0}$*), or a *Ptprc* point mutation that decreases CD45 surface protein by 25-fold (*Ptprc^{loc}*). Like *thunder*, the *Ptprc* point mutant strain *lochy* (*Ptprc^{loc}*) was identified in a flow cytometric blood screen of pedigrees of ENU-mutagenized C57BL/6J mice (21). Several individuals in pedigree ENU134 exhibited a low percentage of CD8$^+$ T cells in the peripheral blood, a high proportion of which were CD44^{hi} activated/memory cells (Fig. 1*A*). Further characterization of progeny revealed two segregating heritable traits (Fig. 1*B*, 1*C*): *lochy*, identified by low B220 expression on CD19$^+$ B cells, and *nessy*, with normal B220 expression. The cause of low T cells in *nessy* was subsequently shown to be a missense mutation in the condensin protein kleisin-β (24). Flow cytometric analysis of (CD45.2 B6^{loc} × CD45.1 NOD) F1 hybrids, where the allelic products can be measured by staining with specific Abs to the CD45 alleles, showed that there was a selective loss of CD45.2 and normal expression of CD45.1 in B and T cells, indicating a *cis*-acting defect in the CD45.2 allele (data not shown). Sequencing of *Ptprc* cDNA from *loc/loc* mice revealed a 5-nt insertion at the start of exon 21 of the *Ptprc* gene (Fig. 1*D*), resulting from an

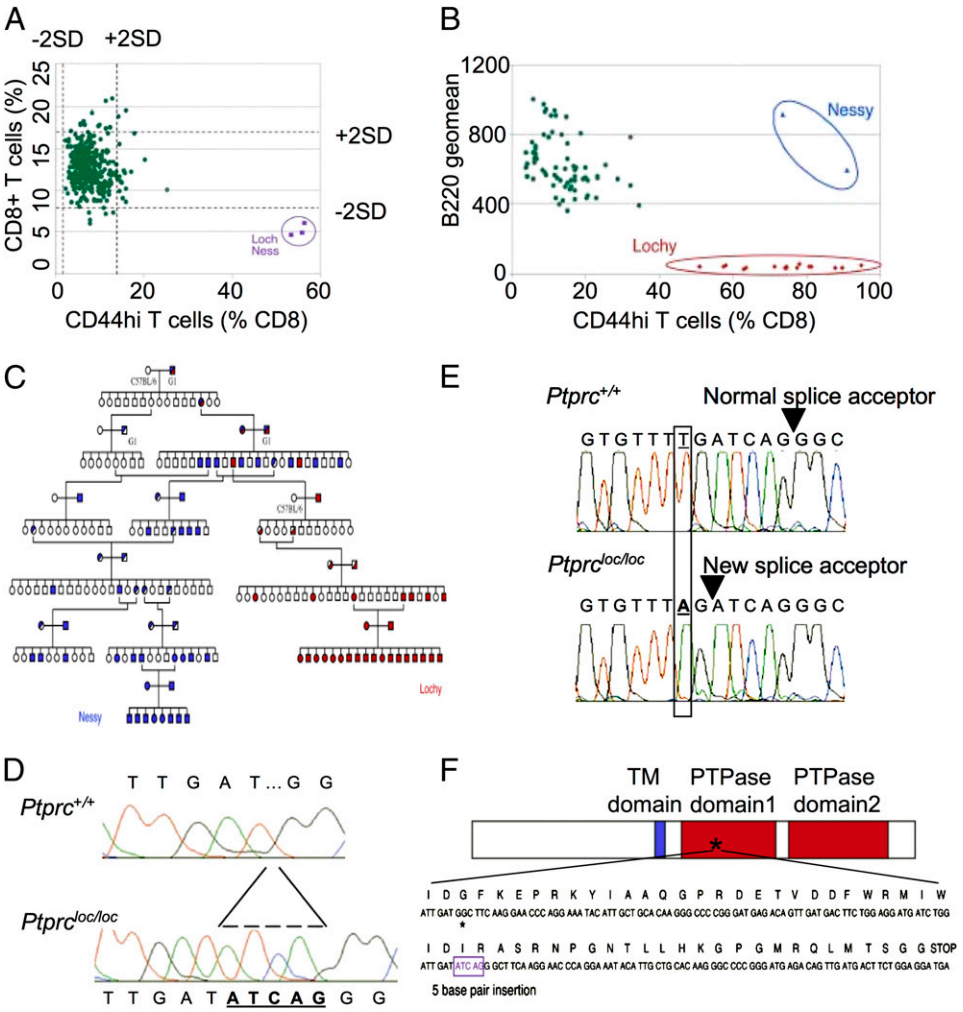

FIGURE 1. Characterization of *Ptprc*$^{loc/loc}$ mutation. *A*, Flow cytometric analysis of peripheral blood. Dots show the percentage of CD8$^+$ T cells and the fraction that are CD44^{hi} in individual mice. Dotted lines delineate the boundaries for ±2 SD from the mean. *B*, Flow cytometric analysis of peripheral blood leukocytes from a separate cohort of mice, showing the percentage of CD8 cells that are CD44^{hi} and the geometric mean fluorescent staining for B220 on CD19$^+$ cells. *C*, Pedigree of the Loch Ness strain segregating the *lochy* and *nessy* mutations into separate lineages. Filled red and blue symbols denote individuals with the *lochy* or *nessy* flow cytometric phenotypes. *D*, *Ptprc* cDNA sequence trace showing five additional nucleotides in *Ptprc*$^{loc/loc}$ mice. *E*, *Ptprc* genomic DNA sequence trace showing T → A nucleotide substitution in the intron preceding *Ptprc* exon 21 and position of the exon 21 splice acceptor determined from cDNA sequencing. *F*, Schematic of CD45 protein showing consequences of *lochy* mis-splicing for the protein sequence.

intronic T → A substitution that created a new splice acceptor 5 bp upstream from the correct splice acceptor (Fig. 1*E*). The insertion caused a frame shift and premature stop codon within the first cytoplasmic protein tyrosine phosphatase domain of CD45 (Fig. 1*F*). No CD45 protein of normal or truncated m.w. was detectable by Western blotting with B220 Abs (data not shown). Flow cytometric staining, which is more sensitive, detected ~2% of wild-type CD45 levels on B cells (data not shown) and 4% of wild-type levels on T cells (Fig. 2*A*, 2*B*). These trace amounts were too little to analyze by Western blotting, but on the basis of the residual thymic differentiation observed below, we assume that the residual CD45 derives from a trace amount of correctly spliced *Ptprc* mRNA.

A pan-CD45 mAb was used to measure the relative amount of total CD45 on CD4$^+$CD8$^+$ double-positive (DP) thymocytes from *Hnrpll*$^{thu/thu}$ and *Hnrpll*$^{+/+}$ controls, either bearing wild-type *Ptprc* genes (*Ptprc*$^{+/+}$) or an allelic series comprising *Ptprc*$^{loc/loc}$ or exon 6 disrupted *Ptprc* (*Ptprc*$^{0/0}$) and their heterozygous intermediates (Fig. 2*A*, 2*B*). The hnRNPLL^{thunder} mutation had no effect on the overall surface levels of CD45, regardless of the *Ptprc* genotype. This assay was nevertheless sensitive to differences in CD45 abundance, because it showed that there was 40–50% less CD45 on *Ptprc*$^{0/+}$ and *Ptprc*$^{loc/+}$ heterozygotes, only 4% of wild-type CD45 on cells from *Ptprc*$^{loc/loc}$ homozygotes, and no detectable CD45 on *Ptprc*$^{0/0}$ animals by flow cytometry. The same was true for CD4 SP spleen cells, except that ~5–10% of *Ptprc*$^{0/0}$ cells express CD45 as shown previously (6). Although the *Hnrpll*$^{thu/thu}$ mutation did not alter the cell surface abundance of CD45, it dramatically altered the isoforms expressed on DP thymocytes (Fig. 2*C*). On DP thymocytes from *Ptprc*$^{+/+}$, *Ptprc*$^{0/+}$, and *Ptprc*$^{loc/+}$ mice, the hnRNPLL mutation increased expression of CD45RA and RB isoforms 20- to 50-fold, and RC isoforms were increased ~200-fold. Despite the large reduction of CD45 expression in *Ptprc*$^{loc/loc}$ mice, it was still possible to detect increased expression of the higher m.w. CD45 isoforms as a result of the *Hnrpll* $^{thu/thu}$ mutation, whereas no expression of any of the CD45 isoforms was detectable on the surface of *Ptprc*$^{0/0}$:*Hnrpll*$^{thu/thu}$ DP cells (Fig. 2*C*).

Effects of Hnrpll*-dependent shift in CD45 isoforms on thymocyte differentiation and Lck phosphorylation*

We used the *Ptprc* allelic series to investigate whether thymocyte selection was altered by the *Hnrpll*$^{thu/thu}$-induced change in CD45 isoforms. The *Ptprc*$^{0/0}$ mutation arrested thymocyte differentiation at the DP cell stage, thus leading to very low frequencies of mature CD4$^+$ and CD8$^+$ SP T cells, and this was not altered when combined with the *Hnrpll*$^{thu/thu}$ mutation (Fig. 3*A*). Higher frequencies of SP thymocytes developed in *Ptprc*$^{loc/loc}$ mice, indicating that the 4% of normal CD45 present on these cells was functional, which is in line with recent studies that showed as little as 3% of total CD45 was sufficient to rescue positive selection in the thymus (4). *Ptprc*$^{loc/loc}$:*Hnrpll*$^{thu/thu}$ mice produced equivalent frequencies of DP and mature CD4$^+$ and CD8$^+$ SP cells compared with those of the *Ptprc*$^{loc/loc}$ mice (Fig. 3*B–D*). The *thunder* mutation also had no effect on frequencies of SP cells in the thymus of *CD45*$^{0/+}$ and *CD45*$^{loc/+}$ mice with half the normal CD45 protein (Fig. 3*B–D*). Measured in this way, the shift to high m.w. CD45 isoforms in *Hnrpll*$^{thu/thu}$ mice had no discernable effect on the efficiency of positive selection.

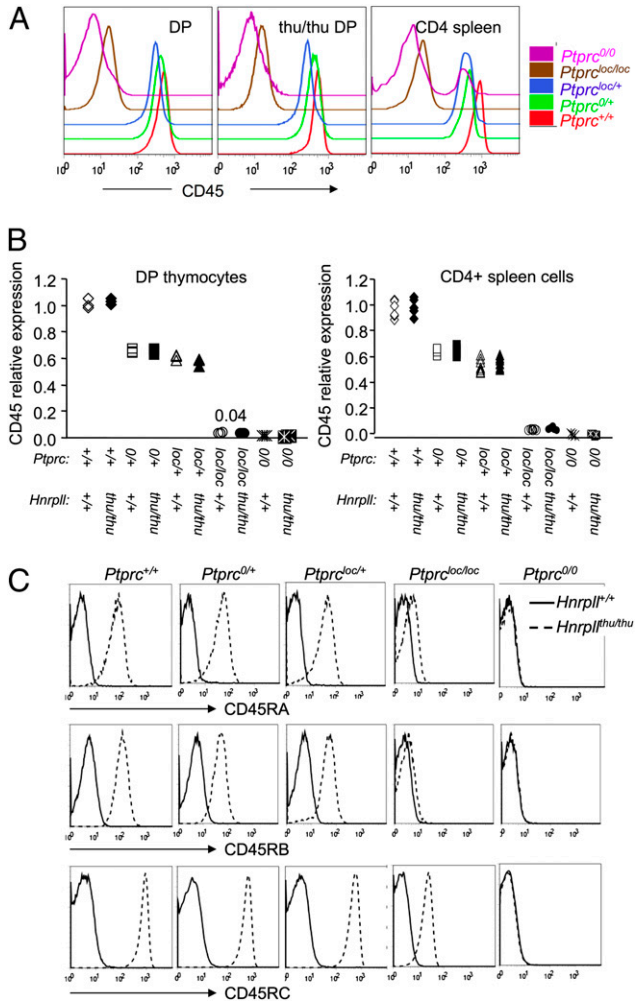

FIGURE 2. Effects of *Hnrpll* mutation on T cell CD45 expression and isoforms in a *Ptprc* allelic series. *A*, Representative flow cytometric histograms comparing staining for total CD45 on DP thymocytes or CD4$^+$ splenocytes from mice with the indicated *Ptprc* genotypes. The animals shown had either wild-type *Hnrpll* (*left* and *right panels*) or *Hnrpll*$^{thu/thu}$ allele (*middle panel*). *B*, Quantitation of data obtained as in *A* from multiple animals. Each symbol represents the value from one individual mouse. CD45 relative expression was calculated using the geometric mean fluorescence on T cells from each test animal divided by the average CD45 geometric mean of CD4$^+$ T cells of $n = 6$ C57BL/6 mice. *C*, Representative flow cytometric histograms from staining with specific Abs to CD45RA, RB, or RC isoforms on DP cells in mice with the indicated *Ptprc* genotypes and either *Hnrpll*$^{+/+}$ (solid lines) or *Hnrpll*$^{thu/thu}$ mice (dotted lines).

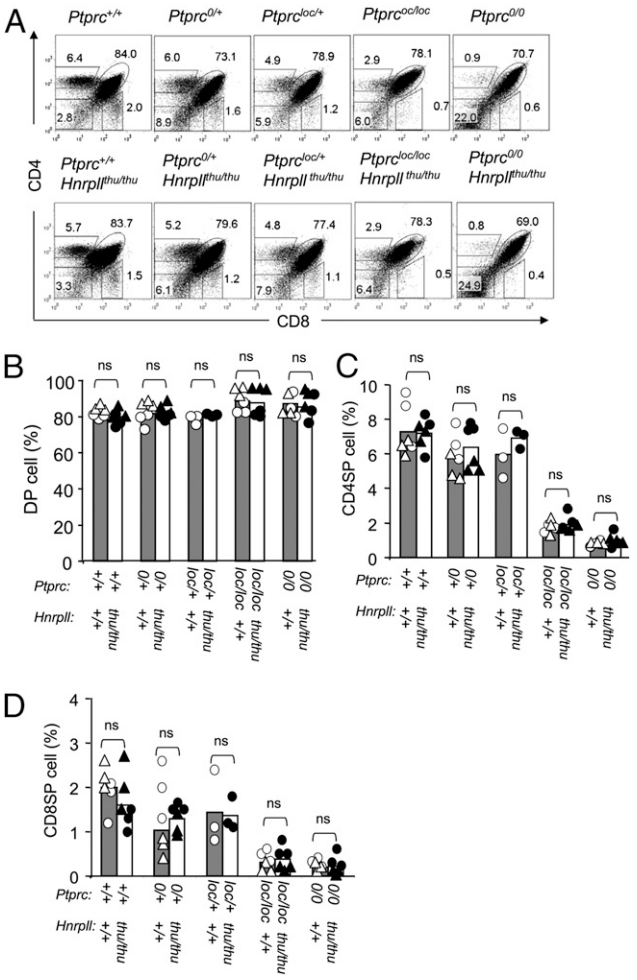

FIGURE 3. Effects of *Hnrpll* mutation on T cell development in animals with differing amounts of CD45. *A*, Representative flow cytometric staining for CD4 and CD8 on thymocytes from mice of the indicated *Ptprc* genotypes that were either *Hnrpll*$^{+/+}$ (*top panel*) or *Hnrpll*$^{thu/thu}$ (*lower panel*). *B–D*, Frequencies of DP cells (*B*), CD4$^+$ SP cells (*C*), and CD8$^+$ SP cells (*D*) in the thymus of individual mice of the indicated *Ptprc* and *Hnrpll* genotypes. Columns show the means, and each dot represents one individual mouse. Each group contains $n = \sim 3$–6 animals pooled from two experiments. Circles and triangles represent data from different experiments. Comparison of *thu/thu* and wild-type groups with the same *Ptprc* genotype by Student *t* test showed no significant differences.

Because *p56*^{lck} is a primary target of CD45 in T cells, we examined whether the *Hnrpll*$^{thu/thu}$ mutation affected the phosphorylation status of the two key tyrosine residues dephosphorylated by CD45, using intracellular flow cytometry of thymocytes with Abs specific for phosphorylated Lck Y505 (pY505) or Lck Y394 (pY394). This assay was sensitive to detect functional differences in CD45 activity against Lck, because it reproducibly detected increased pY505 and pY394 in thymocytes and peripheral T cells from *Ptprc*$^{loc/loc}$ or *Ptprc*$^{0/0}$ mice with low or no CD45 (Figs. 4, 5). As an independent measure of TCR–Lck signal strength, we measured expression of CD5 on DP cells, because this inhibitory receptor is expressed at levels proportional to the strength of CD45-dependent TCR signaling in DP thymocytes (25). CD5 expression was decreased on DP and CD4$^+$ SP thymocytes from *Ptprc*$^{0/0}$ and *Ptprc*$^{loc/loc}$ mice (Fig. 4*G*, 4*H*).

By contrast with the effects of decreasing overall CD45 protein, there was no significant effect of the altered CD45 splicing caused by the *Hnrpll* mutation upon intracellular levels of pLckY505 or pLckY394 in DP or CD4 SP cells with normal levels of CD45 or in the absence of CD45. However, in DP cells from *Ptprc*$^{loc/loc}$ mice, with 4% of normal CD45, there was a consistent 25% increase in pY505 staining ($p = 0.002$) in cells with mutant *Hnrpll* and high m.w. CD45 isoforms (Fig 4*C*). A smaller but consistent increase in pY505 was observed in *Ptprc*$^{loc/loc}$:*Hnrpll*$^{thu/thu}$ CD4 SP thymocytes (Fig. 4*D*). This increase in LckY505 phosphorylation was not accompanied by any change in LckY394 phosphorylation (Fig. 4*E*, 4*F*) nor in CD5 expression (Fig. 4*G*, 4*H*). A more subtle ~10% higher LckY505P mean fluorescence intensity was observed in TCR-stimulated *Ptprc*$^{loc/loc}$:*Hnrpll*$^{thu/thu}$ DP and CD4 SP cells relative to that in *Hnrpll*$^{+/+}$ wild-type counterparts (Supplemental Fig. 1). Total Lck remained unchanged (Supplemental Fig. 2). There was no evidence for an effect of the *Hnrpll*$^{thu/thu}$ mutation upon LckY505 or LckY394 phosphor-

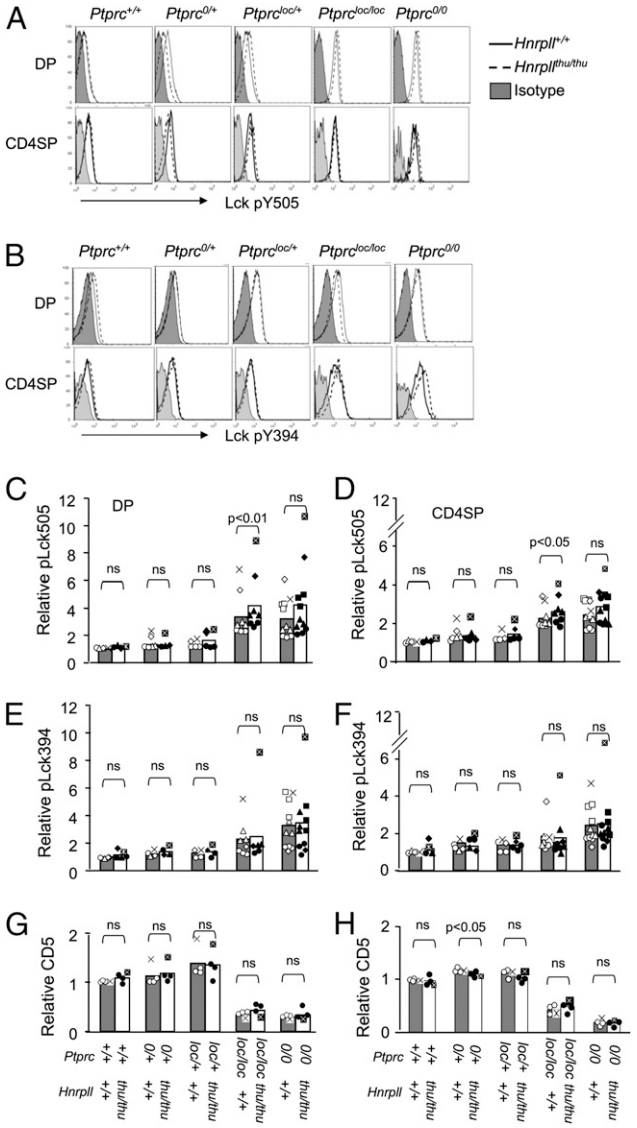

FIGURE 4. Effects of *Hnrpll* mutation on Lck phosphorylation and CD5 expression in thymocytes. *A* and *B*, Representative histogram overlays of *Hnrpll*$^{+/+}$ (solid lines) and *Hnrpll*$^{thu/thu}$ (dotted lines) thymocytes, gated on DP or CD4$^+$ SP cells, showing intracellular staining for Lck pY505 (*A*) or Lck pY394 (*B*). Intracellular staining with isotype control Ab shown by shaded histograms. *C–H*, Relative levels of intracellular Lck pY505, Lck pY394, and cell surface CD5 in DP thymocytes (*C*, *E*, *G*) and CD4$^+$ SP thymocytes (*D*, *F*, *H*) from *Hnrpll*$^{+/+}$ (solid bar with open dots) or *Hnrpll*$^{thu/thu}$ mice (open bars with filled dots) with the indicated *Ptprc* genotype. Bars show means, and symbols show individual animals, with the values calculated using the geometric mean fluorescence on T cells from each test animal divided by the average geometric mean fluorescence of the *Ptprc*$^{+/+}$:*Hnrpll*$^{+/+}$ mice analyzed in that experiment. Each dot represents one individual mouse. Each group contains n = ~4–11 animals pooled from five experiments. Circles, triangles, squares, crosses, and diamonds represent data from different experiments. Results of a statistical comparison of *thu/thu* and their wild-type counterparts with the same *Ptprc* genotype by ratio t test or log-rank paired t test are shown.

ylation in peripheral naive or memory T cells expressing CD45 at normal or 4% of normal levels (Fig. 5*A*, 5*B*). LckY505P levels and CD5 expression tended to be lower in *Hnrpll*$^{thu/thu}$ peripheral CD4 T cells lacking any CD45 (Fig. 5*A*), presumably reflecting the CD45-independent effects of the *thu/thu* mutation on peripheral T cell persistence described below (see Fig. 7).

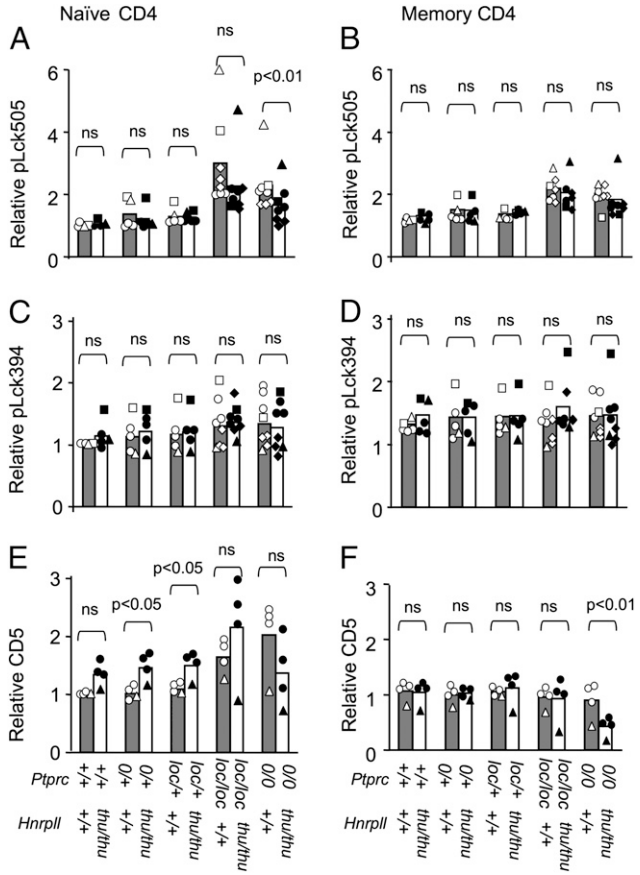

FIGURE 5. Effects of *Hnrpll* mutation on Lck phosphorylation and CD5 expression in peripheral T cells. *A–F*, Relative levels of intracellular Lck pY505 (*A*, *B*), Lck pY394 (*C*, *D*), and cell surface CD5 (*E*, *F*) in naive (CD62L$^+$CD44^{lo}) (*A*, *C*, *E*) or memory (CD62L$^-$CD44^{hi}) (*B*, *D*, *F*) CD4$^+$ splenocytes from *Hnrpll*$^{+/+}$ (solid bars with open dots) or *Hnrpll*$^{thu/thu}$ (open bars with filled dots) mice with the indicated *Ptprc* genotype. Symbols show values for individual animals calculated using the geometric mean fluorescence on T cells from each test animal divided by the average geometric mean of *Ptprc*$^{+/+}$:*Hnrpll*$^{+/+}$ naive CD4$^+$ T cells in the same experiment. Each group contains n = ~4–11 animals pooled from five experiments. Circles, triangles, squares, crosses, and diamonds represent data from different experiments. Results of a statistical comparison of *thu/thu* and their wild-type counterparts with the same *Ptprc* genotype by ratio t test or log-rank paired Student t test are shown.

Changes in CD45 splicing do not affect positive or negative selection of Ag-specific T cells

To examine the effect of the *thunder* mutation on positive and negative selection of Ag-specific T cells in the thymus, we bred the *Hnrpll*$^{thu/thu}$ mutation onto the 3A9 TCR transgenic background. The 3A9 TCR is I-A^k–restricted, directs T cell development toward the CD4$^+$ lineage, and recognizes the immunodominant 46–61 peptide of hen egg lysozyme (HEL) bound to I-A^k (22, 23). The transgene carried by *insHEL* mice encodes a membrane-bound form of lysozyme under transcriptional control of the rat insulin promoter. The *insHEL* gene mirrors the pattern of proinsulin gene expression, with high expression in all pancreatic islet β cells (22) and low expression in medullary thymic epithelial cells. To study negative selection of islet-reactive CD4$^+$ T cells, we bred the 3A9 *TCR* transgenic with *insHEL* transgenic mice to generate double-transgenic mice. The developmental fate of the HEL-specific 3A9 T cells in the thymus and periphery of *TCR* and *TCR* × *insHEL* double-transgenic mice was monitored by flow cytometry using the TCR clonotype-specific mAb 1G12 (23). A modest decrease in TCR signaling due to the

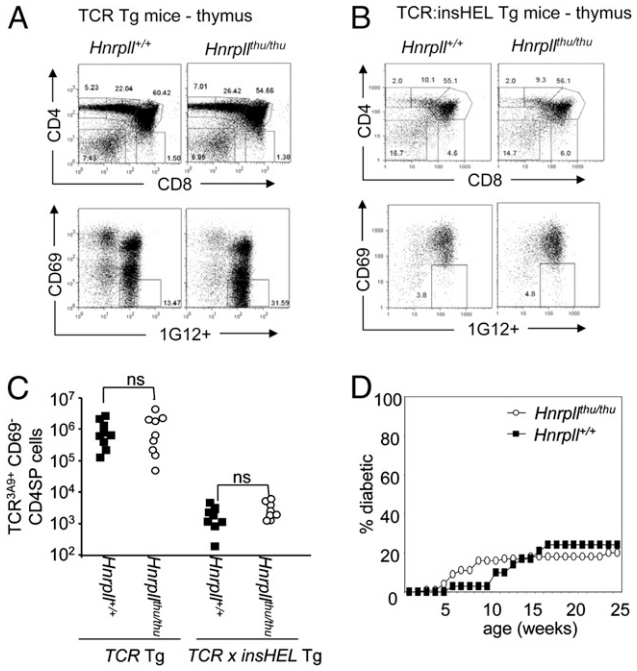

FIGURE 6. Effects of *Hnrpll* mutation on positive and negative selection in 3A9 TCR transgenic mice. *A*, Representative flow cytometric plots of thymocytes from *Hnrpll*$^{+/+}$ and *Hnrpll*$^{thu/thu}$ 3A9 TCR transgenic mice, showing CD4 and CD8 (*upper panel*) or staining for CD69 and 3A9 TCR clonotype (1G12$^+$) gated on CD4$^+$ SP cells (*lower panel*). *B*, Corresponding analysis to *A* in *Hnrpll*$^{+/+}$ (*left lane*) and *Hnrpll*$^{thu/thu}$ (*right lane*) 3A9 *TCR* × *insHEL* double-transgenic mice. *C*, Number of 3A9 TCR clonotype 1G12$^+$CD4$^+$CD69$^-$ mature T cells in the thymus of individual animals of the indicated genotypes *Hnrpll*$^{+/+}$ (filled squares, $n = 9$ TCR$^+$ insHEL$^-$ and $n = 8$ TCR$^+$insHEL$^+$) or *Hnrpll*$^{thu/thu}$ (open circles, $n = 9$ TCR$^+$insHEL$^-$ and $n = 8$ TCR$^+$insHEL$^+$) mice. There was no difference in the means between the TCR$^+$insHEL$^-$ $p = 0.0554$ and TCR$^+$insHel$^+$ mice $p = 0.4529$ using a Student *t* test. *D*, Diabetes incidence in *Hnrpll*$^{+/+}$ (filled square, $n = 18$) or *Hnrpll*$^{thu/thu}$ (open circle, $n = 44$) 3A9 *TCR* × *insHEL* double-transgenic mice. There was no significant difference in diabetes incidence between the *Hnrpll*$^{+/+}$ and *Hnrpll*$^{thu/thu}$ *TCR* × *insHel* double-transgenic mice using a log-rank test ($p = 0.3258$).

Zap70$^{mrd/mrd}$ catalytic site mutation, which caused only a 50% decrease in SP cell formation on a polyclonal TCR gene background, nevertheless caused a 50-fold decrease in positive selection of 1G12$^+$ SP thymocytes when introduced into 3A9 TCR transgenic mice (26). We therefore used this sensitive assay to examine whether changes in CD45 isoform expression influenced positive or negative selection.

In TCR transgenic mice not expressing the *insHEL* transgene, the positive selection of mature CD4$^+$1G12$^+$ cells occurred equally in the presence or absence of the *Hnrpll*$^{thu/thu}$ mutation and there were equivalent numbers of mature CD4$^+$1G12$^+$CD69$^-$ T cells in the thymus of wild-type and *thunder* TCR transgenic mice (Fig. 6*A*, 6*C*). Similarly, there was no difference in thymic deletion of the islet-reactive CD4$^+$1G12$^+$ cells in the thymus of *Hnrpll*$^{+/+}$ and *Hnrpll*$^{thu/thu}$ *TCR* × *insHEL* transgenic mice (Fig. 6*B*, 6*C*). There was also no difference in the percentage of wild-type or *thunder TCR* × *insHEL* transgenic mice that developed diabetes (Fig. 6*D*). Although there appeared to be a trend for earlier onset among the few *Hnrpll* mutant animals that became diabetic, the number of animals that developed diabetes was low in both groups and the interquartile range for their ages at onset (*thu/thu*, 45- to 74-d-old; wild-type, 48- to 83-d-old) overlaps that for a much larger cohort of wild-type TCR × insHEL animals (48- to 93-d-old, $n = 276$). The shift to high m.w. CD45 isoforms in *Hnrpll*$^{thu/thu}$ thymocytes therefore does not demonstrably affect TCR signaling for positive or negative selection or the incidence of autoimmune diabetes.

Diminished accumulation of Hnrpll$^{thu/thu}$ *peripheral T cells is independent of CD45*

In addition to its effects on CD45 isoform expression, the *Hnrpll*$^{thu/thu}$ mutation decreases peripheral T cell survival and accumulation (5). We therefore examined *Ptprc*$^{0/0}$:*Hnrpll*$^{thu/thu}$ animals to see if this effect of the *Hnrpll* mutation was abolished in T cells lacking CD45. In this analysis, the subset of peripheral T cells in *Ptprc*$^{0/0}$ mice that escape the targeted exon 6 insertion and express cell surface CD45 (6) was excluded by flow cytometric staining with a pan-CD45 Ab. Fewer CD4 and CD8 cells were present in *Ptprc*$^{0/0}$ mice with wild-type hnRNPLL, but their numbers were further decreased in *Ptprc*$^{0/0}$ mice that also carried the *Hnrpll*$^{thu/thu}$ mutation (Fig. 7*A–C*). *Hnrpll*$^{thu/thu}$ mutation therefore diminishes peripheral T cell accumulation independently of its effect on CD45 isoforms.

Discussion

In this study, we employed a loss-of-function mutation in the CD45 splicing silencer, hnRNPLL, to examine the consequences of dramatically increasing the proportion of high m.w. CD45 isoforms on

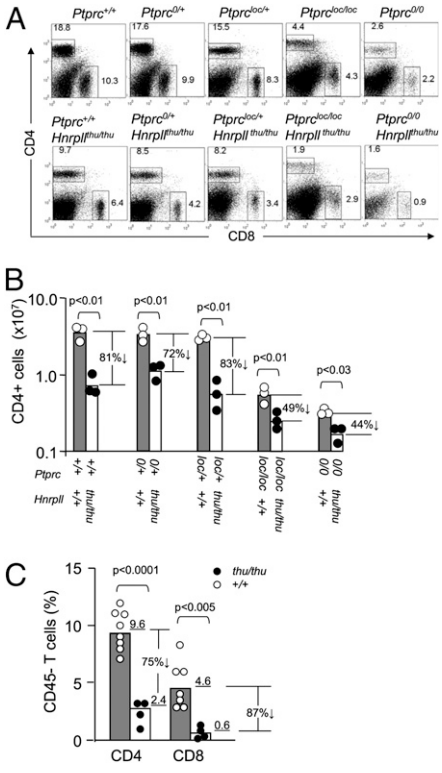

FIGURE 7. Effect of *Hnrpll* mutation on accumulation of peripheral T cells in the absence of CD45. *A*, Representative flow cytometric plots showing spleen cells stained for CD4 and CD8 in mice of the indicated *Ptprc* genotypes that were either *Hnrpll*$^{+/+}$ (*top row*) or *Hnrpll*$^{thu/thu}$ (*bottom row*). *B*, Number of splenic CD4+ cells in the mice of the indicated genotypes. CD45$^+$ cells were excluded from analysis of *Ptprc*$^{0/0}$ animals. Each dot represents one individual mouse, and bars denote means. *Hnrpll*$^{+/+}$ and *Hnrpll*$^{thu/thu}$ counterparts of a given *Ptprc* genotype were compared by Student *t* test. The percentage decrease in mean CD4 cell number in groups of *Hnrpll*$^{thu/thu}$ mice relative to their *Hnrpll*$^{+/+}$ counterpart for a given *Ptprc* genotype is shown. *C*, Percentage of CD45$^-$ T cells in peripheral blood of individual *Ptprc*$^{0/0}$ mice that were either *Hnrpll*$^{+/+}$ (open symbols) or *Hnrpll*$^{thu/thu}$ mice (filled symbols). All *p* values were calculated by Student *t* test. The percentage decrease in mean CD4 or CD8 cell frequency in *Hnrpll*$^{thu/thu}$ mice is shown.

T cell signaling and selection in vivo. This approach allowed the overall cell surface abundance of CD45 to be maintained at physiological levels, whereas combining the mutation with different *Ptprc* mutations tested the consequences of shifting CD45 isoforms when there were limiting amounts of cell surface CD45. Despite the use of assays that were internally validated to detect differences in CD45 activity and a 20- to 200-fold increase in CD45RA- and RC-bearing isoforms on hnRNPLL mutant thymocytes, there was no discernable effect upon whole-cell Lck phosphorylation, CD5 induction, or positive and negative selection. The only setting where an effect of the *Hnrpll* mutation and altered CD45 splicing was observed was a 25% increase in LckY505 phosphorylation in $Ptprc^{loc/loc}$:$Hnrpll^{thu/thu}$ mice thymocytes with 4% of normal CD45. These results reinforce and extend previous studies indicating that the efficiency of TCR signaling for T cell development depends upon the total amount of CD45 protein expressed but is unaffected by the expression of different CD45 isoforms. The limited in vivo effect of the different isoforms constrains models for the function of different isoforms and raises a conundrum of why CD45 alternative splicing has evolved and appears conserved across many species.

CD45 is required throughout all of the stages of T cell development in the thymus, and CD45 mRNA undergoes alternative splicing in a developmental and activation-dependent manner (1). The ectodomain of CD45 is highly glycosylated, and it has been proposed that it could contribute a considerable negative charge to the surface of the thymocytes that could potentially interfere with the interaction between the TCR and the selecting peptide/MHC ligand. In the thymus, DP cells predominantly express the CD45R0 isoform, which may allow CD45 access to the TCR signaling complex to favor positive selection of thymocytes. Our findings showed that expression of the CD45 isoforms was largely irrelevant to the outcome of positive selection, but what mattered was the total amount of CD45 protein expressed at the cell surface. Consistent with McNeill et al. (4), we conclude from our results that the level of CD45 expressed by thymocytes rather than CD45 isoforms is more important for the outcome of T cell differentiation in the thymus.

The 25% increase in LckY505 phosphorylation in $Hnrpll^{thu/thu}$ thymocytes with 4% of normal CD45 ($Ptprc^{loc/loc}$; Fig. 4*C*, 4*D*) indicates that the shift to higher m.w. CD45 isoforms is associated with an apparent decrease in CD45 phosphatase activity against Lck505 that is only detectable in thymocytes and only under conditions of limiting amounts of CD45. This result is consistent with in vitro evidence that high m.w. CD45 isoforms promoted less Lck tyrosine kinase activity and TCR signaling, due to either less efficient association with CD4 and TCR (27, 28) or stronger binding and dimerization by lectins recognizing the O-linked carbohydrates, such as macrophage galactose-like lectin (2). It contrasts with the evidence that high m.w. isoforms promote more TCR signaling of intracellular calcium or inositol 1,4,5-triphosphate compared with CD45RO due to their reduced propensity to homodimerize (29). The failure to detect this difference in LckY505 phosphorylation in cells with normal CD45 levels, nor in peripheral T cells with decreased or normal CD45 levels, and the lack of a discernable effect on SP cell formation indicate that the effect of the different isoforms is subtle and readily compensated. Although we did not detect an effect of altered CD45 splicing upon LckY505 phosphorylation in peripheral T cells, our results do not exclude the possibility that the subtle differences noted in thymocytes become significant under particular stimulation conditions in mature T cells. Given the large number of genes with alternative splicing in *Hnrpll* mutant T cells, we cannot exclude the possibility that splicing changes in other genes might mask the effect of the differences in CD45 isoforms.

Several studies have identified polymorphisms within different CD45 exons and associated these with disease susceptibility (3). The most common mutation is the C77G mutation in a splicing silencer element in exon 4 that prevents excision of the exon so that heterozygous carriers express both CD45RA and CD45R0 isoforms. The C77G allele has been associated with susceptibility to autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and HIV/AIDS (30–35). Other CD45 alleles that have been characterized include an exon 4 allele C59A and an exon 6 allele A138G (36, 37). Alterations in CD45 isoform expression on mature T cells could lead to altered TCR signaling that could lead to the production of effector cytokines and promote autoimmunity (16). The regulation of CD45 activity is thought to be controlled by dimerization, because a mouse expressing a mutation in the cytoplasmic wedge domain on CD45 (CD45E613R) develops autoimmune nephritis and a lymphoproliferative syndrome (38). In this study, we have used a validated model of autoimmune type 1 diabetes to investigate how changes in CD45 isoform expression would affect negative selection of autoreactive $CD4^+$ T cells in vivo. The *TCR* × *insHEL* model is highly sensitized to develop type 1 diabetes, because mice containing only one mutant copy of a diabetes susceptibility gene (e.g., *Aire*) exhibit accelerated onset of type 1 diabetes (39). By contrast, the expression of high m.w. CD45 isoforms in *thunder TCR* × *insHel* double-transgenic mice did not affect the proportion that developed type 1 diabetes. We have also previously shown that *thunder* 3A9 TCR transgenic cells can produce equivalent levels of cytokines compared with those of wild-type cells (5). It may be that human CD45 isoform polymorphisms combine with other genetic mutations to perturb T cell function but on their own may not be sufficient to trigger autoimmune disease. However, it should also be noted that the association of the C77G allele with autoimmunity has not been replicated in a number of studies (40–44).

The *thunder* mouse strain was originally identified by the low frequency of $CD4^+$ and $CD8^+$ T cells circulating in the blood, and we have established that the mutation affects the survival of naive and memory T cells (S. Chan, E. Bertram, and C.C. Goodnow, unpublished observations) (5). Survival and homeostasis of peripheral T cells depends on TCR signals (4, 5, 45), making it attractive to hypothesize that the altered CD45 isoform expression was responsible for diminishing T cell accumulation. The results here nevertheless disfavor that hypothesis, because the *thunder* mutation decreased the number of peripheral T cells, even when no CD45 was present. Future analysis will need to focus on the many other hnRNPLL-regulated targets revealed by microarray analysis (5), which may illuminate a novel mechanism by which hnRNPLL controls the accumulation and longevity of circulating T cells.

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Disclosures

The authors have no financial conflicts of interest.

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