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Alternative Splicing Controlled by Heterogeneous Nuclear Ribonucleoprotein L Regulates Development, Proliferation, and Migration of Thymic Pre-T Cells

Marie-Claude Gaudreau,*† Florian Heyd,‡ Rachel Bastien,* Brian Wilhelm,§ and Tarik Möröy*†

The regulation of posttranscriptional modifications of pre-mRNA by alternative splicing is important for cellular function, development, and immunity. The receptor tyrosine phosphatase CD45, which is expressed on all hematopoietic cells, is known for its role in the development and activation of T cells. CD45 is known to be alternatively spliced, a process that is partially regulated by heterogeneous nuclear ribonucleoprotein (hnRNP) L. To investigate the role of hnRNP L further, we have generated conditional hnRNP L knockout mice and found that LckCre-mediated deletion of hnRNP L results in a decreased thymic cellularity caused by a partial block at the transition stage between double-negative 4 and double-positive cells. In addition, hnRNP L−/− thymocytes express aberrant levels of the CD45RA splice isoforms and show high levels of phosphorylated Lck at the activator tyrosine Y394, but lack phosphorylation of the inhibitory tyrosine Y505. This indicated an increased basal Lck activity and correlated with higher proliferation rates of double-negative 4 cells in hnRNP L−/− mice. Deletion of hnRNP L also blocked the migration and egress of single-positive thymocytes to peripheral lymphoid organs in response to sphingosine-1-phosphate and the chemokines CCL21 and CXCL12 very likely as a result of aberrant splicing of genes encoding GTPase regulators and proteins affecting cytoskeletal organization. Our results indicate that hnRNP L regulates T cell differentiation and migration by regulating pre-TCR and chemokine receptor signaling. The Journal of Immunology, 2012, 188: 5377–5388.

ThehnRNP proteins must pass through a tightly regulated developmental process to mature into effector T cells. First, early lymphocyte precursors migrate from bone marrow to the peripheral blood and enter the thymus, where they undergo differentiation (1, 2). The most immature thymocytes, termed double negative (DN), are characterized by the absence of both CD4 and CD8 coreceptor surface markers and are subdivided based on the expression of CD44 and CD25 into four fractions called DN1 to DN4 (3). In DN3 cells, b-selection takes place, which assures the selection of cells with a productive rearrangement of the TCRγ gene and the correct assembly of a surface pre-TCR complex. From this, DN4 cells emerge and develop further by upregulating both CD4 and CD8 markers to become double-positive (DP) cells (4–9). These cells then undergo a positive and negative selection to eliminate autoreactive T cells and to produce the final single-positive (SP) CD4+ and CD8-expressing T effector cell populations. During these two selection processes, thymocytes receive different signals from the pre-TCR, the TCR, the coreceptors, and probably other receptors that promote cell survival and proliferation (4, 10–16).

One of the critical factors that regulate the strength of TCR or pre-TCR signaling is the transmembrane tyrosine phosphatase CD45, which is not only expressed on T cells, but is found on a wide variety of hematopoietic cells, except platelets and erythrocytes (17, 18). This protein exerts its regulatory function by modulating the activity of the src kinases Lck and Fyn (18–22). Multiple isoforms of CD45 can be generated by alternative splicing of the variable exons 4–6, also called A, B, and C, which code for different extracellular domains of the protein (23). The expression of a specific isoform of CD45 is cell-type specific and changes during thymocyte development. Immature DPs predominantly express CD45RO, which lacks the domains encoded by exons 4–6, whereas CD4SP or CD8SP cells express the high m.w. isoform CD45RB, which contains the domain encoded by exon 5 (24–26). The smaller isoforms of CD45 form predominantly homodimers, whereas the high m.w. isoforms lose this potential, resulting in a less efficient signal transduction (27). A number of studies using CD45-deficient mice have demonstrated a crucial role for this protein, because its absence results in a severely impaired TCR signal transduction and in a differentiation block during positive and negative selection that occurs during the differentiation of DP thymocytes to mature SP cells (28–30).

Several lines of evidence provided by in vitro studies on alternative splicing of CD45 reveal the implication of heterogeneous nuclear ribonucleoprotein (hnRNP) L in mediating this process. The hnRNP proteins belong to a family of RNA-binding factors...
that regulate alternative splicing by binding exonic splicing silencer elements, resulting in exon exclusion from the mature mRNA (31). In the case of hnRNP L, its binding to the exonic splicing silencer elements 1 sequence in the alternatively spliced CD45 exons results in their exclusion from the mature, spliced RNA (23, 31). We show in this study that deletion of hnRNP L in the mouse results in a very early block of embryonic development, emphasizing its crucial role in morphogenesis. To study a role of hnRNP L in T cell development and function, we have restricted hnRNP L deletion to the T cell compartment by using a T cell-specific Cre recombinase transgene (LckCre). This strategy allowed us to reveal new and important roles of alternative splicing mediated by hnRNP L in T cell maturation and migration.

Materials and Methods

Mice

C57BL/6, LckCre transgenic, Gt(Rosa)26Sor tm1(ActB-tdTomato,-EGFP)Jav, and CD45.1 mice were purchased from Jackson ImmunoResearch Laboratories or were maintained at the Institut de Recherches Cliniques de Montreal. For the hnRNP L targeting construct, a phage screen was performed that yielded a clone containing ~18 kb of the genomic hnRNP L locus (performed and provided by R. Waldschütz, University of Duisburg-Essen). An 11-kb EcoRVD-Ndel fragment was cloned into pcDNA3 for further manipulation. A single loxP site was inserted into the EcoRI site, and a neocassette flanked by two loxP sites was inserted into the SfiI site (thereby destroying this site). This construct was found to efficiently recombine in bacteria stably expressing Cre-recombinase. For homologous recombination in embryonic stem cells, the targeting construct was freed from vector sequences by EcoRV-Ndel digestion. After transfection into embryonic stem cells, homologous recombination was confirmed by verifying presence of the neocassette (Stud digestion) as well as the third loxP site (XmnI digestion) by Southern blot. A positive clone was transiently transfected with a plasmid encoding Cre-recombinase, and partially recombined clones with the neocassette removed, leaving the endogenous locus with only two loxP sites inserted, were identified by PCR and confirmed by Southern blot (BclI/SfiI digestion). One clone was used to generate hnRNP L-floxed mice using standard procedures. hnRNP L-floxed mice were backcrossed to C57BL/6 for more than eight generations. All animals were housed under specific pathogen-free conditions at the Institut de Recherches Cliniques de Montreal animal facilities, and all experiments conformed to ethical principles and guidelines approved by the institutional animal care committee.

Abs

Anti-mouse CD4 (RM4-5), CD8a (53-6.7), CD25 (PC61), CD44 (1M7), TCRγδ (GL3), TCRαβ (H57-597), CD3 (145-2C11), B220 (RA3-682), CD45RA (14.8), and CD45RB (16A) Abs and streptavidin fluorescent conjugated forms were purchased from BD Biosciences. The lineage cocktail was made from B220, Ter119, CD3, Mac1, Gr1, NK1.1, CD49b, TCRγδ, and CD8, all biotinylated, and were from either BD Biosciences or eBioscience. Phospho-specific Abs were LckY505 from BD Biosciences, LckY394 from Santa Cruz Biotechnology, ERK and Akt from Cell Signaling Technology. Goat anti-hamster Ig, HRP-, and FITC-conjugated anti-rabbit and anti-goat were purchased from Jackson ImmunoResearch Laboratories, and anti-actin was from Santa Cruz Biotechnology.

Immunoblotting

Cell lysates from equal number of cells were prepared by lysis in buffer (20 mM Tris [pH 7.5], 150 mM NaCl, and 5 mM EDTA), supplemented with complete mini protease inhibitors (Roche Applied Science), 1 mM Na2VO4, and 1% Nonidet P-40. Western blot analysis was done following SDS polyacrylamide electrophoresis and transferred onto nitrocellulose membranes (GE Healthcare). General and specific tyrosine-phosphorylated proteins were detected with Abs, followed by goat anti-rabbit or donkey anti-goat–coupled HRP. All immunoblots were visualized with ECL chemiluminescent (Thermo Scientific) detection system, and images were taken on film.

Cell stimulation

Thymocytes were preincubated with 10 μg/ml anti-CD3 (2C11) for 10 min on ice. Bound Abs were cross-linked with goat anti-hamster Ig at 20 μg/ml and immediately incubated at 37°C for the indicated time. Cells were processed for surface and intracellular staining according to standard procedure.

Proliferation assays

For in vivo proliferation assays, mice were injected i.p. with 200 μl 10 mg/ml solution 5-bromo-2-deoxyuridine (Sigma-Aldrich) 16 h before sacrifice. Thymocytes were stained with specific fluorescent Abs, fixed, and treated with Perm/Wash (BD Biosciences). DNase I (Sigma-Aldrich) treatment at 30 μg/ml was applied for 1 h at 37°C. Anti-BrdU FITC conjugated (BD Biosciences) was added for 30 min at room temperature, and events were acquired on a LSR (BD Biosciences).

RT-PCR

Total RNA was extracted from thymocytes using RNAeasy mini kit (Qiagen), according to the manufacturer’s instructions. For RT-PCR analysis, RNA was used to prepare cDNA using SuperScript II reverse transcriptase (Invitrogen). PCR for CD45 and GAPDH were done according to previous report (32). To analyze CD45 alternative splicing, an established radioactive RT-PCR protocol was used (33). Briefly, 0.5 μg purified RNA of sorted thymic subpopulations was used for reverse transcription with a gene-specific primer, and PCR was performed with a 32P-labeled forward primer. Products were separated on a denaturing PAGE and quantified by Phosphoimager analysis (Typhoon 9400, ImageQuant; both GE Healthcare).

Quantitative PCR

DN3 and DN4 or DN, DP, CD4SP, and CD8SP thymocyte subsets were sorted using a MoFlo (Cytofmetry), and RNA was extracted using RNAeasy mini kit (Qiagen), according to the manufacturer’s instructions. cDNA was prepared from RNA using SuperScript II reverse transcriptase (Invitrogen) and detected using TaqMan probes (Applied Biosystems) for Ptcra and Gapdh as an internal control. A Mx-3005 system (Stratagene) was used, and relative expression was calculated via the ΔΔCT cycle threshold (CT) method. Exon usage of genes identified in the RNA-Seq analysis was validated using quantitative PCR. Validation was performed using two sets of primers spanning a variable exon junction and a constant exon junction from the same transcript. Primer sequences will be made available on request. Quantitative PCR was performed using a MasterMix for SYBR Green (Quanta Biosciences) and a Mx-3005 system. Melting curve was used to confirm the amplicon specificity. The exon usage was calculated via the ΔΔCT (variable exon-constant exon) and the ΔΔCT method.

Migration assay

Total thymocytes were harvested and resuspended in migration media (RPMI 1640 containing 0.5% BSA) at 2.5 × 106 cells/ml. Migration assays were performed in Transwell plates (Corning; 5 μm pore). Chemokines CXCL12 (PeproTech), CCL21 (PeproTech), and sphingosine-1-phosphate (SIP; Avanti Polar Lipids) were diluted in migration media at different concentrations and added to the lower chamber. After resting for 2 h at 37°C, 100 μl cell suspension was added to the upper chamber and cells were allowed to migrate for 3 h at 37°C. Cells were collected, stained for CD4 and CD8, and resuspended in 200 μl PBS supplemented with 2% FCS, and a fixed number of countbright beads (Beckman Coulter) was added. The percentage of migrating cells was calculated by dividing the number of cells that migrated into lower chamber by the input cell number.

Actin polymerization

Thymocytes were collected and resuspended in RPMI 1640 supplemented with 0.5% BSA and rest at 37°C for 2 h. Cells were then activated with different concentrations of SIP (CXCL12, or CCL21) for 30 s at 37°C. Cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 10 min on ice and Perm/Wash (BD Biosciences). Cells were then incubated for 30 min at room temperature with 1 μl phalloidin-Alexa488 (Invitrogen), followed by surface staining for CD4 and CD8. Events were acquired on a LSR (BD Biosciences). Relative F-actin polymerization was calculated as the ratio of mean fluorescence of phalloidin-Alexa488 of activated cells over non-stimulated cells.

Recent thymic emigrants

Four- to 5-wk-old mice were anesthetized with isoflurane. The shaved skin at the thoracic inlet was cut open by a 1-cm incision, one-third of the sternum was longitudinally bisected, and the thymus was exposed. Each thymic lobe was injected with 10 μl FITC (Sigma-Aldrich) dissolved in...
RNA-Seq
RNA was extracted from total thymocytes using Tri Reagent (Molecular Research Center), followed by a purification using RNeasy mini kit and RNase-free DNase on column (Qiagen) for 15 min at room temperature, both following manufacturer’s instructions. RNA integrity and quality have been confirmed using a bioanalyzer (Agilent). rRNA from each biological sample was depleted from total RNA using a RiboMinus kit (Invitrogen), and the treated RNA was then fragmented using RNase III. Ligation of the adaptor mix A and reverse transcription were performed following the manufacturer’s protocol. Libraries were size selected for fragments between 150 and 300 bp, amplified for 12 cycles of PCR, and purified using PureLink PCR micro kit (Invitrogen). Barcoded library concentrations were determined by quantitative PCR using a standard curve of template at known concentrations (DH10B), and ~0.25 ng each library was used for each full-emulsion PCR (4 full-emulsion PCR/sample). Approximately 200 million beads from each two samples were deposited on single full slides (2 slides for 4 samples in total) and sequenced using the Opti200 million beads from each two samples were deposited on single full slides (2 slides for 4 samples in total) and sequenced using the Opti

Statistical analysis
Two-tailed Student t test was used to calculate p values, where indicated. A p value ≤0.05 was considered statistically significant, as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Two-way ANOVA was used to calculated p value for the migration assay and the actin polymerization.

Results
Constitutive deletion of hnRNP L impedes early embryonic development
To be able to analyze the consequences of a constitutive or inducible ablation of hnRNP L for the immune system and hematopoietic differentiation, we used a previously published strategy (34) to generate mice carryingloxP( fl) sites flanking coding exons 2–6 of the hnRNP L gene (Supplemental Fig. 1A, 1B). Animals carrying two hnRNP L-floxed alleles (hnRNP L fl/fl) were bred with germline deleter strains (CMV Cre) to generate constitutively hnRNP L-deficient mice. However, live pups or embryos beyond stage 3.5 days postcoitus could not be generated with this strategy (data not shown), suggesting that the ablation of hnRNP L is lethal at a very early stage of murine development.

To overcome this problem and because RT-PCR analyses revealed expression of hnRNP L in a wide range of hematopoietic cells, including lymphoid progenitors and thymocyte subsets (Supplemental Fig. 1C), we chose to restrict our analysis to the T cell compartment. To determine the function of hnRNP L in T lymphocytes, we deleted the hnRNP L-floxed alleles using mice expressing Cre recombinase driven by the Lck proximal promoter. In these mice, Cre expression starts at the pro-T cell DN2/DN3 stages and is most efficient at and after the DN4 stage. As expected, the floxed alleles in LckCre, hnRNP L fl/fl mice efficiently deleted in pro-T cells, and hnRNP L protein expression is almost eliminated in the thymus of these animals (Supplemental Fig. 1D, 1E).

Loss of hnRNP L expression affects thymic cellularity in a cell-autonomous manner
Flow cytometric analysis revealed a normal frequency of the different thymocyte subsets in LckCre’hnRNP L fl/fl mice with a slight increase of DN cells compared with wild-type (wt) thymus (Fig. 1A, 1B). However, loss of hnRNP L protein led to a decrease in overall thymic cellularity by 75% compared with wt mice (Fig. 1C). Without affecting the absolute DN cell number, deletion of hnRNP L resulted in the loss of DP thymocytes and the more mature CD4 and CD8 SP cells in about the same proportions (Fig. 1C). In addition, the loss of thymic cellularity in LckCre’hnRNP L fl/fl mice was specific to TCRβ cells and did not affect TCRγδ cells because their absolute number remained unchanged (Fig. 1D, 1E). Consistent with this, the DN4 population of LckCre’hnRNP L fl/fl mice had a reduced frequency of cells expressing surface TCRβ, but all hnRNP L-deficient DN4s expressed the cytoplasmic form of TCRβ. In addition, the expression of Ptcra was not disturbed by the ablation of hnRNP L, suggesting that a pre-TCR can be made in hnRNP L-deficient mice (Fig. 1F, 1G).

To test whether the reduced thymic cellularity of LckCre’hnRNP L fl/fl mice could be due to an effect of hnRNP L deletion in thymic epithelial cells, we generated mixed bone marrow chimeras. Congenic CD45.1+ recipient mice that were reconstituted with bone marrow cells from LckCre’hnRNP L fl/fl mice (CD45.2+) showed the same defect in thymocyte development as was observed in the original hnRNP L-deficient mice (data not shown), suggesting that the phenotype observed upon hnRNP L deletion is cell autonomous.

To discriminate between cells before and after positive and negative selection, we stained thymocytes from both wt and hnRNP L-deficient mice for CD4, CD8, CD69, and TCRβ expression, which enables the definition of four maturation stages (35). This analysis showed that the relative percentages of the mature subsets as defined by CD69 and TCRβ expression were not different with regard to the relative frequency of cells between wt and hnRNP L-deficient mice. In this analysis, CD4 cells appear before CD8 cells, and this sequential appearance is also unaffected by hnRNP L deletion (Supplemental Fig. 2A, 2B). In addition, to confirm whether the absence of hnRNP L affects positive selection, we introduced the OTI or OTII TCR transgene into LckCre’hnRNP L fl/fl mice. We observed that CD8SP cells or CD4SP cells were formed in OTI tg LckCre’hnRNP L fl/fl and in OTII tg LckCre’hnRNP L fl/fl mice, respectively, at a similar rate than in controls (Supplemental Fig. 2C, 2D). This suggests that there are no major defects in either positive or negative selection or in CD4/CD8 lineage choice due to the loss of hnRNP L.

hnRNP L regulates the splicing of CD45 in vivo
Because it has been suggested that hnRNP L regulates the alternative splicing of CD45 in vitro (23, 31), we tested whether the expression of this transmembrane phosphatase was affected by hnRNP L ablation in vivo. It has been described that CD45RO and CD45RB isoforms are expressed in thymocytes, whereas the CD45RABC isoform is absent in these cells (26). We detected an increased expression of CD45RB on hnRNP L−/− DP and CD8SP cells compared with the respective wt controls and found the expression of CD45RA upregulated 4-fold over the wt in all hnRNP L−/− thymocyte subsets except DN3 (Fig. 2A, 2B). An assessment of the mRNA expression level of all possible isoforms of CD45 by RT-PCR indicated that the mRNAs for the CD45RABC isoform that is absent from wt thymus are now present in hnRNP L-deficient DN4 and CD8SP cells and to a lesser extent also in DP and CD4SP cells compared with their respective controls (Fig. 2C). In addition, the same analysis also suggested that the mRNA encoding CD45RB and CD45RBC are enriched in hnRNP L−/− CD4 and DP cells over wt controls (Fig. 2C). A more quantitative RT-PCR method using the incorporation of radioactive nucleotides confirmed an enrichment of the
CD45RB mRNA over the CD45ROΔ7 mRNA isoforms in DN4 and DP cells (Fig. 2D). These data indicate that absence of hnRNP L leads to an enrichment of mRNA isoforms that encode the larger isoforms of CD45, which is consistent with the role of hnRNP L as one of the proteins that regulate the exclusion of CD45 exons.

hnRNP L modulates the TCR signal in thymocytes

It is known that CD45 is a tyrosine phosphatase that controls the duration of pre-TCR or TCR signaling by removing the inhibitory phosphate group at tyrosine (Y) 505 in the Lck kinase that is associated with the cytoplasmic domains of the TCR complex (22, 36). The removal of the Y505 phosphate enables Lck to phosphorylate its substrate, the ZAP70 kinase (Fig. 3A). It has been shown that larger CD45 isoforms are more efficient phosphatases than the smaller variants, such as CD45RO, probably owing to their different potential to form homodimers (27). Because the LckCre+hnRNP L fl/fl thymocytes, in particular DN4 cells, expressed aberrant level of the larger CD45 isoforms, we tested whether this affected the phosphorylation status of LckY505. We found a lower level of Lck phosphorylation at the inhibitory Y505 in hnRNP L-deficient DN4 cells both at steady state and after anti-CD3 activation (Fig. 3B, 3C). Interestingly, the phosphorylation of the activating tyrosine Y394 of Lck as well as the downstream tyrosine kinases ERK and Akt was increased in these DN4 cells over wt controls at steady state levels (Fig. 3C, 3D). These results suggest that hnRNP L controls TCR signaling intensity through the alternative splicing of CD45.

Deletion of hnRNP L in thymocytes results in aberrant cell proliferation

Next, we assessed whether the aberrant expression of CD45 isoforms and the ensuing deregulation of Lck phosphorylation had any effect on cell proliferation or cell death, which could explain the

**FIGURE 1.** Deletion of hnRNP L and its effect on thymocyte populations. Flow cytometric analysis of wt and hnRNP L-deficient thymocyte populations assessed by Lin−/CD44+/CD25− (A) and CD4+/CD8− staining (B). (C) Histograms representing the absolute cell number of total thymus and different thymocyte subsets from LckCre+hnRNP L fl/fl and wt mice at 6 wk of age. A minimum of six mice per group was analyzed. Flow cytometry (D) and absolute cell number (E) of TCRα/β and TCRγ/δ thymocyte populations. (F) Intracellular and surface expression of TCRβ by flow cytometry performed on DN3- and DN4-gated cells. The plot is representative for three independent analyses. (G) Expression of Ptcra measured by quantitative RT-PCR on DN3 and DN4 cells sorted by flow cytometry. Expression was measured and normalized to the expression of the Gapdh gene and is presented as the fold increase relative to wt cells (set as 1-fold). Data represent three independent experiments, each done in triplicate. All error bars are means ± SEM (*p < 0.01, **p < 0.001, ***p < 0.0001).
lower thymic cellularity in hnRNP L−/− mice. Staining with annexin V for apoptotic cells did not show any differences between hnRNP L-deficient pre-T cell subsets compared with wt controls (Fig. 3E). However, in vivo BrdU pulse labeling clearly indicated that DN4 and CD4SP cells from hnRNP L mice progressed faster through S phase than their wt counterparts (Fig. 3F). This result indicated that thymocytes from LckCre+hnRNP L fl/fl mice are not apoptotic, but have shortened cell cycle phases resulting in higher proliferation in stages following the β-selection checkpoint.

**Deletion of hnRNP L results in loss of peripheral T cells**

We next investigated whether hnRNPL deletion and the differential expression of CD45 isoforms affected CD4 or CD8 SP cells. We noted that the frequency of peripheral CD3-positive cells was considerably reduced in blood and spleen of LckCre+hnRNP L fl/fl mice.
mice compared with wt animals and that this reduced frequency corresponded to a significantly reduced absolute number of peripheral CD3+ T cells (Fig. 4A, 4B). Importantly, the loss of peripheral T cells affected both CD4 and CD8 cells equally (Fig. 4B). A genotyping PCR analysis showed the presence of both floxed and excised hnRNP L alleles in sorted peripheral CD3+ T cells of LckCre+hnRNP L fl/fl mice, although only fully excised alleles were found in CD4SP and CD8SP subsets in the thymus (Supplemental Fig. 1D). This suggests that peripheral T cells are counterselected for complete excision, or that CD4SP or CD8SP thymocytes that have completely excised hnRNP L are not migrating to the periphery. To investigate this hypothesis further, we quantified recent thymic emigrants by injecting FITC into the thymus of wt or LckCre+hnRNP L fl/fl mice. Four days after injection, the mice were sacrificed and blood and spleen were analyzed for the presence of FITC-labeled CD4 or CD8 T cells by FACS. A quantification showed clearly that FITC-labeled cells were significantly reduced in the periphery of hnRNP L-deficient mice.

**FIGURE 3.** Loss of hnRNP L in thymus does not affect cell death, but causes aberrant TCR activation. (A) Schematic representation of CD45 phosphatase activity on Lck following TCR activation. (B) Phosphorylation levels of Lck in total thymus from the indicated mice were assessed by Western blot or (C) by flow cytometry on gated DN4 cells using Abs specifically recognizing LckY505 or LckY394 at basal level and following 2-min anti-CD3 stimulation (gray = isotype control, black = nonstimulated, dotted line = 2-min CD3 activation). (D) Basal phosphorylation of ERK and Akt in wt and hnRNP L-deficient DN4-gated cells by flow cytometry analysis. Data are representative of three independent experiments. (E) Flow cytometric analysis of cell death by annexin V staining on different thymocyte subpopulations and bar graph representing the percentage of annexin V-positive cells. (F) Proliferation was assessed by incorporation of BrdU in all thymocyte subsets after 16 h postadministration and quantified by flow cytometry. All graphs represent the mean ± SEM from *n = 6 mice (*p < 0.01).
mice over wt controls (Fig. 4D), suggesting a reduction of thymic egress caused by the deletion of hnRNP L.

Next, LckCre+hnRNP L fl/fl and LckCre+hnRNP L wt/wt were crossed with Gt(ROSA)26Sor tm4(ACTB-tdTomato,-EGFP)Luo mice (hereafter ROSAfltomatofl-GFP). These animals allow the tracking of cells by measuring red fluorescence (tomato) and Cre activity by measuring green fluorescence (GFP). Both ROSAfltomato-GFP and ROSA+ LckCre+hnRNP L fl/fl mice had 95–98% GFP-positive thymocytes (data not shown). We found 73–85% GFP-positive T cells (CD8+ or CD4+) in blood or spleen in ROSAfltomatofl-GFP LckCre+hnRNP L wt/wt mice, indicating efficient deletion by the Lck promoter-driven Cre recombinase (Fig. 4E). However, T cells from blood or spleen of ROSAfltomato-GFP LckCre+hnRNP L fl/fl mice were mostly tomato

FIGURE 4. hnRNP L-deficient mice show a loss of peripheral T cells. (A) Flow cytometric analysis of blood and splenic T cells assessed by CD3 and CD4/CD8 staining from both control and hnRNP L-deficient mice. (B) Absolute cell numbers of splenic CD4 and CD8 T cell subsets. (C) Excision of floxed hnRNP L alleles visualized by PCR on sorted CD3-positive cells from blood and spleen from LckCre+hnRNP L fl/fl and control mice. (D) Recent thymic emigrant assay. Briefly, 10 μg FITC was injected intrathymically, and mice were sacrificed 4 d later. Blood and spleen were harvested and analyzed by flow cytometry for side/forward light scatter and FITC expression on gated CD4 and CD8 cells. Graphs show the numbers of CD4 and CD8 FITC-positive cells in blood and spleen. (E) ROSAfltomato-GFP mice were crossed with LckCre+hnRNP L fl/fl or LckCre+hnRNP L wt/wt. Cells expressing the LckCre transgene delete the floxed tomato allele and switch the expression from tomato (red) to GFP (green), allowing to track cells with active Cre deletion. The numbers of GFP-positive cells were assessed in blood and spleen by flow cytometry after staining for CD4 and CD8 on CD3-gated cells. Absolute cell numbers are shown in the bar graphs. All error bars are means ± SEM (*p < 0.01, **p < 0.001), and a minimum of six mice was used in all experiments.
positive (81–93%), and only very few (0.2–2.5%) were GFP positive (Fig. 4E), indicating that cells that have deleted the tomato marker in the thymus, and by inference also the floxed hnRNP L alleles and are thus GFP+, do not leave the thymus or fail to migrate into the blood and settle in the peripheral lymphoid organs.

**hnRNP L regulates the migration of thymocytes**

It is known that the motility of pre-T cells within the thymus and their egress into the bloodstream are tightly regulated by different chemokines such as CCL21, CXCL12, or S1P (37, 38). Because the previous experiments suggested an impaired motility or egress

**FIGURE 5.** hnRNP L-deleted thymocytes show defects in chemotaxis. (A) Cell surface expression of chemokine receptors CXCR4, CCR7, and S1P1 on CD4- and CD8-gated thymocytes by flow cytometry. Histograms show cells from wt (gray) or LckCre+hnRNP L fl/fl (black) mice, and dashed line the isotype control. (B) Transwell migration assay: chemotactic response of CD4SP and CD8SP to CXCL12, CCL21, and S1P after 3-h incubation depicted as the percentage ± SEM of cells that migrated into the lower chamber normalized to the corresponding input cells. At least three different mice were used for each measurement. (C) hnRNP L-deficient CD4SP and CD8SP cells have reduced actin polymerization compared with wt control cells. To detect F-actin, cells were stained with phalloidin-Alexa488 and with Abs for CD4 or CD8 after 30-s stimulation with different concentrations of CXCL12, CCL21, or S1P. Cells were analyzed by flow cytometry. Relative F-actin content was calculated as the mean fluorescence intensity of phalloidin-Alexa488 after stimulation over nonstimulated cells. Graphs show means ± SEM of at least three different mice.
of hnRNP L-deficient thymic T cells, we tested the chemotactic response of hnRNP L\(^{-/-}\) thymocytes toward these chemokines. The expression of CXCR4, CCR7, and S1P receptor 1 (S1P1), which are the receptors for CXCL12, CCL21, and S1P, respectively, was not altered in hnRNP L-deficient thymocytes, excluding a role of hnRNP L in the regulation of chemokine receptor expression (Fig. 5A). However, a Transwell assay, which allows a quantification of thymocyte migration toward chemokine-enriched media, demonstrated that hnRNP L\(^{-/-}\) CD4SP and CD8SP thymocytes migrated poorly toward the chemokines compared with wt cells (Fig. 5B). In addition, hnRNP L-deleted cells were deficient compared with wt controls to polymerize actin upon chemokine treatment, suggesting a defect in the relay of signals from the chemokine receptor to actin polymerization, which is required for cell migration (Fig. 5C).

**hnRNP L regulates alternative splicing of other gene targets**

The aberrant splicing of CD45 in hnRNP L-deficient thymocytes may not account for all phenotypes observed in this mouse mutant. To gain more insight into the global effects of hnRNP L deletion, we undertook a genome-wide analysis of all splicing events through next-generation RNA sequencing of thymus from wt and LckCre\(^{+}\)hnRNP L\(^{fl/fl}\) mice. The RNA-Seq analysis did not reveal any major changes in the expression level of genes between hnRNP L-deficient thymocytes and controls (Supplemental Fig. 3A, 3B); hence, the differences between the two samples are thus

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### Table

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<tr>
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<td>0.0015</td>
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<tr>
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<td>0.0133</td>
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<tr>
<td>Protein localization</td>
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<td>2.03</td>
<td>0.0025</td>
</tr>
<tr>
<td>Regulation of protein kinase cascade</td>
<td>5</td>
<td>2.24</td>
<td>0.18</td>
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</tbody>
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**FIGURE 6.** RNA-Seq analysis of splicing events affected by hnRNP L ablation. (A) Scatterplot of all junction-spanning sequence reads from either normal control thymocytes (WT) or hnRNP L-deficient thymocytes (KO), in which the sum of one or the other condition (or both) is at least 1. Green lines indicate a ±2-fold difference between WT and KO samples, and blue dotted lines at 5 represent a minimum of 32 (2\(^5\)) reads spanning either WT or KO junctions. Differentially spliced junctions with low absolute counts (below the blue lines) may be valid, but were not considered in the analysis to avoid inclusion of junctions resulting from sampling noise. (B) For differential splice junction usage, two different approaches were assessed. First, splice junctions were filtered based on those that had at least 10 reads in both WT and KO samples and were then sorted based on the ratio of reads in KO versus WT. Second, the threshold of reads in either KO or WT samples was set to 32, and genes were then sorted based on a KO versus WT ratio. The Venn diagram shows the ~80% overlap between both approaches, indicating robust detection of differentially spliced junctions. For gene ontology (GO) pathway analysis, gene lists were generated from data using former threshold (32 reads). (C) Gene ontology categories enriched in a set of 250 genes with differential exon junction usage between WT and hnRNP L-deficient thymocytes. (D) Quantitative PCR validation of the alternate exon usage. Two sets of primers were designed for each gene, one corresponding to a constant exon junction and a second specific for an exon junction that showed alternate usage between WT and hnRNP L-deficient thymocytes in the RNA-Seq analysis. Quantification was calculated using the \(\Delta\Delta\) method with the constant junction used as the normalizer and is presented as the fold increase relative to the DN subset from WT cells (set as 1-fold). Bar graphs are representative of three independent experiments.
due to differential splicing. To survey the differences in splicing between control and hnRNP L-deficient thymocytes, sequence reads were mapped against all possible annotated splice junction combinations within a gene locus, regardless of prior evidence of their usage in vivo. The total number of spliced reads in the sum of the two biological replicates was scaled using all mapped sequence reads to account for differences in sequencing depth. Scatterplots comparing exon junction usage between wt and hnRNP L\(^{-/-}\) cells were generated with evidence of junction usage in at least one cell type as a threshold for inclusion. Green diagonal lines indicate a ±2-fold difference between wt and hnRNP L\(^{-/-}\), and blue dotted lines on either axis represent a minimum of 32 (2\(^5\)) reads spanning junction for either wt or hnRNP L-deleted cells (Fig. 6A). To avoid evidence from low confidence junctions, gene lists were generated from data over the blue dotted lines (e.g., \(\geq32\) junction-spanning reads) for gene ontology (GO)/Annotation pathway analysis (Supplemental Table I). Alternative methodologies of filtering splice junctions yielded similar results (Fig. 6B), but were not used.

The gene lists were analyzed for enrichment of genes of specific functions using the DAVID tool (http://david.abcc.ncifcrf.gov/ summary.jsp). The most significant annotation cluster generated for hnRNP L-deficient cells was for genes involved in cytoskeleton/microtubule organization, and no elements of this annotation cluster were contained in analysis of the wt cells (Fig. 6C). This cytoskeleton/microtubule category includes genes such as \(\text{Wasf2, Dia}1\), and \(\text{Arhgef2}\) (Table I). Another GO category that is significantly enriched in hnRNP L\(^{-/-}\) thymocytes concerns genes involved in G protein-mediated signaling (Fig. 6C). This suggests that hnRNP L may affect thymocyte egress by regulating chemokine receptors that are implicated in cell migration and bind GTPases and GTPase regulators like Dock9, Arhgef2, and Arhgap17 (Fig. 6C, Table I). Another GO category that was suspected from previous in vitro studies, but also indicates that hnRNP L controls the alternative splicing of hundreds of other genes implicated in early T cell differentiation and migration.

**Discussion**

In this study, we provide evidence for an important role for the splicing factor hnRNP L in the development, intrathymic migration, and thymic egress of pre-T cells. In particular, our study shows that hnRNP L regulates the alternative splicing of CD45 in vivo, as was suspected from previous in vitro studies, but also indicates that hnRNP L controls the alternative splicing of hundreds of other genes implicated in early T cell differentiation and migration.

CD45 expression is crucial for T cell development as well as in controlling the threshold of Ag-mediated activation in peripheral T lymphocytes (22, 29, 39, 40). Larger CD45 isoforms (e.g., CD45RABC, -RAB, -RBC, or -RB) facilitate the activation through the TCR, whereas T cells expressing CD45RB need a stronger signal to obtain the same level of activation (27, 41). Our observation that both the basal and TCR-induced inhibitory phosphorylation of Lck at tyrosine Y505 is reduced in LckCre\(^{+}\) hnRNP L\(^{+/+}\) DN4 pre-T cells is in agreement with this, because we show that hnRNP L-deficient cells express higher CD45RB and RA levels than wt cells. This is also consistent with the increased basal-activating phosphorylation of Lck at Y394 in hnRNP L-deficient cells compared with wt controls. It is known that Lck mediates activation of T cells through recruitment and phosphorylation of its substrates, such as Zap70, but also activates If3 (42–45). Hence, the higher basal Akt and Erk phosphorylation and related increased proliferation rate observed in hnRNP L-deficient DN4 cells are likely to be a consequence of the aberrant Lck signaling in these cells. We conclude that alternative splicing of CD45 is regulated by hnRNP L and represents a mechanism to control pre-TCR signaling in DN4 cells, and thus, the early steps of pre-T cell differentiation at the DN to DP transition. However, in absence of hnRNP L, thymocytes proliferate in a higher rate than wt cells, but their absolute number is still reduced without showing an increased apoptosis or necrosis. Further experimentation is needed to clarify this phenomenon. In this study, we can only suggest that either the hnRNP L-deficient thymocytes are dying from an unidentified mechanism, the dying cells are not detectable by either propidium iodide or annexin V staining, or the increased BrdU incorporation seen in absence of hnRNP L is due to an increased rate of DNA repair.

Recently, new studies suggested that instead of hnRNP L, the related protein hnRNP L-like (hnRNP LL) plays a major role in controlling the alternative splicing of CD45 (46, 47). Both ENU-induced mutations in the hnRNP LL gene and shRNA knockdown showed the same shift in CD45 isoform expression as our hnRNP L-deficient model. It is possible that hnRNP L and hnRNP LL may have overlapping functions, but with different mRNA-binding requirements. Alternatively, hnRNP L may be mediating the basal CD45 alternative splicing, whereas hnRNP LL may be necessary in cells that receive a TCR signal to induce exon skipping (46–48). Further investigation has to clarify the shared or specific role of both proteins in TCR signaling and CD45 alternative splicing.

Our observation that hnRNP L deficiency correlates with a loss of peripheral T cells was unexpected and suggested a role of alternative splicing in thymic egress or the intrathymic migration of pre-T cells. Indeed, a number of experiments support this view.

### Table I. Selected genes that contain exon(s) showing preferential splicing in hnRNP L-deleted thymocytes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene Description</th>
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<tbody>
<tr>
<td>(\text{Ptprc})</td>
<td>Protein tyrosine phosphatase, receptor type C (CD45)</td>
</tr>
<tr>
<td>(\text{Dia}1)</td>
<td>Diaphanous homolog 1, related formin</td>
</tr>
<tr>
<td>(\text{Wasf2})</td>
<td>WAS protein family member 2</td>
</tr>
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<td>(\text{Arhgef2})</td>
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<td>Dedicator of cytokinesis 9</td>
</tr>
<tr>
<td>(\text{Cd86})</td>
<td>CD8 Ag (\beta)-chain 1</td>
</tr>
<tr>
<td>(\text{Akt3})</td>
<td>Thymoma viral proto-oncogene 3</td>
</tr>
<tr>
<td>(\text{Tbl1})</td>
<td>Talin 1</td>
</tr>
<tr>
<td>(\text{Arhgap17})</td>
<td>Rho GTPase-activating protein 17</td>
</tr>
<tr>
<td>(\text{Bc110})</td>
<td>B cell leukemia/lymphoma 10</td>
</tr>
<tr>
<td>(\text{Hdac4})</td>
<td>Histone deacetylase 4</td>
</tr>
<tr>
<td>(\text{Tbk1})</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>(\text{Fn1})</td>
<td>Fibronectin 1</td>
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First, our Transwell assays showed that hnRNP L-deficient CD4SP and CD8SP cells failed to adequately respond to chemotactic signals provided by CCL21, CXCL12, or S1P, although they express normal levels of the respective CCR7, CXCR4, or S1P1 receptors. Second, hnRNPL-deficient mice show very low yields of labeled T cells in the periphery after intrathymic injection of FITC. Third, almost no peripheral T cells in LckCre*hnRNP L2/2 mice show complete excision, which have been verified by both genotyping PCR and GFP expression in the ROSAfltomatofl-GFP LckCre*hnRNP L2/2 mice. This suggests that thymic egress or intrathymic migration selects against cells that lack hnRNP L.

Stage-dependent migration of thymocytes is important to obtain the right signal at the right time (37). It has been shown in Jurkat cell lines that CD45 and CXCR4 colocalize to mediate signal transduction following CXCL12 treatment (49). Furthermore, a recent study linked CD45 expression in DN1 cells with the migration toward CXCL12 (50). Absence of CD45 results in a deficiency in CXCL12-induced migration of DN1 cells in the cortex, which is opposite of our model because CD45 is present on cell surface. Finally, it has been shown that ablation of SC35, which also regulates alternative splicing of CD45, correlates with a reduced number of peripheral CD4 and CD8 T cells in the spleen. However, in contrast to our findings, the CD45RO isoform was predominant and CD45RB was undetectable in SC35-deficient mice (51). Although our hnRNP L−/−-deficient SP cells show a similar migration defect as SC35-deficient animals, higher m.w. isoforms of CD45 are upregulated in the absence of hnRNP L, which differs from the situation seen in SC35-deficient mice. These observations suggest that aberrant CD45 alternative splicing may not account for the migratory deficiency seen in hnRNP L-deficient mice.

One of the first steps in the chemotactic response after binding of the chemokine to its receptor is the activation of GTPase-dependent events and the polymerization of F-actin. Rho-GTPases such as cdc42 and Rac are converted upon receptor activation from the inactive form to the active GTP-bound form to control F-actin polymerization through interaction with Arp2/3, WASP, and the Wave complex (52, 53). Our evidence suggests that hnRNP L controls the steps between chemokine receptor engagement and F-actin polymerization, and thereby affects cell motility, leading to the phenotype observed in hnRNP L-deficient mice. Two sets of data support this notion, as follows: first, we show that F-actin polymerization is indeed disturbed by the ablation of hnRNP L in CD4SP and CD8SP thymic cells. Second our next-generation RNA sequencing data identified different alternatively spliced RNAs belonging to GO categories, such as GTPase binding and regulation and cytoskeletal proteins, among them the genes Arhgef1, GEF-Hi, Wave2, and Diaph1.

Previous studies have already shown the importance of these molecules in immune cell chemotaxis (54–56). For instance, macrophage migration toward CSF-1 requires the Wave2 complex and its phosphorylation by MAPKs because the reduction of its expression through irRNA abrogated F-actin–rich membrane protrusions (54). In addition, GEF-H1 plays a pivotal role in urzpord formation during transendothelial migration of T cells (57). Among these potential hnRNP L targets, the Diaph1 gene, which is involved in actin nucleation and polymerization, might be the key regulator of lymphocyte migration in the thymus (56, 58). Similarly to the results presented in this study, it has been reported that mDia1−/− mice show reduced numbers of peripheral T cells and an impaired chemotactic response to CXCL12 and CCL21. Moreover, the block in migration in mDia1−/− mice was due to a suppressed production of F-actin (58), which is very similar to the results we obtained with hnRNP L-deficient cells. It remains to be shown how the ablation of hnRNP L leads to a loss or alteration of mDia1 function, but it can be speculated that hnRNP L does not necessarily need to affect the protein expression of the target gene candidates identified in this work. Altered mRNA isoforms generated by hnRNP L-controlled alternative splicing might either encode proteins that undergo differential posttranslational modification, lose their binding capacity to specific interaction partners, or produce truncated proteins with altered functions.

Our study not only reveals a new, critical role of the splicing factor hnRNP L in the differentiation and migration or thymic egress of pre-T cell, it also illustrates to which extent alternative splicing serves as a regulatory mechanism of biological processes at a posttranscriptional level. The fine-tuning of CD45 isoform expression at differential stages of thymocyte development is one example of how alternative splicing regulates the generation of a functional T cell-mediated immune response. The regulation of alternative splicing of genes involved in relaying signals from chemokine receptors to F-actin polymerization is another example. Our findings link alternative splicing controlled by hnRNP L to define cellular processes in the acquired immune system, and thus, ascribe this factor important new biological roles.

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Disclosures
The authors have no financial conflicts of interest.

References