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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 Ptprcex/+/ex 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.
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 dephosphorylation of the activating Lck Y394 site, whereas much larger decreases are needed to compromise dephosphorylation of the inhibitory Lck Y505 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)), 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) 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. Conjugates were from BD Pharmingen (San Diego, CA), including anti-mouse pLck Y505, or from Caltag Laboratories (Burlingame, CA). Phospho-Src family (Tyr102/104) (100F9) rabbit Ab was from Cell Signaling Technology (Danvers, MA) with the sheep anti-rabbit IgG (H+L) (FITC-conjugated secondary Ab from Chemicon International (North Ryde, New South Wales, Australia). Cell surface staining followed 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

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 thunder mutant mice that also carried either a normal Ptprc gene, a targeted knockout of Ptprc exon 6 (6) (Ptprc(0)), or a Ptprc point mutation that decreases CD45 surface protein by 25-fold (Ptprc(0)). Like thunder, the Ptprc point mutant strain lochy (Ptprc(0)) was identified in a flow cytometric blood screen of pedigrees of ENU-mutagenized C57BL/6f 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(high) activated/memory cells (Fig. 1A). Further characterization of progeny revealed two segregating heritable traits (Fig. 1B, 1C). lochy, identified by low B220 expression on CD19(B) B cells, and nesxy, with normal B220 expression. The cause of low T cells in nesxy was subsequently shown to be a missense mutation in the condensin protein kleisin-β (24). Flow cytometric analysis of CD45.2 B6foc × 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 locfloc mice revealed a 5-nt insertion at the start of exon 21 of the Ptprc gene (Fig. 1D), resulting from an
intrinsic T → A substitution that created a new splice acceptor 5 bp upstream from the correct splice acceptor (Fig. 1E). The insertion caused a frame shift and premature stop codon within the first cytoplasmic protein tyrosine phosphatase domain of CD45 (Fig. 1F). 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. 2A, 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 Hnrpllthu/thu and Hnrpll+/− controls, either bearing wild-type Ptprc genes (Ptprc+/+) or an allelic series comprising Ptprcloc/loc or exon 6 disrupted Ptprc (Ptprc0/0) and their heterozygous intermediates (Fig. 2A, 2B). The hRNPLL 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 were 40–50% less CD45 on Ptprc0/0 and Ptprcloc/loc heterozygotes, only 4% of wild-type CD45 on cells from Ptprc0/0 homozygotes, and no detectable CD45 on Ptprcloc/loc animals by flow cytometry. The same was true for CD4 SP spleen cells, except that ~5–10% of Ptprc0/0 cells express CD45 as shown previously (6). Although the Hnrpllthu mutation did not alter the cell surface abundance of CD45, it dramatically altered the isoforms expressed on DP thymocytes (Fig. 2C). On DP thymocytes from Ptprc+/+, Ptprc0/0, and Ptprcloc/loc mice, the hRNPLL 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 Ptprcloc/loc mice, it was still possible to detect increased expression of the higher m.w. CD45 isoforms as a result of the Hnrpll thunder mutation, whereas no expression of any of the CD45 isoforms was detectable on the surface of Ptprc0/0,Hnrpllthu/homozygous DP cells (Fig. 2C).

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 thunder-induced change in CD45 isoforms. The Ptprc0/0 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 Hnrpllthu mutation (Fig. 3A). Higher frequencies of SP thymocytes developed in Ptprcloc/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). Ptprcloc/loc:Hnrpllthu mice produced equivalent frequencies of DP and mature CD4+ and CD8+ SP cells compared with those of the Ptprcloc/loc mice (Fig. 3B–D). The thunder mutation also had no effect on frequencies of SP cells in the thymus of CD45+/+ and CD45loc/loc mice with half the normal CD45 protein (Fig. 3B–D). Measured in this way, the shift to high m.w. CD45 isoforms in Hnrpllthu mice had no discernable effect on the efficiency of positive selection.
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).

By contrast with the effects of decreasing overall CD45 protein, there was no significant effect of the altered CD45 splicing caused by the Hnrpll^thu/thu 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 4C). A smaller but consistent increase in pY505 was observed in Ptprc^loc/loc:Hnrpll^thu/thu CD4 SP thymocytes (Fig. 4D). This increase in LckY505 phosphorylation was not accompanied by any change in LckY394 phosphorylation (Fig. 4E, 4F) nor in CD5 expression (Fig. 4G, 4H). A more subtle \( \sim 10\% \) 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 phosphorylation.
ylation in peripheral naive or memory T cells expressing CD45 at normal or 4% of normal levels (Fig. 5A,5B). LckY505P levels and CD5 expression tended to be lower in Hnrplthuru/thu peripheral CD4 T cells lacking any CD45 (Fig. 5A), presumably reflecting the CD45-independent effects of the thu/thu mutation on peripheral T cell persistence described below (see Fig. 7).

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 Hnrplthuru mutation onto the 3A9 TCR transgenic background. The 3A9 TCR is I-Ak–restricted, directs T cell development toward the CD4+ lineage, and recognizes the immunodominant 46–61 peptide of hen egg lysozyme (HEL) bound to I-Ak (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 ß 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 TCR transgenic 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
Effects of Hnrpol mutation on positive and negative selection in 3A9 TCR transgenic mice. A, Representative flow cytometric plots of thymocytes from Hnrpol+/+ and Hnrpolthu/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 Hnrpol+/+ (left lane) and Hnrpolthu/thu (right lane) 3A9 TCR x 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 that were either Hnrpol+/+ or Hnrpolthu/thu (open circles, n = 8 TCR+insHEL+) or Hnrpol+/+ (filled squares, n = 9 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. The percentage decrease in mean CD4 or CD8 cell frequency in Hnrpolthu/thu−/− relative to Hnrpol+/+ was 44% (open circle, n = 44). D, Diabetes incidence in Hnrpol+/+ (open square, n = 18) or Hnrpolthu/thu (open circle, n = 44) 3A9 TCR × insHEL double-transgenic mice. Each dot represents one individual mouse, and bars denote means.

FIGURE 6. Effects of Hnrpol mutation on positive and negative selection in 3A9 TCR transgenic mice. A, Representative flow cytometric plots of thymocytes from Hnrpol+/+ and Hnrpolthu/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 Hnrpol+/+ (left lane) and Hnrpolthu/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 Hnrpol+/+ (filled squares, n = 9 TCR+ insHEL− and n = 8 TCR+insHEL+) or Hnrpol+/+ (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. The percentage decrease in mean CD4 or CD8 cell frequency in Hnrpolthu/thu−/− relative to Hnrpol+/+ was 44% (open circle, n = 44). D, Diabetes incidence in Hnrpol+/+ (open square, n = 18) or Hnrpol+/+ (open circle, n = 44) 3A9 TCR × insHEL double-transgenic mice. Each dot represents one individual mouse, and bars denote means.

Zap70 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+ T cells in the absence of CD45. We therefore examined 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 equal-
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 Ptprcloc/cloc;Hnrpllpha/btha 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 CD45RO isoform, which may allow CD45 access to the TCR MHC ligand. In the thymus, DP cells predominantly express the CD45RO isoform, which may allow CD45 access to the TCR MHC ligand. However, the only setting where an effect of the Hnrpll mutation and altered CD45 splicing was observed was a 25% increase in LckY505 phosphorylation in Ptprcloc/cloc;Hnrpllpha/btha 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.

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 CD45RO 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 (30, 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 autoimmunity 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.

References


