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Leucine-rich repeat containing 8A (LRRC8A) is essential for T lymphocyte development and function

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LRRC8A spans the cell membrane four times and its extracellular C terminus contains 17 LRRs (Sawada et al., 2003; Smits and Kajava, 2004). A 17-yr-old female patient with congenital facial abnormalities, absent B cells, and agammaglobulinemia, but normal numbers of T cells, had a balanced t(9;20)(q33.2;q12) translocation, resulting in the deletion of the C-terminal two-and-a-half LRRs of LRRC8A (91 aa) and the addition of 35 aa derived from an intronic sequence (Sawada et al., 2003). The truncated LRRC8A product was co-expressed with the intact product of the normal LRRC8A allele at comparable levels (Sawada et al., 2003). Reconstitution of irradiated recipient mice with...
protein tyrosine kinase (LCK)–ZAP-70–GAB2–PI3K pathway. Our work demonstrates an essential role for LRRC8A in T cell development and function.

RESULTS

Lrrc8a is widely expressed and LRRC8A is highly expressed on thymocytes compared with other immune cells

Lrrc8a mRNA was detected in all 13 tissues tested (Fig. 1 A). We examined cellular expression of LRRC8A using a rabbit polyclonal antibody to the C-terminal 18-aa-long peptide of LRRCA, and a mAb, 4D10, directed against the region between the second and third putative transmembrane domains (aa 147–262) of LRRC8A. FACS analysis using these two antibodies readily detected LRRC8A on the surface of 293T cells transfected with a vector encoding LRRC8a, but not empty vector (Fig. S1 A), indicating that LRRC8A can be expressed on the cell surface, and that both the N and C termini

syngeneic CD34+ BM progenitors transduced with a retroviral vector overexpressing the mutant LRRC8A resulted in a severe block in B cell development at the pro–B cell to pre–B cell transition and reduced numbers of T cells (Sawada et al., 2003). The phenotype was attributed to the dominant negative effect of the co-expressed mutant LRRC8A allele (Conley, 2003; Sawada et al., 2003). No developmental or functional analysis of the T cells was conducted in these mice, and the expression level of the mutant protein in hematopoietic cells was not documented (Sawada et al., 2003).

To understand the role of LRRCA in the adaptive immune system, we generated Lrrc8a−/− mice that expressed no LRRC8A protein. Unlike the patient, Lrrc8a−/− mice have peripheral B cells and normal immunoglobulin levels but display a severe cell-intrinsic block in thymic development and impaired peripheral T cell function. We demonstrate that thymic epithelial cell (TECs) express ligands for LRRC8A and that LRRCA ligation activates AKT via the lymphocyte-specific protein tyrosine kinase (LCK)–ZAP-70–GAB2–PI3K pathway. Our work demonstrates an essential role for LRRC8A in T cell development and function.

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of the molecule are extracellular, rather than intracellular as has been suggested recently (Abascal and Zardoya, 2012). This conclusion was further supported by the observation that 293T cells transfected with a C-terminally FLAG-tagged LRRC8A demonstrated surface staining with anti-FLAG mAb (Fig. S1 B). FACS analysis using C18 antibody revealed that LRRC8A was expressed on the surface of mouse splenic CD3+ T cells, B220+ B cells, DX5+ NK cells, CD14+ macrophages, and CD11c+ dendritic cells (Fig. 1 B and not depicted). FACS analysis of permeabilized splenic T and B cells revealed that a substantial amount of LRRC8A was intracellular (Fig. 1 B). Thymocytes and B cells in BM expressed surface LRRC8A at all stages of development, except for minimal, if any, expression on pro–B cells (Fig. 1, C and D). Thymocytes at all stages had the highest surface expression of LRRC8A of all immune cells studied. Similar results were obtained for all cell lineages using 4D10 mAb (unpublished data).

**Figure 2. B cell development and function in Lrrc8a−/− mice.** (A and B) FACS analysis (A) and percentage (B) of B cell subpopulation in the BM (Immat.: immature, Recirc.: recirculating). (C–E) Gross appearance and H&E staining (bars, 200 µm; C), numbers of B220+ cells in spleens (D), and FACS analysis of CD21 and CD24 expression by slgM+ cells (E, left), of slgM and CD21 expression by slgM+CD23+ cells (E, middle), and of CD21 and CD23 expression by slgM+ cells (E, right). (F) FACS analysis of peritoneal lavage fluid for IgM+CD5+ B1 cells (top) and for CD5 and CD11b (bottom), within the gated B220+ cell population. (G) 3H-thymidine incorporation purified splenic B cells after anti-IgM, LPS, and anti-CD40 stimulation for 72 h. med.: medium. (H) Serum levels of immunoglobulin isotypes in 4–6-wk-old Lrrc8a−/− mice and WT littermates determined by ELISA. (I) IgM and IgG3 serum antibody levels after immunization with TNP-LPS and TNP-Ficoll. Mice were immunized intraperitoneally with 10 µg TNP-LPS or 10 µg TNP-Ficoll on day 0 and bled on day 14. The level of antigen-specific antibody response in mice sera were analyzed by TNP-specific ELISA using 96-well plates coated with TNP-conjugated BSA at 10 µg/ml in PBS. Data are representative of three independent experiments with one mouse per group (A–C, E, and F), two independent experiments with three mice per group (D and G), six independent experiments with one mouse per group in five experiments and one Lrrc8a−/− mouse and two WT littermates in one experiment (H), and four independent experiments with one mouse per group in two experiments and two mice per group in two experiments (I). ELISAs were run on all samples simultaneously and were repeated twice. Each symbol represent mean OD value of an individual mouse in H. Mean and SEM are shown in B, D, and G–I, * P < 0.05; ** P < 0.01; and *** P < 0.001 (Student’s t test). NS = not significant.
embryos at E14.5 was \( \sim 7.9\% \) (\( n = 38 \)), indicating increased early mortality in utero. \( Lrc8a^{+/−} \) mice had increased postnatal lethality; very few survived beyond 4 wk and none beyond 16 wk (Fig. 1 G). \( Lrc8a^{+/−} \) mice appeared normal at birth, but by the end of the first week of life, they showed persistent growth retardation (Fig. 1, H and I) although they fed normally. \( Lrc8a^{+/−} \) exhibited curly hair, hind limb weakness, progressive hydrolephosisis, and sterility. Histological examination revealed epidermal hyperkeratosis, thin skeletal muscle bundles, vacuolated renal tubular cells, and absence of ovarian corpora lutea (Fig. 1 J). \( Lrc8a^{+/−} \) mice were comparable in appearance, size, and weight to WT littermates (Fig. 1, H and I) and had normal tissue histology (not depicted).

**LRRC8A deficiency modestly impairs B cell development but not function**

BM from \( Lrc8a^{+/−} \) mice had normal cellularity, modestly increased percentage of CD43+ B220\(^{+/−}\) IgM\(^−\) pro–B cells, and modestly decreased percentages of CD43+ B220\(^{+/−}\) IgM\(^−\) pre–B cells, CD43+ B220\(^{+/−}\) IgM\(^−\) immature B cells, and B220\(^{hi}\) IgM\(^{hi}\) recirculating B cells (Fig. 2 A and B). \( Lrc8a^{+/−} \) mice had small spleens with well-preserved architecture (Fig. 2 C). The number of B220\(^{+}\) cells in the spleen was approximately fourfold lower in \( Lrc8a^{+/−} \) mice compared with WT controls (Fig. 2 D). The percentage of splenic B220\(^{+}\)AnnexinV\(^{+}\) cells was comparable in \( Lrc8a^{+/−} \) mice and WT controls (unpublished data). To exclude the potential contribution of extrinsic factors to the B cell lymphopenia in \( Lrc8a^{+/−} \) mice, we examined \( Rag2^{−/−} \) chimeras reconstituted with either \( Lrc8a^{+/−} \) or WT BM cells. Splenic B cell numbers were similarly decreased in \( Lrc8a^{+/−} \)→\( Rag2^{−/−} \) chimeras compared with WT→\( Rag2^{−/−} \) chimeras (11.2 \( \pm \) 1.8 \( \times \) 10\(^6\) versus 47.9 \( \pm \) 3.6 \( \times \) 10\(^6\) cells, \( n = 3 \), \( P < 0.01 \)), indicating that the peripheral B cell lymphopenia in \( Lrc8a^{+/−} \) mice is cell intrinsic.

FACS analysis of splenic B cell subsets (Carsetti et al., 2004) revealed comparable percentages of follicular B cells, but modestly decreased percentages of transitional B cells and marginal zone B cells in \( Lrc8a^{+/−} \) mice compared with WT littermates (Fig. 2 E). The numbers and subset distribution of peritoneal B220\(^{+}\) B cells were normal in \( Lrc8a^{+/−} \) mice (Fig. 2 F).

Splenic B cells from \( Lrc8a^{+/−} \) mice proliferated normally to anti-IgM, anti-CD40, and LPS (Fig. 2 G). Except for a higher level of IgG2a, \( Lrc8a^{+/−} \) mice had normal levels of serum IgM, IgA, and IgG isotypes (Fig. 2 H) and mounted a normal antibody response to the type I T independent (TI) antigen TNP-LPS and the type II TI antigen TNP-Ficoll (Fig. 2 I). These results suggest that LRRC8A plays a minor role in B cell development and is important for peripheral B cell homeostasis but not B cell function.

**LRRC8B deficiency results in decreased thymic cellularity and impaired thymocyte viability**

The thymus was markedly smaller in \( Lrc8a^{+/−} \) mice compared with WT littermates (Fig. 3 A) and had an \( \sim 10\)-fold reduction
in cellularity (Fig. 3 B). Examination of H&E-stained thymus sections demonstrated effacement of the corticomedullary junction and numerous pyknotic and karyorrhectic nuclei in Lm8a<sup>−/−</sup> mice (Fig. 3 C). TdT-mediated dUTP nick end labeling (TUNEL) demonstrated significantly increased numbers of apoptotic cells in Lm8a<sup>−/−</sup> thymi (Fig. 3 D). This was confirmed by the presence of increased numbers of CD3<sup>+</sup> cells that co-stained for activated caspase 3 (Fig. 3 E). These results suggest that LRR.C8A is important for thymocyte survival.

To exclude the effect of environmental factors on T cell development in Lm8a<sup>−/−</sup> mice, we examined thymi from Rag2<sup>−/−</sup> mice reconstituted with either Lm8a<sup>−/−</sup> or WT BM cells. Thymi of Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras were smaller and contained approximately fourfold fewer cells compared with thymi from WT→Rag2<sup>−/−</sup> control chimeras (Fig. 3, H and I). Histological analysis revealed impaired corticomedullary differentiation with increased numbers of karyorrhectic and apoptotic nuclei in thymi from Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras compared with thymi from control chimeras (Fig. 3, H and I).

**Lm8a<sup>−/−</sup> mice have a cell-autonomous early block in thymocyte development**

The distribution of double negative (DN), double positive (DP), and single positive (SP) subsets was comparable between Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> and control chimeras (Fig. 4 A). However, as expected from the reduced thymic cellularity, the numbers of CD4<sup>−</sup>CD8<sup>−</sup> DN, CD4<sup>+</sup>CD8<sup>+</sup> DP, and CD4<sup>+</sup> and CD8<sup>+</sup> SP thymocytes were reduced by approximately threefold in Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras compared with controls (Fig. 4 B). Analysis of DN subsets revealed a significant reduction in the numbers of CD44<sup>+</sup>CD25<sup>−</sup> DN1 cells, CD44<sup>−</sup>CD25<sup>−</sup> DN2, CD44<sup>−</sup>CD25<sup>+</sup> DN3, and CD44<sup>−</sup>CD25<sup>−</sup> DN4 cells in Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras compared with controls (Fig. 4 C). The numbers of CD44<sup>+</sup>CD25<sup>−</sup> DN1 cells were decreased, but not significantly, in thymi from Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras. Irradiation could drive transiently the development of RAG2-deficient thymocytes in a restricted manner generating DP cells that express no surface CD3, but no SP cells, in the absence of donor-derived hematopoietic cells (Zúñiga-Pflücker et al., 1994). The DP and SP cells in the thymi of both chimeras were all CD3<sup>+</sup> (unpublished data).

Furthermore, irradiated Rag2<sup>−/−</sup> mice did not harbor DP or CD3<sup>+</sup> thymocytes when examined at 8 wk (unpublished data). These results indicate that the defect in thymocyte development in Lm8a<sup>−/−</sup> mice is cell intrinsic.

The defect in the development of Lm8a<sup>−/−</sup> thymocytes could be due to increased cell death and/or decreased cell proliferation. The percentage of annexin V<sup>+</sup> cells was significantly increased in Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras (Fig. 4 D), consistent with the increased number of apoptotic nuclei noted by TUNEL staining. In addition, BrdU incorporation in vivo was significantly decreased in thymocytes from Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras compared with controls (Fig. 4 E). Thymocyte proliferation to anti-CD3+IL-2 was significantly decreased Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras compared with control chimeras, but proliferation to PMA+ionomycin was comparable in the two groups (Fig. 4 F). Thus, LRR.C8A expression by thymocytes is essential for their survival and proliferation.

**Lm8a<sup>−/−</sup> mice exhibited a more exaggerated block in thymocyte development than Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras and a substantial decrease in the percentage of DP cells, reflected by a drastic decrease in their number compared with WT controls (Fig. 5, A and B). The decreased percentage of DP thymocytes and the resulting greater reduction in thymocyte numbers in Lm8a<sup>−/−</sup> mice compared with Lm8a<sup>−/−</sup>→Rag2<sup>−/−</sup> chimeras**

Figure 4. Cell-autonomous defect in thymocyte maturation in Lm8a<sup>−/−</sup> mice. (A–C) Representative FACS analysis of thymocytes with the percentage of cells found in each quadrant indicated (A), number of thymocyte subsets (B), and number of DN cell subsets (C) in the chimeras. (D and E) Percentage of annexin V<sup>+</sup> cells (D) and of BrdU<sup>+</sup> cells 3 h after i.p. injection of BrdU (E) in total thymocytes from the chimeras. (F) H<sup>3</sup>-thymidine incorporation in thymocytes from the chimeras in response to medium (med), anti-CD3+IL-2, and PMA+ionomycin (P+I) stimulation after 72 h in culture. Data are representative of three independent experiments with one mouse per group (A–F). Mean and SEM are shown in B–F. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student’s t test). NS = not significant.
chimeras suggest that extrinsic factors exacerbate the cell-intrinsic thymic phenotype in Lrrc8a−/− mice. DP thymocytes are exquisitely sensitive to damage by cytokines and hormones (Screpanti et al., 1989; Cohen, 1992; Ivanov and Nikolich-Zugic, 1998; Gruver and Sempowski, 2008). Serum chemistry profile and levels of TNF and cortisol levels were normal in Lrrc8a−/− mice (unpublished data). As in the Lrrc8a−/− → Rag2−/− chimeras, the numbers of DN2-DN4, but not DN1, cells were significantly lower in Lrrc8a−/− mice than in WT controls (Fig. 5 C). The distribution of DN1a-e subsets, including the DN1a and DN1b early thymic progenitors (ETPs; Porritt et al., 2004), and the percentage of Lin−Sca1+c-Kit+ (LSK) cells in the BM which contain thymic multipotent progenitors (Ikuta and Weissman, 1992; Schwarz and Bhandoola, 2004) were comparable in Lrrc8a−/− and WT mice (Fig. 5, D and E). As in Lrrc8a−/− → Rag2−/− chimeras, the percentage of annexin V+ apoptotic thymocytes was increased and the BrdU incorporation in thymocytes was decreased significantly in Lrrc8a−/− mice compared with WT controls (Fig. 5, F and G). The percentage of TCR-γ/δ cells and the mean fluorescence intensity of the TCR-β chain on phenotypically mature thymocytes were comparable in Lrrc8a−/− mice and WT controls (Fig. 5, H and I). Unexpectedly, Lrrc8a−/− thymi had a significant increase in FOXP3+ regulatory T (T reg) cells by immunohistochemistry staining and flow cytometry (Fig. 5, J and K).

LRRC8A deficiency impairs peripheral T cell expansion and function

Spleens of Lmr8a−/− → Rag2−/− chimeras were smaller (Fig. 6 A) and had an approximately fourfold decrease in the number of CD3+ T cells compared with WT → Rag2−/− control chimeras (Fig. 6 B). The splenic CD4/CD8 ratio was comparable in Lmr8a−/− → Rag2−/− and control chimeras (Fig. 6 C). Lmr8a−/− → Rag2−/− chimeras had a significant decrease in the percentage of splenic CD4−CD62LhiCD44hi T effector memory cells compared with control chimeras, and a compensatory increase in the percentage of CD4−CD62LloCD44lo naive T cells (Fig. 6 D). The proliferation of splenic T cells to immobilized anti-CD3 was significantly impaired in Lmr8a−/− → Rag2−/− chimeras compared with controls and was not increased by the addition of anti-CD28 mAb (Fig. 6 E). T cells from Lmr8a−/− → Rag2−/− chimeras proliferated normally in response to stimulation with PMA and ionomycin, indicating that they do not have a general intrinsic proliferative defect. These results indicate that LRRC8A is important for peripheral T cell expansion and function.

Like Lmr8a−/− → Rag2−/− chimeras, Lmr8a−/− mice had a significant reduction in the number of splenic T cells compared with WT controls, with a normal CD4/CD8 ratio (Fig. 6, F and G). The percentage of splenic CD3+Annexin V− cells was comparable in Lmr8a−/− and WT mice (unpublished data).

Figure 5. Defective thymocyte development in Lrrc8a−/− mice. (A) FACS analysis of CD4 and CD8 expression by thymocytes from a 3-wk-old Lrrc8a−/− mouse and WT littermate. The percentage of cells found in each quadrant is indicated. (B and C) Number of DN, DP, and SP thymocytes (B) and of Lineage-negative DN1, DN2, DN3, and DN4 thymocytes (C) in 3–6-wk-old Lrrc8a−/− mice and WT littermates. (D) FACS analysis of ETPs (DN1a-e) in the thymus. Lineage-negative DN1 thymocytes were resolved into ETP subpopulations by staining with c-Kit and CD24. The percentage of cells found in each gate is indicated. (E) FACS analysis of Lin−Sca1+c-Kit+ cells in the BM. (F and G) Percentage of annexin V− cells (F) and BrdU− cells 3 h after i.p. injection of BrdU (G) in thymocytes from 3–6-wk-old Lrrc8a−/− mice and WT littermates. (H) Percentage of TCR-γ/δ cells in the thymus of Lrrc8a−/− mice and WT controls. Each symbol represents an individual mouse and the small horizontal line indicates the mean. (I) Mean fluorescence intensity (MFI) of surface TCR-β chain expressed on phenotypically mature thymocytes. (J and K) Immunostain of FOXP3+ cells (bars = 200 µm) and its quantitation (J) and percentage of FOXP3+ cells in the CD4+ cell population (K) in thymi from Lrrc8a−/− mice and WT littermates. Data are representative of six independent experiments with one mouse per group (A, B, C, and H), and three independent experiments with one mouse per group (D–G and I–K). Mean and SEM are shown in B, C, F, G, and I–K. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student’s t test). NS = not significant.
LRRC8A is dispensable for the development and function of thymic epithelium

TECs play a critical role in thymic development (Rodewald, 2008). Because Lrcc8a is ubiquitously expressed, we examined TECs from Lrcc8a−/− mice. FACS analysis revealed that the percentages of CD4+CD62L−CD44− naive T cells and CD4+CD62L+CD44+ T effector memory cells (G) and proliferation of T cells (H) from Lrcc8a−/− mice and WT control littersmates. (I) Spectratyping analysis of CD3 diversity of selected TCR-β chains in splenic T cells from a 6-wk-old Lrcc8a−/− mouse and its WT littermate. med = medium. P + I = PMA+ionomycin. Data are representative of three independent experiments with one mouse per group (A–H), and two independent experiments with one mouse per group (I). Mean and SEM are shown in B, D–F, and H, * , P < 0.05; ** , P < 0.001 (Student’s t test). NS = not significant.

However, splenic T cells from Lrcc8a−/− mice, like those from Lmrca8a−/−→Rag2−/− mice, had significantly impaired proliferation to immobilized anti-CD3, which was not increased by the addition of anti-CD28 mAb or IL-2 (Fig. 6 H). Analysis of TCR-β CD3 diversity at 6 wk of age showed partial restriction of the T cell repertoire in Lrcc8a−/− mice compared with age-matched WT littermates, as indicated by skewed distribution for some (~25%), but not all, of the TCR-β families analyzed (Fig. 6 I). The limited restriction of the TCR repertoire in Lmrca8a−/− mice is compatible with an abnormal TCR repertoire selection in the thymus and/or with abnormal clonal expansion/maintenance in the periphery.
architectural abnormalities, mTECs showed signs of maturation with expression of autoimmune regulator (AIRE), Claudin-4, and the ligand for ulex europeus agglutinin 1 (UEA-1; Fig. 7 C). Implantation of fetal thymic tissue from WT and Lrrc8a<sup>−/−</sup> fetal thymi 8 wk after engraftment into the renal subcapsular space of WT recipient mice (bars = 100 µm). Data are representative of three independent experiments with one mouse per group (A–D). Mean and SEM are shown in A. NS = not significant.

Specifically, the grafts demonstrated corticomedullary differentiation with generation of SP T cell residents in the medulla (Fig. 7 D). Thus, LRRC8A is dispensable for the development of TECs and for their ability to support T cell development. However, a role for LRRC8A in dendritic cell–thymocyte interactions cannot be ruled out.

**A ligand for LRRC8A is expressed by TECs and is important for the maturation of DN into DP thymocytes**

We tested the hypothesis that a ligand for LRRC8A is expressed by TECs and is important for thymocyte maturation. Because of the kidney tubule abnormalities in Lrrc8a<sup>−/−</sup> mice, we initially examined whether the human embryonic kidney epithelial cell line 293T expresses an LRRC8A ligand. FACS analysis revealed increased binding of glutathione S-transferase (GST)–LRRC8A₃₄₃₋₈₁₀ fusion protein (GST-LRRC8A) to 293T cells, compared with GST (Fig. 8 A). This binding was specific because it was displaced by MBP-LRRC8A, but not by MBP (Fig. 8 A). Conversely, MBP-LRRC8A bound to 293T cells and was displaced by GST-LRRC8A but not GST (unpublished data). GST-LRRC8A did not bind to splenocytes (Fig. 8 B), further indicating the specificity of its binding to 293T cells. GST-LRRC8A bound to WT CD45<sup>−</sup> TECs, but not CD45<sup>+</sup> thymocytes, including DN, DP, and SP cells (Fig. 8, C and D). Both CD45<sup>+</sup> classII<sup>+</sup>BP1<sup>+</sup> cTECs and CD45<sup>−</sup> classII<sup>−</sup>BP1<sup>−</sup> mTECs bound GST-LRRC8A (Fig. 8 C). These results indicate that an LRRC8A ligand is expressed on non-hematopoietic cells, including TECs.

The BM-derived stromal cell line OP9 stably transfected with the Notch ligand Delta-like 1 (OP9-DL1) supports the differentiation and expansion of DN thymocytes into DP cells in the presence of IL-7 and Flt-3 ligand (Schmitt and Zúñiga-Pflücker, 2002). GST-LRRC8A specifically bound to OP9-DL1 (Fig. 8 E). Addition of GST-LRRC8A, but not GST alone, significantly inhibited the maturation of WT DN thymocytes into DP thymocytes in co-cultures with OP9-DL1 cells in the presence of IL-7 and Flt-3L (Fig. 8, F and G) and resulted in a higher percentage of annexin V<sup>+</sup> apoptotic DN and DP cells (Fig. 8 H). Inhibition of the DN to DP maturation by GST-LRRC8A was dose dependent (Fig. 8 I). These results suggest that interaction of LRRC8A in thymocytes with its ligand on OP9-DL1 cells is important for the in vitro maturation and survival of DN thymocytes into DP thymocytes.

**LRRC8A associates with GRB2, GAB2, and LCK and activates AKT in thymocytes via the LCK–ZAP-70–GAB2–PI3K pathway**

The kinase AKT has been implicated in the survival and proliferation of thymocytes (Chen et al., 2001; Juntilla et al., 2007). Given the increased cell death of LRRC8-deficient thymocytes, we examined whether LRRC8A activates AKT. Cross-linking of LRRC8A with anti-LRRC8A mAb resulted in AKT phosphorylation in WT thymocytes (Fig. 9 A), including DN thymocytes (Fig. 9 B). LRRC8A cross-linking failed to cause AKT phosphorylation in Lrrc8a<sup>−/−</sup> thymocytes (Fig. 9 C), but TCR/CD3 cross-linking caused normal AKT phosphorylation
structurally different SRC kinase inhibitors PP2 and SU6656, and SYK/ZAP-70 inhibitors Piceatannol and R406, but not the MEK1/2 inhibitor GSK1120212, blocked LRRC8A-driven AKT phosphorylation in thymocytes (Fig. 9 J and not depicted). Furthermore, LRRC8A-driven AKT phosphorylation was diminished in Zap70−/− thymocytes (Fig. 9 K).

These results indicate that LRRC8A constitutively associates with the GRB2–GAB2 complex and LCK, and activates AKT via the LCK–ZAP-70–GAB2–PI3K pathway.

We examined whether the lack of LRRC8A impairs AKT phosphorylation in thymocytes. Immunostaining sections of thymi immediately after sacrifice revealed the presence of pAKT throughout the thymus in WT mice, with the subcapsular area giving the highest signal, but less intense pAKT staining in the thymus in Lrrc8a−/− mice (Fig. 10 A). pAKT staining was specific because it was abolished by preincubation with the specific phosphopeptide used for immunization (Fig. 10 B). Compared with WT thymi, Lrrc8a−/− thymi had a reduced percentage of pAKT-positive thymocytes, and a lower pAKT/AKT staining intensity ratio with a normal in these cells. LRRC8A-driven AKT phosphorylation in WT thymocytes was completely inhibited by LY294002 (Fig. 9 A), indicating that it was dependent on PI3 kinase (PI3K).

Both intracellular loops of LRRC8A lack the YXXM binding motif for binding PI3K. Receptors whose intracellular domain lacks this motif activate AKT by associating with the GRB2–GAB2 complex (Gu and Neel, 2003; Caron et al., 2009). GAB2 associates with the SRC kinases and is tyrosine phosphorylated by these kinases and ZAP-70 (Gu and Neel, 2003; Palacios and Weiss, 2007) on Y452. This residue is part of the YXXM motif in GAB2 that recruits the p85 regulatory subunit of PI3K by interacting with its SH2 domain (Nishida et al., 1999; Zhao et al., 1999; Crouin et al., 2001). The first intracellular loop of LRRC8A contains a proline-rich region that could potentially interact with the SH3 domain of the adaptor GRB2 and SRC kinases. LRRC8A was found to be constitutively associated in thymocytes with GRB2, GAB2, and LCK (Fig. 9, D–F). Furthermore, LRRC8A ligation on thymocytes caused tyrosine phosphorylation of GAB2 at residue Y452, LCK, and its substrate ZAP-70 (Fig. 9, G–I). The structurally different SRC kinase inhibitors PP2 and SU6656, and SYK/ZAP-70 inhibitors Piceatannol and R406, but not the MEK1/2 inhibitor GSK1120212, blocked LRRC8A-driven AKT phosphorylation in thymocytes (Fig. 9 J and not depicted). Furthermore, LRRC8A-driven AKT phosphorylation was diminished in Zap70−/− thymocytes (Fig. 9 K). These results indicate that LRRC8A constitutively associates with the GRB2–GAB2 complex and LCK, and activates AKT via the LCK–ZAP-70–GAB2–PI3K pathway.

We examined whether the lack of LRRC8A impairs AKT phosphorylation in thymocytes. Immunostaining sections of thymi fixed immediately after sacrifice revealed the presence of pAKT throughout the thymus in WT mice, with the subcapsular area giving the highest signal, but less intense pAKT staining in the thymus in Lrrc8a−/− mice (Fig. 10 A). pAKT staining was specific because it was abolished by preincubation with the specific phosphopeptide used for immunization (Fig. 10 B). Compared with WT thymi, Lrrc8a−/− thymi had a reduced percentage of pAKT-positive thymocytes, and a lower pAKT/AKT staining intensity ratio with a normal
percentage of AKT-positive thymocytes (Fig. 10, C and D). The decreased AKT phosphorylation in Lm8a−/− thymi is specific because Lm8a−/− and WT thymi had comparable staining for pSTAT3 and STAT3 (Fig. 10, E and F). The percentage of thymocytes that stained with pAKT, but not of those that stained with AKT, was also markedly reduced in thymi from Lm8a−/−→Rag2−/− chimeras compared with those from control chimeras (Fig. 10 G).

**DISCUSSION**

The present study demonstrates that LRRCA8A plays a critical cell-autonomous role in T lymphocyte development and function. The thymus of Lm8a−/−→Rag2−/− chimeras, like that of Lm8a−/− mice, had decreased cellularity, disorganized architecture, increased apoptosis, and decreased proliferation, indicating that these defects are T cell intrinsic. The decreased proliferation of thymocytes from Lm8a−/−→Rag2−/− chimeras to anti–CD3+IL-2, but not to the TCR-independent stimuli PMA+ionomycin, suggests that LRRCA8A signaling contributes to TCR-driven thymocyte proliferation. The numbers of thymocytes at the DN2 stage and beyond were significantly reduced in Lm8a−/−→Rag2−/− chimeras, as in Lm8a−/− mice, indicating that the early block in thymocyte development is cell autonomous. Despite their defective T cell development and function, Lm8a−/−→Rag2−/− chimeras had no increase in mortality, indicating that the running and premature death of Lm8a−/− mice is likely due to their multiple organ abnormalities.

Our studies demonstrate that LRRCA8A activates AKT via the LCK–ZAP-70–GRB2–PI3K pathway. LRRCA8A constitutively associates with the GRB2–GAB2 complex and LCK. These associations may be direct, via interactions between the proline-rich region in the first intracellular domain of LRRCA8A and the SH3 domain of GRB2 and LCK, and/or indirectly via the interaction of GAB2 with GRB2 and LCK (Gu and Neel, 2003). LRRCA8A ligation caused phosphorylation of LCK and its substrate ZAP-70, and of their target GAB2. The recruited PI3K undergoes phosphorylation by LCK–ZAP-70–GAB2–PI3K pathway. LRRCA8A constitutively associates with the GRB2–GAB2 complex and LCK. These associations may be direct, via interactions between the proline-rich region in the first intracellular domain of LRRCA8A and the SH3 domain of GRB2 and LCK, and/or indirectly via the interaction of GAB2 with GRB2 and LCK (Gu and Neel, 2003). LRRCA8A ligation caused phosphorylation of LCK and its substrate ZAP-70, and of their target GAB2. The recruited PI3K undergoes phosphorylation by LCK–ZAP-70–GAB2–PI3K pathway. LRRCA8A constitutively associates with the GRB2–GAB2 complex and LCK. These associations may be direct, via interactions between the proline-rich region in the first intracellular domain of LRRCA8A and the SH3 domain of GRB2 and LCK, and/or indirectly via the interaction of GAB2 with GRB2 and LCK.
Juntilla et al., 2007), the significant reduction in tonic AKT phosphorylation may play an important role in the defective thymic maturation of Lrrc8a−/− mice. Decreased AKT activation could also explain the relative increase in CD4+FOXP3+ cells in thymus of these mice because AKT inhibits the generation of these cells (Haxhinasto et al., 2008; Merkenschlager and von Boehmer, 2010). The block in thymocyte maturation occurs earlier in Lrrc8a−/− mice than in Akt1−/−/Akt2−/− mice, in which the DN3:DN4 transition is blocked (Juntilla et al., 2007). This could be explained by the fact that thymocytes from Akt1−/−/Akt2−/− still express Akt3 (Juntilla et al., 2007) and that LRRCA8 ligation may deliver signals in addition to AKT that are important for thymocyte development and survival.

In addition to Akt, several genes have been implicated in early thymic development. They include Notch 1 and its downstream targets Hes1, Delta, Niap, and Ptcra (Deftos et al., 1998, 2000; Krebs et al., 2001; Lamar et al., 2001), as well as Bcl-2 (Deftos et al., 1998) and Bcl11b (Wakabayashi et al., 2003; Li et al., 2010). qPCR analysis revealed that the expression of these genes was either unaffected, or in a few cases increased, in DN1-DN4 thymocytes from Lrrc8a−/− mice compared with WT littermates (unpublished data). IL-7R signaling is important for the survival of early thymocytes (Peschon et al., 1994; Akashi et al., 1997; Kim et al., 1998). Surface expression of IL-7Rα by thymocyte subpopulations (DN1-4, DP, and SP) was comparable between Lrrc8a−/− mice and controls (unpublished data). These results rule out a role for abnormalities in the above pathways in the thymic developmental block caused by LRRCA8 deficiency.

A ligand for LRRCA8 was detected on TECs and on the stromal cell line OP9. A fusion protein containing GST and the extracellular domain of LRRCA8 inhibited OP9-DL1 cell–dependent maturation of DN cells into DP cells in vitro. This finding, together with the decreased pAKT content of the thymus in Lrrc8a−/− mice, suggests that the putative LRRCA8 ligand expressed by TECs delivers a critical survival signal via AKT to thymocytes. In addition to 293T cells, GST-LRRCA8 bound to keratinocytes and fibroblasts (unpublished data), suggesting that disruption of LRRCA8 interaction with a ligand expressed by epithelial and mesenchymal cells may contribute to the tissue pathology in Lrrc8a−/− mice. Identification of this ligand is currently the subject of investigation.

The reduced number of T cells and decreased percentage of CD4+ T effector memory cells in the spleen of Lrrc8a−/−→Rag2−/− chimeras suggest that cell-intrinsic expression of LRRCA8 in T cells is important for their homeostatic expansion in the periphery. The decreased proliferation of splenic T cells from these chimeras in response to TCR/CD3 ligation, but intact response to PMA+ionomycin, suggest that LRRCA8 delivers a co-stimulatory signal to antigen-activated T cells. Indeed, ligation of LRRCA8 causes AKT activation in normal splenic T cells (unpublished data), as it does in thymocytes. Lack of LRRCA8-driven AKT activation and/or maturation in an abnormal thymic environment may contribute to the decreased homeostatic proliferation and impaired function of LRRCA8-deficient peripheral T cells. Selective deletion of Lrrc8a in mature T cells is needed to distinguish between these two possibilities.
**Role of LRRC8A in the immune system | Kumar et al.**

*Facility at the Harvard Medical School. TUNEL staining of thymic sections tissue histopathology was performed at the Rodent Histopathology Core Histology, immunohistochemistry, and immunofluorescence.*

**Anti-LRRC8A antibodies and immunoblotting.** A polyclonal antibody (C18) was raised in rabbits against a C-terminal 18-aa-long peptide (NH\_2-FSTLPPEVKERLWRADKE-COOH) sequence (aa 791–808) of LRRC8A and purified from using LRRC8A peptide Sepharose column chromatography. An LRRC8A-specific mouse mAb (4D10) was raised against the first extracellular loop (aa 147–262) of LRRC8A using standard protocols and purified from ascites by protein G column chromatography. Cell lysates were immunoblotted using C18 or 4D10 Abs, followed by HRP-conjugated goat anti–rabbit antibody or goat anti–mouse antibody conjugated to horseradish peroxidase–HRP and ECL.

**Histology, immunohistochemistry, and immunofluorescence.** Mouse tissue histopathology was performed at the Rodent Histopathology Core facility at the Harvard Medical School. TUNEL staining of thymic sections was performed as per the instructions of the manufacturer (BD). 2-μm-thick formalin-fixed paraffin-embedded sections were subjected to hematoxylin and eosin staining and immunohistological analysis. In brief, sections were de-waxed, rehydrated, and endogenous peroxidase activity blocked by 0.3% H\_2O\_2 methanol. Heat-induced antigen retrieval was performed when needed. Single immunostains were revealed by Real EnVision rabbit or mouse HRP Labeled Polymer system (Dako) or by preabsorbed biotinylated rabbit anti–rat mouse Ab (1:200; Vector), followed by Strepavidin–SA–HRP conjugated and Diaminobenzidine (DAB; Dako), and nuclei were counterstained with hematoxylin. Bright field double immunostains were performed using Real EnVision Rabbit HRP (Dako) and MACH4 Universal AP Polymer kit (Biocare Medical) for the detection of CD3 and Caspase 3, respectively, and developed by either DAB or Ferangi Blue (DAKO). Nuclei were counterstained with methyl green. Double immunofluorescence analysis has been performed using secondary swine anti–rabbit FITC-conjugated antibody (1:30; Dako) for CK5 and rabbit anti–rat biotinylated antibody (1:200; Vector Laboratories), followed by Strepavidin–Texas red (1:100; Southern-Biotech) for CK8. Sections were then counterstained with DAPI. Digital images were acquired by a DP70 camera (Olympus) mounted on a Bx60 microscope (Olympus), using CellF Imaging software (Soft Imaging System GmbH). The following primary antibodies were used: rabbit anti-caspase 3 active (clone AF835, 1:600; R&D Systems), anti-CD3 (clone 2C11-145, 1:100; Dako), anti–cytokeratin-5 (clone D5/16 B4, 1:100; Covance), anti-AIRE (provided by P. Peterson, University of Tartu, Tartu, Estonia; 1:2,000), anti-pAKT (Ser 473; clone 763E11, 1:30; Cell Signaling Technology), anti-AKT (clone 11E7, 1:100; Cell Signaling Technology), anti-pSTAT3 (Tyr 705; clone D3A7, 1:80; Cell Signaling Technology), STAT3 (clone 7D6, 1:100; Cell Signaling Technology), rat anti-cytokeratin 8 (clone TR0MA-I, 1:200; Developmental Studies Hybridoma Bank), anti-FOXP3 (clone F9, 1:100; Santa Cruz Biotechnology, Inc.), and mouse anti-CL4 (clone 3E2C1, 1:100; Invitrogen). In addition, biotinylated UEA-1 ligand (1:600; Vector Laboratories) was used to detect mature mTECs. The pAKT peptide used for immunization was used as a blocking peptide (Cell Signaling Technology) as a control for pAKT specificity.

**Preparation of cells and flow cytometry.** Single-cell suspensions from BM, thymus, and spleen of 3–6-wk-old mice were prepared as described earlier (de la Fuente et al., 2006). TECs were prepared as described by Gray et al. (2002). Cells were stained with the appropriate fluorochrome-labeled mAbs and analyzed on a FACSCalibur or FACSanto (BD). Fluorescent-labeled or biotinylated monoclonal antibodies to B220 (clone RA3-6B2), CD1 (clone 6C3), CD3e (clone 145-2C11), CD4 (clone L3T4), CD8 (clone 53-6.7), CD11b (clone M1/70), CD11c (clone N418), CD21/23 (clone eBio81D9), CD23 (clone B3B4), CD24 (clone M1/69), CD25 (clone 3C7), CD43 (clone eBioR260), CD44 (clone IM7), CD62L (clone MEL-14), CD71 (clone R17217), CD127 (clone A7R34), c-kit (clone 2B8), FOXP3 (clone 23C1, 1:100; Invitrogen). In addition, biotinylated UEA-1 ligand (1:600; Vector Laboratories) was used to detect mature mTECs. The pAKT peptide used for immunization was used as a blocking peptide (Cell Signaling Technology) as a control for pAKT specificity.
in TBS tissue-freezing liquid (Triangular Biomedical Sciences), and stored at −80°C. Sections of 30-µm thickness were mounted on Superfrost Plus slides (Thermo Fisher Scientific) and stained with fluorescent mouse CD4 (GK1.5, mouse CD8α (53–67) antibodies (BD), and UEA-1 in a humidified chamber after Fc-receptor blockade with 1 µg/ml antibody 2.4G2 (Bio X Cell). Samples were mounted in FluorSave reagent solution (EMD Millipore) and stored at 4°C until analysis. Images were collected with a confocal microscopy system (Bio-Rad Laboratories) using a microscope (BX50WI; Olympus) and 10×/0.4 numerical aperture or 60×/1.2 numerical aperture water-immersion objective lenses. Images were analyzed with LaserSharp2000 software (Bio-Rad Laboratories), Velocity (Perkin Elmer), and Imaris (Bitplane).

Proliferation and activation of thymocytes and splenic T and B cells.
Thymocytes were prepared as described earlier (de la Fuente et al., 2006). Stained T and B cells were purified by negative selection using kits from Miltenyi Biotec. Thymocytes and purified T cells were cultured in medium alone or in wells coated with 2 µg/ml anti-CD3 monoclonal antibody (clone KT3; Abcam) with or without 2 µg/ml anti-CD28 (clone L293; BD) or 40 ng/ml IL-2 (PeproTech). PMA (Sigma-Aldrich) was used at 50 ng/ml, and ionomycin (Sigma-Aldrich) was used at 0.5 µM. Purified B cells were cultured in medium alone or in the presence of goat Fab(‘)2 mouse–IgM (Jackson Immunoresearch Laboratories, Inc.), 2 µg/ml anti-CD40 (R&D Systems), or 10 µg/ml LPS (Sigma-Aldrich). 72 h later, the cells were pulsed with 1 µCi [3H]thymidine and counted.

TCR-Vβ repertoire analysis.
TCR-Vβ repertoire clonality determination on splenic T cells was performed using spectratyping by BioMed Immunotech, Inc.

Generation of LRRC8A fusion proteins and analysis of binding of LRRC8A fusion protein to cells.
LRRC8A C-terminal polypeptide (aa 343–810) was fused downstream of GST and MBP (maltose binding protein) in pGEX-4T1 (GE Healthcare) and pMAL-c2G (New England Biolabs, Inc.) expression vectors, respectively. Fusion proteins were expressed in recombinant bacterial hosts. Expressed GST-LRRC8A and MBP-LRRC8A fusion proteins were purified as per the manufacturer’s instructions. Target cells were incubated with GST/GST-LRRC8A or MBP/MBP-LRRC8A on ice for 30 min. The binding of LRRC8A fusion proteins was detected using flow cytometry by staining cells with the appropriate fluorochrome-labeled anti-tag antibody (anti-GST antibody [clone 26H1; Cell Signaling Technology] or anti-MBP antibody [clone MBP-17; Sigma-Aldrich]).

In vitro maturation of DN to DP thymocytes.
Purified DN thymocytes were cultured on monolayers of OP-9-DL1 cells (gift from J.C. Zúñiga-Pflücker, University of Toronto, Toronto, Canada) in recombinant IL-7 and Flt3L (PeproTech) with or without goat F(ab’2) antibody (Jackson Immunoresearch Laboratories, Inc.) or anti-MBP antibody [clone MBP-17; Sigma-Aldrich].

LRRCA8A signaling studies.
Cell stimulation, purified cells were incubated with the respective antibodies on ice for 20 min and cross-linked with Fab(‘)2 fragments of appropriate secondary antibodies for indicated time points at 37°C. Immediately after stimulation, the cells were lysed in SDS sample buffer by adding one-fourth volume of 5× SDS lysis buffer directly into the cell suspensions. Samples were boiled for 5 min and separated by 4–15% SDS-PAGE and evaluated by immunoblotting using anti-pAKT (clone DE9, 1:1,000; Cell Signaling Technology), anti-AKT (clone 11E7, 1:1,000; Cell Signaling Technology), anti-pGAB2 (Y10, 1:1,000; Cell Signaling Technology), anti-pSRC (Y416, clone D494G, 1:1,000; Cell Signaling Technology), anti-p-AKT (clone 11E7, 1:1,000; Cell Signaling Technology), or anti-GRB2 (clone c-23; Santa Cruz Biotechnology, Inc.) antibody. PP2 and Su6656 (SRC inhibitors) were purchased from EMD Millipore. Pecstatin and R406 (SYK inhibitors) were purchased from Selleckchem Chemicals, LLC. Wortmannin and Ly294002 (PI3K inhibitors) were purchased from Sigma-Aldrich. GSK1120212 (MEK1/2 inhibitor) was purchased from BioVision Technology, Inc. LRRCA8A and LCK immunoprecipitations were performed as described previously (de la Fuente et al., 2006) using anti-LRRCA8A antibodies and anti-LCK mouse monoclonal antibody (clone 3AS; Santa Cruz Biotechnology, Inc.), respectively. Cell activation marker expression, thymocytes, and splenic B and T cell proliferation and cytokine production assays were performed as described previously (de la Fuente et al., 2006).

Serum immunoglobulins and antibody measurements.
Serum immunoglobulins and antibody levels were determined by previously described methods. Mice were immunized with KLH prepared with Inject Alum (Thermo Fisher Scientific), TNP-Ficoll, or TNP-LPS (Bio-Rad Laboratories). ELISA assays were performed to estimate specific immunoglobulins levels in the serum samples of the immunized mice as earlier (de la Fuente et al., 2006).

Quantitative RT-PCR.
Total RNA was prepared from the flow cytometrically–sorted WT and KO DN1–4 thymocytes using the RNeAqueous extraction kit (Ambion). RT-PCR was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Carboxyfluorescein (FAM)–labeled specific TaqMan primers were purchased from Applied Biosystems. Quantitative PCR reactions were run on an ABI Prism 7300 sequence detection system platform (Applied Biosystems). The housekeeping gene β2-microglobulin was used as a control. The relative gene expression among the different samples was determined using the method described by Pfaffl (2001). Quantities of all targets in test samples were normalized to the corresponding β2-microglobulin levels.

Statistical analysis.
Statistical analysis of the data using the Student’s t test or analysis of variance (ANOVA) was performed with Prism software (GraphPad Software Inc.).

Online supplemental material.
Fig. S1 shows surface expression of LRRC8A in untransfected and LRRC8A–transfected 293T cells by FACS using LRRC8A and FLAG tag–specific antibodies and the strategy to generate Lnr8a−/− mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131379/DC1.

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