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

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LRRC8A is a ubiquitously expressed gene that encodes a leucine-rich repeat (LRR)–containing protein detected at higher levels on the surface of thymocytes than on other immune cells. We generated Lrrc8a−/− mice to investigate the role of LRRC8A in lymphocyte development and function. Lrrc8a−/− mice had increased prenatal and postnatal mortality, growth retardation, and multiple tissue abnormalities. Lrrc8a−/− mice displayed a modest block in B cell development but intact intrinsic B cell function. In contrast, both Lrrc8a−/− mice and Lrrc8a−/− → Rag2−/− bone marrow chimeras exhibited a severe cell–intrinsic block in early thymic development, with decreased proliferation and increased apoptosis of thymocytes, and impaired peripheral T cell function. Thymic epithelial cells expressed an LRRC8A ligand that was critical for double-negative to double-positive thymocyte differentiation and survival in vitro. LRRC8A constitutively associated with the GRB2–GAB2 complex and lymphocyte–specific protein tyrosine kinase (LCK) in thymocytes. LRRC8A ligation activated AKT via the LCK–ZAP–70–GAB2–PI3K pathway, and AKT phosphorylation was markedly reduced in the thymus of Lrrc8a−/− mice. These findings reveal an essential role for LRRC8A in T cell development, survival, and function.
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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 LRRCA8A, 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

protein tyrosine kinase (LCK)–ZAP-70–GAB2–PI3K pathway. Our work demonstrates an essential role for LRRC8A in T cell development and function.

Figure 1. Expression of LRRC8A in C57BL/6 mice and survival, morphology, and tissue histology of Lrrc8a−/− mice. (A) Q-PCR analysis of Lrc8a mRNA expression in tissues. Lrc8a mRNA levels are expressed relative to Gapdh mRNA levels. (B) FACS analysis of LRRC8A surface and intracellular expression on electronically gated splenic CD3+ cells B220+ cells using polyclonal antibody C18. Perm: permeabilized. (C and D) FACS analysis of LRRC8A surface expression by subpopulations of thymocytes (C) and BM B cells (D) using polyclonal antibody C18. (E) FACS analysis of LRRC8A expression on gated splenic CD3+ cells B220+ cells from Lrc8a−/− mice and WT littermates. (F) Frequency of WT, Lrc8a+/−, and Lrc8a−/− pups obtained from the matings of Lrc8a−/− mice (n = 622 pups). (G) Kaplan-Meier analysis of survival of 120 F2 offspring born from matings of Lrc8a−/− mice, which included 8 Lrc8a−/−, 72 Lrc8a+/−, and 40 Lrc8a+/+ littermates. (H) Body weight of Lrc8a−/− mice and of Lrc8a+/− and WT littermates [6 mice per group]. (I) Gross appearance of WT, Lrc8a−/−, and Lrc8a+/− mice at 5 wk of age. (J) Representative H&E-stained tissue sections from skin, skeletal muscle, and ovary (bars, 200 µm), and kidney (bars, 100 µm) of Lrc8a−/− mice and WT littermates. FC: follicle, CL: corpus luteum. Data are representative of three independent experiments with one mouse per group (A, C, and D), six independent experiments with one mouse per group (B and E), two independent experiments with 3 mice per group (H and I), and two independent experiments with two mice per group (J). Mean and SEM are shown in A and H. ***, P < 0.001 (Student’s t test).

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 LRRCA8A in the adaptive immune system, we generated Lrrc8a−/− mice that expressed no LRRC8A protein. Unlike the patient, Lrc8a−/− 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 LRRC8A ligation activates AKT via the lymphocyte-specific
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).

**Generation and characterization of Lrrc8a−/− mice**

The strategy for generating Lrrc8a−/− mice is depicted in Fig. S1 (C–F). LRRC8A was not detectable by immunoblotting of cell lysates from Lrrc8a−/− mice (Fig. S1 G) or by FACS analysis of splenic T and B cells from these mice (Fig. 1 E). Lrrc8a−/− mice were bred for 10 generations on the C57BL/6 background. Similar findings were obtained in Lrrc8a−/− mice generated from two independently targeted ES clones. The frequency of live Lrrc8a−/− pups obtained from mating Lrrc8a+/− mice was 5.5% (Fig. 1 F). The frequency of Lrrc8a−/−...
embryos at E14.5 was \(~\sim 7.9\%\) \((n = 38)\), indicating increased early mortality in utero. \(Lrrc8a^{−/−}\) mice had increased postnatal lethality; very few survived beyond 4 wk and none beyond 16 wk (Fig. 1 G). \(Lrrc8a^{−/−}\) 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. \(Lrrc8a^{−/−}\) exhibited curly hair, hind limb weakness, progressive hydronephrosis, and absence of ovarian corpora lutea (Fig. 1 J). \(Lrrc8a^{−/−}\) 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 \(Lrrc8a^{−/−}\) 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^- recirculating B cells (Fig. 2 A and B). \(Lrrc8a^{−/−}\) mice had small spleens with well-preserved architecture (Fig. 2 C). The number of B220^+ cells in the spleen was approximately fourfold lower in \(Lrrc8a^{−/−}\) mice compared with WT controls (Fig. 2 D). The percentage of splenic B220^+ AnnexinV^- cells was comparable in \(Lrrc8a^{−/−}\) mice and WT controls (unpublished data). To exclude the potential contribution of extrinsic factors to the B cell lymphopenia in \(Lrrc8a^{−/−}\) mice, we examined \(Rag2^{−/−}\) chimeras reconstituted with either \(Lrrc8a^{−/−}\) or WT BM cells. Splenic B cell numbers were similarly decreased in \(Lrrc8a^{−/−}\rightarrow Rag2^{−/−}\) chimeras compared with WT \(→\)Rag2^{−/−} chimeras (11.2 ± 1.8 \times 10^6 versus 47.9 ± 3.6 \times 10^6 cells, \(n = 3\), \(P < 0.01\)), indicating that the peripheral B cell lymphopenia in \(Lrrc8a^{−/−}\) 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 \(Lrrc8a^{−/−}\) mice compared with WT littermates (Fig. 2 E). The numbers and subset distribution of peritoneal B220^+ B cells were normal in \(Lrrc8a^{−/−}\) mice (Fig. 2 F).

Splenic B cells from \(Lrrc8a^{−/−}\) mice proliferated normally to anti-IgM, anti-CD40, and LPS (Fig. 2 G). Except for a higher level of IgG2a, \(Lrrc8a^{−/−}\) 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 T 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.

**LRRC8 deficiency results in decreased thymic cellularity and impaired thymocyte viability**
The thymus was markedly smaller in \(Lrrc8a^{−/−}\) 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 Lrn8a−/− mice (Fig. 3 C). TdT-mediated dUTP nick end labeling (TUNEL) demonstrated significantly increased numbers of apoptotic cells in Lrn8a−/− thymi (Fig. 3 D). This was confirmed by the presence of increased numbers of CD3+ cells that co-stained for activated caspase 3 (Fig. 3 E). These results suggest that LRRCA is important for thymocyte survival.

To exclude the effect of environmental factors on T cell development in Lrn8a−/− mice, we examined thymi from Rag2−/− mice reconstituted with either Lrn8a−/− or WT BM cells. Thymi of Lrn8a−/−→Rag2−/− chimeras were smaller and contained approximately fourfold fewer cells compared with thymi from WT→Rag2−/− control chimeras (Fig. 3, H and I). Histological analysis revealed impaired corticomedullary differentiation with increased numbers of karyorrhectic and apoptotic nuclei in thymi from Lrn8a−/−→Rag2−/− chimeras compared with thymi from control chimeras (Fig. 3, H and I).

**Lrn8a−/− 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 Lrn8a−/−→Rag2−/− and control chimeras (Fig. 4 A). However, as expected from the reduced thymic cellularity, the numbers of CD4+CD8− DN, CD4+CD8+ DP, and CD4+ and CD8+ SP thymocytes were reduced by approximately threefold in Lrn8a−/−→Rag2−/− chimeras compared with controls (Fig. 4 B). Analysis of DN subsets revealed a significant reduction in the numbers of CD44+CD25− DN1, CD44−CD25+ DN3, and CD44−CD25− DN4 cells in Lrn8a−/−→Rag2−/− chimeras compared with controls (Fig. 4 C). The numbers of CD44+CD25− DN1 cells were decreased, but not significantly, in thymi from Lrn8a−/−→WT chimeras. Irradiation can 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+ (unpublished data). Furthermore, irradiated Rag2−/− mice did not harbor DP or CD3+ thymocytes when examined at 8 wk (unpublished data). These results indicate that the defect in thymocyte development in Lrn8a−/− mice is cell intrinsic.

The defect in the development of Lrn8a−/− thymocytes could be due to increased cell death and/or decreased cell proliferation. The percentage of annexin V+ cells was significantly increased in Lrn8a−/−→Rag2−/− 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 Lrn8a−/−→Rag2−/− chimeras compared with controls (Fig. 4 E). Thymocyte proliferation to anti-CD3+IL-2 was significantly decreased Lrn8a−/−→Rag2−/− chimeras compared with control chimeras, but proliferation to PMA+ionomycin was comparable in the two groups (Fig. 4 F). Thus, LRRCA expression by thymocytes is essential for their survival and proliferation.

Lrn8a−/− mice exhibited a more exaggerated block in thymocyte development than Lrn8a−/−→Rag2−/− 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 Lrn8a−/− mice compared with Lrn8a−/−→Rag2−/−

![Figure 4](image-url)
chimeras suggest that extrinsic factors exacerbate the cell-intrinsic thymic phenotype in \textit{Lrrc8a}−/− mice. DP thymocytes are exclusively sensitive to damage by cytokines and hormones (Screpanti et al., 1989; Cohen, 1992; Ivanov and Nikolić-Zugić, 1998; Gruver and Sempowski, 2008). Serum chemistry profile and levels of TNF and cortisol levels were normal in \textit{Lrrc8a}−/− mice (unpublished data). As in the \textit{Lrrc8a}−/−→\textit{Rag}2−/− chimeras, the numbers of DN2-DN4, but not DN1, cells were significantly lower in \textit{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 \textit{Lrrc8a}−/− and WT mice (Fig. 5, D and E). As in \textit{Lrrc8a}−/−→\textit{Rag}2−/− chimeras, the percentage of annexin V+ apoptotic thymocytes was increased and the BrdU incorporation in thymocytes was decreased significantly in \textit{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 \textit{Lrrc8a}−/− mice and WT controls (Fig. 5, H and I). Unexpectedly, \textit{Lrrc8a}−/− thymus was comparable in \textit{Lrrc8a}−/− mice compared with WT controls, with a normal CD4/CD8 ratio (Fig. 6, F and G). The proliferation of splenic T cells to immobilized anti-CD3 was significantly impaired in \textit{Lrrc8a}−/−→\textit{Rag}2−/− chimeras compared with controls and was not increased by the addition of anti-CD28 mAb (Fig. 6 E). T cells from \textit{Lrrc8a}−/−→\textit{Rag}2−/− 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 \textit{Lrrc8A} is important for peripheral T cell expansion and function.

Like \textit{Lmr8a}−/−→\textit{Rag}2−/− chimeras, \textit{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 \textit{Lrrc8a}−/− and WT mice (unpublished data).

\textbf{LRRC8A deficiency impairs peripheral T cell expansion and function}

Spleens of \textit{Lmr8a}−/−→\textit{Rag}2−/− chimeras were smaller (Fig. 6 A) and had an approximately fourfold decrease in the number of CD3+ T cells compared with WT→\textit{Rag}2−/− control chimeras (Fig. 6 B). The splenic CD4/CD8 ratio was comparable in \textit{Lmr8a}−/−→\textit{Rag}2−/− and control chimeras (Fig. 6 C). \textit{Lmr8a}−/−→\textit{Rag}2−/− chimeras had a significant decrease in the percentage of splenic CD4+CD62L+CD44+T effector memory cells compared with control chimeras, and a compensatory increase in the percentage of CD4+CD62L−CD44+ naive T cells (Fig. 6 D). The proliferation of splenic T cells to immobilized anti-CD3 was significantly impaired in \textit{Lmr8a}−/−→\textit{Rag}2−/− chimeras compared with controls and was not increased by the addition of anti-CD28 mAb (Fig. 6 E). T cells from \textit{Lmr8a}−/−→\textit{Rag}2−/− 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 \textit{Lmr8A} is important for peripheral T cell expansion and function.

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\textbf{Figure 5. Defective thymocyte development in \textit{Lrrc8a}−/− mice.} (A) FACS analysis of CD4 and CD8 expression by thymocytes from a 3 wk-old \textit{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 \textit{Lrrc8a}−/− mice and WT controls. Each symbol represents an individual mouse and the small horizontal line indicates the mean. (D) 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 \textit{Lrrc8a}−/− mice and WT littermates. (H) Percentage of TCR−γ/δ cells in the thymus of \textit{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 chain on phenotypically mature thymocytes were comparable in \textit{Lrrc8a}−/− mice and WT littermates. (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 \textit{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 (D), and proliferation of T cells (E) from spleens of Lrcc8a−/− → Rag2−/− and control WT → Rag2−/− chimeras. FACS analysis of T cell numbers (F), and proliferation of splenic T cells (H) from Lrcc8a−/− mice and WT control littermates. Spectratyping analysis of CD3 diversity of selected TCR-Vβ families in splenic T cells from a 6-wk-old Lrcc8a−/− mouse and its WT littermate. 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 Lrcc8a−/− → Rag2−/− chimeras, 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-Vβ CDR3 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-Vβ families analyzed (Fig. 6 I). The limited restriction of the TCR repertoire in Lrcc8a−/− mice is compatible with an abnormal TCR repertoire selection in the thymus and/or with abnormal clonal expansion/maintenance in the periphery.

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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 cell line 293T expresses an LRRC8A ligand. FACS analysis revealed increased binding of glutathione S-transferase (GST)–LRRC8A<sub>343-810</sub> 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 (Flt3L; 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

Figure 7. LRRC8A is dispensable for the development and function of thymic epithelium. (A) FACS analysis of MHC class II and BP-1 expression by CD45<sup>-</sup> cells. Numbers represent the percentage of cells. (B) Immunofluorescence staining of thymic sections (scale bars = 100 µm) for the cTEC antigen CK8, the mTEC antigen CK5, and the nuclear marker DAPI. (C) Expression of AIRE, claudin-4, and UEA-1 in thymi from Lrc8a<sup>-/-</sup> and WT littermates (scale bars = 50 µm). (D) Immunofluorescence staining of WT and Lrc8a<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.
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 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
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The present study demonstrates that LRRC8A plays a critical role in T lymphocyte development and function. The thymus of *Lrrc8a*−/− mice is specifically due to their multiple organ abnormalities. Our studies demonstrate that LRRC8A activates AKT via the LCK–ZAP-70–GRB2–PI3K pathway. LRRC8A 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 LRRC8A and the SH3 domain of GRB2 and LCK, and/or indirectly via the interaction of GAB2 with GRB2 and LCK (Gu and Neel, 2003). LRRC8A ligation caused phosphorylation of GRB2 and its substrate GAB2, and LCK immunoprecipitates were immunoblotted for GRB2, and GAB2 and LCK immunoprecipitates were immunoblotted for LRRC8A. Isotype-matched irrelevant antibodies were used in immunoprecipitation as controls. (G–I) Co-immunoprecipitation of LRRC8A with GRB2 (G), GAB2 (H), and ZAP-70 (I) after LRRC8A ligation on thymocytes. (J) AKT phosphorylation after LRRC8A ligation of thymocytes pretreated with the SRC kinase inhibitor PP2, the MEK1/2 inhibitor GSK1120212, and the SYK/ZAP-70 inhibitor Piceatannol. (K) LRRC8A- and TCR/CD3-driven AKT phosphorylation in thymocytes from WT or *Zap70−/−* mice after LRRC8A or TCR/CD3 ligation. Data are representative of three independent experiments with cells derived from one mouse per experiment (A, B, and D–J), and two independent experiments with cells derived from one mouse per group (C and K). The numbers below the blots in A, C, and J represent the mean ratio of pAKT/AKT in two (C) and three (A and J) experiments.

**DISCUSSION**

The present study demonstrates that LRRC8A plays a critical cell-autonomous role in T lymphocyte development and function. The thymus of *Lrrc8a*−/− mice has decreased cellularity, disorganized architecture, increased apoptosis, and decreased proliferation, indicating that these defects are T cell intrinsic. The decreased proliferation of thymocytes from *Lrrc8a*−/−→*Rag2*−/− chimeras to anti–CD3+IL-2, but not to the TCR-independent stimuli PMA+ionomycin, suggests that LRRC8A signaling contributes to TCR-driven thymocyte proliferation. The numbers of thymocytes at the DN2 stage and beyond were significantly reduced in *Lrrc8a*−/−→*Rag2*−/− chimeras, as in *Lrrc8a*−/− mice, indicating that the early block in thymocyte development is cell autonomous. Despite their defective T cell development and function, *Lrrc8a*−/−→*Rag2*−/− chimeras had no increase in mortality, indicating that the runting and premature death of *Lrc8a*−/− mice is likely due to their multiple organ abnormalities.

Our studies demonstrate that LRRC8A activates AKT via the LCK–ZAP-70–GRB2–PI3K pathway. LRRC8A 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 LRRC8A and the SH3 domain of GRB2 and LCK, and/or indirectly via the interaction of GAB2 with GRB2 and LCK (Gu and Neel, 2003). LRRC8A ligation caused phosphorylation of GRB2 and its substrate GAB2, and LCK immunoprecipitates were immunoblotted for GRB2, and GAB2 and LCK immunoprecipitates were immunoblotted for LRRC8A. Isotype-matched irrelevant antibodies were used in immunoprecipitation as controls. (G–I) Co-immunoprecipitation of LRRC8A with GRB2 (G), GAB2 (H), and ZAP-70 (I) after LRRC8A ligation on thymocytes. (J) AKT phosphorylation after LRRC8A ligation of thymocytes pretreated with the SRC kinase inhibitor PP2, the MEK1/2 inhibitor GSK1120212, and the SYK/ZAP-70 inhibitor Piceatannol. (K) LRRC8A- and TCR/CD3-driven AKT phosphorylation in thymocytes from WT or *Zap70−/−* mice after LRRC8A or TCR/CD3 ligation. Data are representative of three independent experiments with cells derived from one mouse per experiment (A, B, and D–J), and two independent experiments with cells derived from one mouse per group (C and K). The numbers below the blots in A, C, and J represent the mean ratio of pAKT/AKT in two (C) and three (A and J) experiments.

Percentage of AKT-positive thymocytes (Fig. 10, C and D). The decreased AKT phosphorylation in *Lrrc8a*−/−→*Rag2*−/− thymus is specific because *Lrrc8a*−/− 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 *Lrrc8a*−/−→*Rag2*−/− chimeras compared with those from control chimeras (Fig. 10 G).
Juntilla et al., 2007), the significant reduction in tonic AKT phosphorylation may play an important role in the defective thymic maturation of Lrrc8a<sup>−/−</sup> mice. Decreased AKT activation could also explain the relative increase in CD4<sup>+</sup>FOXP3<sup>+</sup> cells in thymus of these mice because AKT inhibits the generation of these cells (Flaxhina et al., 2008; Merkenschlager and von Boehmer, 2010). The block in thymocyte maturation occurs earlier in Lrrc8a<sup>−/−</sup> mice than in Akt<sup>1<sup>−/−</sup>/Akt2<sup>−/−</sup></sup> mice, in which the DN3:DN4 transition is blocked (Juntilla et al., 2007). This could be explained by the fact that thymocytes from Akt<sup>1<sup>−/−</sup>/Akt2<sup>−/−</sup></sup> still express Akt3 (Juntilla et al., 2007) and that LRRC8A 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, Deltox, Nkap, and pTCRα (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<sup>−/−</sup> 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<sup>−/−</sup> mice and controls (unpublished data). These results rule out a role for abnormalities in the above pathways in the thymic developmental block caused by LRRC8A deficiency.

A ligand for LRRC8A was detected on TECs and on the stromal cell line OP9. A fusion protein containing GST and the extracellular domain of LRRC8A 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<sup>−/−</sup> mice, suggests that the putative LRRC8A ligand expressed by TECs delivers a critical survival signal via AKT to thymocytes. In addition to 293T cells, GST-LRRC8A bound to keratinocytes and fibroblasts (unpublished data), suggesting that disruption of LRRC8A interaction with a ligand expressed by epithelial and mesenchymal cells may contribute to the tissue pathology in Lrrc8a<sup>−/−</sup> mice. Identification of this ligand is currently the subject of investigation.

The reduced number of T cells and decreased percentage of CD4<sup>+</sup>T effector memory cells in the spleen of Lrrc8a<sup>−/−</sup>→ Rag2<sup>−/−</sup> chimeras suggest that cell-intrinsic expression of LRRC8A 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 LRRC8A delivers a co-stimulatory signal to antigen-activated T cells. Indeed, ligation of LRRC8A causes AKT activation in normal splenic T cells (unpublished data), as it does in thymocytes. Lack of LRRC8A-driven AKT activation and/or maturation in an abnormal thymic environment may contribute to the decreased homeostatic proliferation and impaired function of LRRC8A-deficient peripheral T cells. Selective deletion of Lrrc8a in mature T cells is needed to distinguish between these two possibilities.
**Lm***8a<sup>−/−</sup> mice had a modest block in B cell development and normal B cell function. However, **Lm***8a<sup>−/−</sup> and **Lm***8a<sup>−/−→Rag2<sup>−/−</sup></sup> chimeras had a fourfold decrease in splenic B cells, suggesting that LRRCA8A is important for peripheral B cell homeostasis. Ligation of LRRCA8A caused AKT phosphorylation in B cells (unpublished data), and AKT is important for maintaining normal numbers of peripheral B cells (Juntila et al., 2007). Thus, loss of LRRCA8A–mediated AKT activation in B cells may have contributed to the peripheral B cell lymphopenia in **Lm***8a<sup>−/−</sup> mice.

In contrast to the **Lm***8a<sup>−/−</sup> mouse, the patient with the heterozygous LRRCA8A mutation had no circulating B cells and agammaglobulinemia but normal numbers of circulating T cells (Sawada et al., 2003). The function of these T cells was not tested, but no opportunistic infections were reported in the patient despite an age of 17 yr. The difference in the two phenotypes most likely reflects the difference between the presence of a truncated mutant protein in the patient, which is thought to have acted as dominant negative (Conley, 2003; Sawada et al., 2003), and the complete absence of the protein in the knockout mouse. Given the 99.9% sequence homology between human and mouse LRRCA8A, loss of LRRCA8A expression in humans would likely present as severe combined immunodeficiency associated with multiple organ abnormalities.

### MATERIALS AND METHODS

**Generation of **Lm***8a<sup>−/−</sup> mice.** We designed a gene–targeting construct for replacing the exon 3, which encodes the first 719 aa of LRRCA8A. DNA fragments 4,809 and 3,375 bp in length were PCR amplified from a BAC clone DNA encoding the entire **Lm***8a gene (RP23-31SH12) and cloned 5′ and 3′ in the pLNTK gene targeting vector. The linearized targeting construct was then electroporated into CJ7 ES cells, which were then selected in medium containing 0.4 mg/ml G418 and 10 mg/ml Gancyclovir. Of the three ES clones identified with targeted deletion of one of the two alleles of **Lm***8a, two were injected into C57BL/6 blastocysts for the generation of chimeric mice. ES cell clones and mice were genotyped by Southern blot analysis.

**Generation of **Lrrc8a<sup>−/−</sup> mice and **Lrrc8a<sup>−/−→EII Cre</sup> chimeric mice.** ES cell clones and mice were genotyped by Southern blot analysis. The LoxP-flanked **Lrrc8a**<sup>−/−</sup> was then electroporated into CJ7 ES cells, which were then selected in medium containing 0.4 mg/ml G418 and 10 mg/ml Gancyclovir. Of the 11 ES cell clones with targeted deletion, two were injected into C57BL/6 blastocysts for the generation of chimeric mice. ES cell clones and mice were genotyped by Southern blot analysis. The Animal Care and Use Committee of the Boston Children’s Hospital. All experiments used 4–6-wk-old **Lm***8a<sup>−/−</sup> mice and WT littermates. Due to the limited availability of **Lm***8a<sup>−/−</sup> mice and high pre- and postnatal mortality, many experiments were performed with one **Lm***8a<sup>−/−</sup> and one WT littermate and were repeated at least three times.

**Anti-LRRCA8A antibodies and immunoblotting.** A polyclonal antibody (C18) was raised in rabbits against a C-terminal 18-aa-long peptide (NH<sub>2</sub>-FSTLPVEKRWRADKE-COOH) sequence (aa 791–808) of LRRCA8A and purified from using LRRCA8A peptide Sepharose column chromatography. An LRRCA8A–specific mouse mAb (4D10) was raised against the first extracellular loop (aa 147–262) of LRRCA8A 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 immunohistochemical analysis. In brief, sections were dehydrated, rehydrated, and endogenous peroxidase activity blocked by 0.3% H<sub>2</sub>O<sub>2</sub> 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 antibody (1:200; Vector), followed by Streptavidin–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 Ferang Blue (DAKO). Nuclei were counterstained with methyl green. Double immunofluorescence analysis was 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 Streptavidin–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 736E11, 1:30; Cell Signaling Technology), anti-AKT (clone 11E7, 1:100; Cell Signaling Technology), anti-pSTAT3 (705; clone D3A7, 1:80; Cell Signaling Technology), STAT3 (clone 70D7, 1:100; Cell Signaling Technology), rat anti–cytokeratin 8 (clone TRKOM-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 FACSCanto (BD). Fluorescent-labeled or biotinylated monoclonal antibodies to B220 (clone RA3-682, BP-1 clone 6C3), CD3e (clone 145-2C11), CD4 (clone L3T4), CD8 (clone 53–6.7), CD11b (clone M1/70), CD11c (clone N418), CD21/23 (clone eB08109), 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 FJK-16G), IgD (clone 11-26C3), IgM (clone EB121–15P9), Sca-1 (clone D7), Ter-119 (clone Ter119), and Thy-1.2 (clone 30-H12) were purchased from BioSource. Anti–T–CR–β (clone H57-597) was purchased from BD. Lineage-negative cells were identified by excluding cells stained with single fluorochrome-labeled cocktail of biotinylated B220 (clone RA3-682), CD3e (clone 145-2C11), CD4 (clone L3T4), CD8 (clone 53–6.7), CD11b (clone M1/70), CD11c (clone N418), and Ter-119 (clone Ter119). Annexin-V staining kit from BioVision was used for the detection of apoptotic cells. Anti–FLAG mAb (clone M2) was purchased from Sigma-Aldrich. Intracellular staining was done as per the instructions provided with Cytoperm/Cytofix cell permeabilization and staining kit (BD).

**Brdu incorporation assays.** Mice were injected intraperitoneally with 1 mg BrdU in 100 μl PBS and, 3 h later, tissues were harvested and BrdU incorporation was analyzed by using a BrdU Flow kit (BD).

**Thymus transplantation and immunofluorescence.** Individual thymus lobes from E14.5–18.5 WT and **Lm***8a<sup>−/−</sup> embryos were transplanted under the kidney capsule of anesthetized recipient mice. For staining cryosections, thymi were harvested, fixed in phosphate-buffered 1%–lysin with 1% paraformaldehyde/peroxide (PLP) and dehydrated in 30% sucrose in PBS, snap-frozen.
Proliferation and activation of thymocytes and splenic T and B cells. Thymocytes were prepared as described earlier (de la Fuente et al., 2006). 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 (Pepro-Tech). 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(‘), anti-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 OP9-DL1 cells (gift from J.C. Zúñiga-Pflucker, University of Toronto, Toronto, Canada) in recombinant IL-7 and Flt3L (PeproTech) containing medium in the presence of either GST/GST-LRRC8A or MBP/MBP-LRRC8A for 4–6 d as described earlier (Schmitt and Zúñiga-Pflucker, 2002), after which cells were harvested and analyzed by FACS.

LRRC8A signaling studies. For cell stimulation, purified cells were incubated with the respective antibodies on ice for 20 min and cross-linked with F(ab’)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 D9E9, 1:1,000; Cell Signaling Technology), anti-pGAB (clone 11E7, 1:1,000; Cell Signaling Technology), anti-pGAB2 (Y892, clone C33G1, 1:1,000; Cell Signaling Technology), anti-GAB2 (clone 26B6, 1:1,000; Cell Signaling Technology), anti-GAB (Y416, clone D49G4, 1:1,000; Cell Signaling Technology), anti-LCK (clone L22B1, 1:1,000; Cell Signaling Technology), anti-pZAP (Y105, 1:1,000; Cell Signaling Technology), anti-ZAP (clone DI1C0E, 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. Piceatannol 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. LRRC8A and LCK immunoprecipitations were performed as described earlier (de la Fuente et al., 2006) using anti-LRRC8A 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 Injet Alum (Thermo Fisher Scientific), TNP-Ficol, 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 R.NaQueous 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 Lmr8a−/− mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131379/DC1.

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