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

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.1084/jem.20131379

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:13454721

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Leucine-rich repeat containing 8A (LRRC8A) is essential for T lymphocyte development and function

Lalit Kumar,1,3 Janet Chou,1,3 Christina S.K. Yee,1,3 Arturo Borzutzky,1,3 Elisabeth H. Vollmann,4 Ulrich H. von Andrian,4 Shin-Young Park,2,5 Georg Hollander,6,7 John P. Manis,2,5 P. Luigi Poliani,8 and Raif S. Geha1,3

LRRC8A (LRR containing 8A) is a 94-kD LRR-containing protein highly conserved between human and mouse (Sawada et al., 2003). 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...
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 LRRC8A 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 LRRC8A 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.

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 LRRC8A, 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 of the protein are required for surface expression.
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 splgM+ cells (E, left), of splgM and CD21 expression by splgM+CD23+ cells (E, middle), and of CD21 and CD23 expression by splgM+ 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 in 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 < 0.001; **, P < 0.01; and ***, P < 0.001 (Student’s t test). NS = not significant.
embryos at E14.5 was ~7.9% (n = 38), indicating increased early mortality in utero. Lmr8a−/− mice had increased postnatal lethality; very few survived beyond 4 wk and none beyond 16 wk (Fig. 1 G). Lmr8a−/− 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. Lmr8a−/− exhibited curly hair, hind limb weakness, progressive hydrencephrosis, and sterility. Histological examination revealed epidermal hyperkeratosis, thin skeletal muscle bundles, vacuolated renal tubular cells, and absence of ovarian corpora lutea (Fig. 1 J). Lrn8a−/− mice were comparable in appearance, size, and weight to WT littersmates (Fig. 1, H and I) and had normal tissue histology (not depicted).

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

BM from Lmr8a−/− mice had normal cellularity, modestly increased percentage of CD43−B220loIgM− pro-B cells, and modestly decreased percentages of CD43−B220hiIgM− pre-B cells, CD43−B220loIgM− immature B cells, and B220hi IgMhi recirculating B cells (Fig. 2 A and B). Lmr8a−/− mice had small spleens with well-preserved architecture (Fig. 2 C). The number of B220+ cells in the spleen was approximately fourfold lower in Lmr8a−/− mice compared with WT controls (Fig. 2 D). The percentage of splenic B220+AnnexinV+ cells was comparable in Lmr8a−/− mice and WT controls (unpublished data). To exclude the potential contribution of extrinsic factors to the B cell lymphopenia in Lmr8a−/− mice, we examined Rag2−/− chimeras reconstituted with either Lmr8a−/− or WT BM cells. Splenic B cell numbers were similarly decreased in Lmr8a−/−→Rag2−/− chimeras compared with WT→Rag2−/− chimeras (11.2 ± 1.8 × 10^6 versus 47.9 ± 3.6 × 10^6 cells, n = 3, P < 0.01), indicating that the peripheral B cell lymphopenia in Lmr8a−/− mice is cell intrinsic.

FACS analysis of splenic B cell subsets (Caresetti et al., 2004) revealed comparable percentages of follicular B cells, but modestly decreased percentages of transitional B cells and marginal zone B cells in Lmr8a−/− mice compared with WT littersmates (Fig. 2 E). The numbers and subset distribution of peritoneal B220+ B cells were normal in Lmr8a−/− mice (Fig. 2 F).

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

The thymus was markedly smaller in Lmr8a−/− mice compared with WT littersmates (Fig. 3 A) and had an ~10-fold reduction in thymic cellularity (Fig. 3 B). The thymus was markedly smaller in Lmr8a−/− mice compared with WT littermates (Fig. 1 J). The thymus was markedly smaller in Lmr8a−/− mice compared with WT littermates (Fig. 1 J).
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−/− mice (Fig. 3 C). TdT-mediated dUTP nick end labeling (TUNEL) demonstrated significantly increased numbers of apoptotic cells in Lm8a−/− 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 LRRC8A is important for thymocyte survival.

To exclude the effect of environmental factors on T cell development in Lm8a−/− mice, we examined thymi from Rag2−/− chimeras reconstituted with either Lm8a−/− or WT BM cells. Thymi of Lm8a+/−→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 Lm8a−/−→Rag2−/− chimeras compared with thymi from control chimeras (Fig. 3, H and I).

**Lm8a−/− 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−/−→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 Lm8a−/−→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+ DN2, CD44−CD25− DN3, and CD44+CD25− DN4 cells in Lm8a−/−→Rag2−/− chimeras compared with controls (Fig. 4 C). The numbers of CD44+CD25− DN1 cells were decreased, but not significantly, in thymi from Lm8a−/−→Rag2−/− 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 (Zühiga-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 Lm8a−/− mice is cell intrinsic.

The defect in the development of Lm8a−/− thymocytes could be due to increased cell death and/or decreased cell proliferation. The percentage of annexin V+ cells was significantly increased in Lm8a−/−→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 Lm8a−/−→Rag2−/− chimeras compared with controls (Fig. 4 E). Thymocyte proliferation to anti-CD3+IL-2 was significantly decreased Lm8a−/−→Rag2−/− chimeras compared with control chimeras, but proliferation to PMA+ionomycin was comparable in the two groups (Fig. 4 F). Thus, LRRC8A expression by thymocytes is essential for their survival and proliferation.

**Lm8a−/− mice exhibited a more exaggerated block in thymocyte development than Lm8a−/−→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 Lm8a−/− mice compared with Lm8a−/−→Rag2−/−...**

---

**Figure 4. Cell-autonomous defect in thymocyte maturation in Lm8a−/− 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+ cells (D) and of BrdU+ cells (E) after i.p. injection of BrdU (E) in total thymocytes from the chimeras. (F) 3H-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 \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}} mice. DP thymocytes are exquisitely 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{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}} mice (unpublished data). As in the \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}} chimeras, the numbers of DN2-DN4, but not DN1, cells were significantly lower in \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}} 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\textsuperscript{−} Sca1\textsuperscript{+} c-kit\textsuperscript{+} (LSK) cells in the BM which contain thymic multipotent progenitors (Ikuta and Weissman, 1992; Schwarz and Bhandoola, 2004) were comparable in \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}} and WT mice (Fig. 5, D and E). As in \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}} chimeras, the percentage of annexin V\textsuperscript{+} apoptotic thymocytes was increased and the BrdU incorporation in thymocytes was decreased significantly in \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}} 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{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}} mice and WT controls (Fig. 5, H and I). Unexpectedly, \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}} thymus had a significant increase in the percentage of Lin\textsuperscript{−} c-kit\textsuperscript{−} Sca1\textsuperscript{−} T effector memory cells compared with controls and was not increased by the addition of anti-CD28 mAb (Fig. 6 E). T cells from \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}} 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{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}} chimeras, \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}} 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 proliferation of splenic T cells to immobilized anti-CD3 antibodies 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 deficiency impairs peripheral T cell expansion and function**

Spleens of \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}} chimeras were smaller (Fig. 6 A) and had an approximately fourfold decrease in the number of CD3\textsuperscript{+} T cells compared with \textit{WT→\textit{Rag2\textsuperscript{−/−}} control chimeras} (Fig. 6 B). The splenic CD4/CD8 ratio was comparable in \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}}} and control chimeras (Fig. 6 C). \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}}} chimeras had a significant decrease in the percentage of splenic CD4\textsuperscript{+}CD62L\textsuperscript{−}CD44\textsuperscript{+} T effector memory cells compared with control chimeras, and a compensatory increase in the percentage of CD4\textsuperscript{+}CD62L\textsuperscript{+}CD44\textsuperscript{+} naive T cells (Fig. 6 D). The proliferation of splenic T cells to immobilized anti-CD3 was significantly impaired in \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}} chimeras compared with controls and was not increased by the addition of anti-CD28 mAb (Fig. 6 E). T cells from \textit{Lr\textit{r}rc\textit{8}a\textsuperscript{−/−}→\textit{Rag2\textsuperscript{−/−}} 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.

**Figure 5. Defective thymocyte development in \textit{Lrrc8a\textsuperscript{−/−}} mice.** (A) FACS analysis of CD4 and CD8 expression by thymocytes from a 3-wk-old \textit{Lrrc8a\textsuperscript{−/−}} 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\textsuperscript{−/−}} 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\textsuperscript{−} Sca1\textsuperscript{+} c-Kit\textsuperscript{+} cells in the BM. (F and G) Percentage of annexin V\textsuperscript{+} cells (F) and BrdU\textsuperscript{+} cells 3 h after i.p. injection of BrdU (G) in thymocytes from \textit{Lrrc8a\textsuperscript{−/−}} mice and WT littermates. (H) Percentage of TCR-γ/δ cells in the thymus of \textit{Lrrc8a\textsuperscript{−/−}} 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\textsuperscript{+} regulatory T (T reg) cells by immunohistochemistry staining and flow cytometry (Fig. 5, J and K).
LRRC8A is dispensable for the development and function of thymic epithelium

TECs play a critical role in thymic development (Rodewald, 2008). Because Lr88a is ubiquitously expressed, we examined TECs from Lr88a−/− mice. FACS analysis revealed that the percentages of CD4+CD8−CD44+ naive T cells and CD4+CD8−CD44+ T effector memory cells (D) and, proliferation of T cells (E) from spleens of Lr88a−/− → Rag2−/− and control WT → Rag2−/− chimeras. (F-H) Splenic T cell numbers (F), FACS analysis of CD4+ and CD8+ cells in gated splenic CD3+ T cells (G), and proliferation of splenic T cells (H) from Lr88a−/− mice and WT control littermates. (I) Spectratyping analysis of CD3 diversity of selected TCR-Vβ families in splenic T cells from a 6-wk-old Lr88a−/− 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 Lr88a−/− mice, like those from Lr88a−/− → 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 Lr88a−/− mice compared with age-matched WT littermates, as indicated by a 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 Lr88a−/− mice is compatible with an abnormal TCR repertoire selection in the thymus and/or with abnormal clonal expansion/maintenance in the periphery.
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 \( \text{Lrrc8a}^{-/-} \) 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)–LRRC8A\(^{343-810} \) 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\(^{-}\)TECs, but not CD45\(^{+}\)thymocytes, including DN, DP, and SP cells (Fig. 8, C and D). Both CD45\(^{-}\)classII\(^{+}\)BP1\(^{-}\)cTECs and CD45\(^{-}\)classII\(^{-}\)BP1\(^{+}\)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\(^{+}\) 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 \( \text{Lrrc8a}^{-/-} \) 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 \( \text{Zap70}^{-/-} \) 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 \( \text{Lrrc8a}^{-/-} \) 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, \( \text{Lrrc8a}^{-/-} \) thymi had a reduced percentage of pAKT-positive thymocytes, and a lower pAKT/AKT staining intensity ratio with a normal

Figure 8. DN thymocyte maturation requires interaction of LRRC8A with a ligand expressed on TECs. (A–D) FACS analysis of the binding of GST-LRRC8A fusion protein to 293T cells (A), splenocytes (B), electronically gated CD45+ and CD45− thymus cells, CD45− MHCII+BP-1+ cTECs and CD45− MHCII+BP-1− mTECs from WT mice (C), DN, DP, and SP thymocytes from WT mice (D), and the OP9-DL1 stromal cell line (E). (F–H) FACS analysis of CD4 and CD8 expression by purified CD4−CD8− DN thymocytes from WT mice after 4 d after co-culture with OP9-DL1 cells in the presence of medium, GST-LRRC8A, or GST as control (F), quantitation of the percentages and numbers of CD4+CD8− DP cells recovered (G), and percentage of apoptotic cells in the DN and DP populations at the end of the 4 d co-culture as measured by Annexin V staining followed by flow cytometry (H). (I) Dose response curve of the effect of GST-LRRC8A on the in vitro differentiation of DN thymocytes co-cultured with OP9-DL1 cells. DN thymocytes were cultured on OP9-DL1 cells with GST or GST-LRRC8A at the indicated doses. The graph depicts frequency of DN cells after 4 d of culture. Data are representative of six independent experiments with one sample (A) and one mouse (B) per group, three independent experiments with one mouse per group (C and D), and one sample per group (E), and three independent experiments with three samples per group (F–I). Mean and SEM are shown in G–I. ***, P < 0.001 (Student’s t test).

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 Y\(^{452} \). 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 Y\(^{452} \), 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 \( \text{Zap70}^{-/-} \) 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 \( \text{Lrrc8a}^{-/-} \) 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, \( \text{Lmr8a}^{-/-} \) thymi had a reduced percentage of pAKT-positive thymocytes, and a lower pAKT/AKT staining intensity ratio with a normal
The role of LRRC8A in the immune system | Kumar et al.

Despite their defective T cell development and function, Lrrc8a−/− mice had decreased cellularity, disorganized architecture, increased apoptosis, and decreased proliferation, indicating that the early block in thymocyte development is cell autonomous. Although these defects are T cell intrinsic, the decreased proliferative capacity of thymocytes from Lrrc8a−/− mice is likely due to their multiple organ abnormalities.

Our studies demonstrate that LRRC8A activates AKT via the LCK–ZAP–70–GAB2–PI3K pathway. LRRC8A constitutively associates with the GRB2–GAB2 complex and LCK. These associations may be direct, via interactions between the LCK–ZAP-70–GAB2–PI3K pathway. LRRC8A constitutively associates with the GRB2–GAB2 complex and LCK, and activates AKT via the LCK–ZAP–70–GAB2–PI3K pathway in thymocytes. (A) AKT phosphorylation in WT thymocytes after LRRC8A ligation and the effect of the PI3 kinase inhibitor LY294002 on LRRC8A-mediated AKT phosphorylation. AKT phosphorylation in thymocytes pretreated with LY294002 or DMSO was determined by immunoblotting after LRRC8A ligation for the indicated times. (B) Immunoblot analysis of the phosphorylation of AKT in total DN thymocytes from WT mice after anti-LRRC8A stimulation. (C) AKT phosphorylation in thymocytes from Lrrc8a−/− mice in response to anti-LRRC8A and anti-CD3 cross-linking. CD3-stimulated WT thymocytes were used as controls. (D–F) Co-immunoprecipitation of LRRC8A with GRB2 (D), GAB2 (E), and LCK (F). Ctrl. = control. The asterisk in D indicates a nonspecific band in the control IgG lane. The total cell lysates were prepared from WT thymocytes. LRRC8A 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) Phosphorylation of GAB2 (G), LCK (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.
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 LRRCA8A 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, Deltex, Nup, and pTCRα (Deftos et al., 1998, 2000; Krebs et al., 2001; Lamar et al., 2001), as well as Bcl-2 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 LRRCA8A deficiency.

A ligand for LRRCA8A was detected on TECs and on the stromal cell line OP9. A fusion protein containing GST and the extracellular domain of LRRCA8A 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 LRRCA8A ligand expressed by TECs delivers a critical survival signal via AKT to thymocytes. In addition to 293T cells, GST-LRRCA8A bound to keratinocytes and fibroblasts (unpublished data), suggesting that disruption of LRRCA8A 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 LRRCA8A 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 LRRCA8A delivers a co-stimulatory signal to antigen-activated T cells. Indeed, ligation of LRRCA8A causes AKT activation in normal splenic T cells (unpublished data), as it does in thymocytes. Lack of LRRCA8A–driven AKT activation and/or maturation in an abnormal thymic environment may contribute to the decreased homeostatic proliferation and impaired function of LRRCA8A–deficient peripheral T cells. Selective deletion of Lrrc8a in mature T cells is needed to distinguish between these two possibilities.
Lrrc8a<sup>−/−</sup> mice had a modest block in B cell development and normal B cell function. However, Lrrc8a<sup>−/−</sup> mice and Lrrc8a<sup>−/−</sup>→Rag2<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 (Juntilla et al., 2007). Thus, loss of LRRCA8A–mediated AKT activation in B cells may have contributed to the peripheral B cell lymphopenia in Lrrc8a<sup>−/−</sup> mice.

In contrast to the Lrrc8a<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), and the complete absence of the protein in the knock-out mouse. Given the 99% aa 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 Lrrc8a<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 kb in length were PCR amplified from a BAC clone DNA encoding the entire Lrrc8a 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 Lrrc8a, two were injected into C57BL/6 blastocysts for the generation of chimeric mice. ES cell clones and mice were genotyped by Southern blot analysis. Single-cell suspensions from BM, thymus, and spleen of 3–6-wk-old mice were prepared as described earlier (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 FACS Canto (BD). Fluorescence-labeled or biotinylated monoclonal antibodies to B220 (clone RA3-68B2, clone 6C3), CD3ε (clone 145-2C11), CD4 (clone L3T4), CD8 (clone 53-6.7), CD11b (clone M1/70), CD11c (clone N418), CD21/35 (clone eBio8D9), 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 D3A7, 1:80; Cell Signaling Technology), STAT3 (clone 79D7, 1:100), pAKT (Ser 473; clone 736E11, 1:30; Cell Signaling Technology), anti-pAKT (Ser 473, Tyr 470; clone 736E11, 1:30; Cell Signaling Technology), rat anti-cytokeratin 8 (clone TRKOMA-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 (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 FACS Canto (BD). Fluorescence-labeled or biotinylated monoclonal antibodies to B220 (clone RA3-68B2, clone 6C3), CD3ε (clone 145-2C11), CD4 (clone L3T4), CD8 (clone 53-6.7), CD11b (clone M1/70), CD11c (clone N418), CD21/35 (clone eBio8D9), 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 145-2C11), IgD (clone 11-26C), IgM (clone eB121-15F9), Sca-1 (clone D7), 6C3), CD3<sup>+</sup> (clone 145-2C11), CD4 (clone L3T4), CD8 (clone 53-6.7), CD11b (clone M1/70), CD11c (clone N418), and Ter-119 (clone Ter119), and T lymphocytes were identified by excluding cells stained with single fluorochrome-labeled mAbs. Preparations of splenic T cells were cultured with anti-CD3/CD28, anti-CD28 mAbs (clone 145-2C11), CD4 (clone L3T4), CD8 (clone 53-6.7), CD11b (clone M1/70), CD11c (clone N418), and Ter-119 (clone Ter119), and Annexin-V-FITC was used as a control for pAKT specificity.

**Thymus transplantation and immunofluorescence.** Individual thymus lobes from E14.5–18.5 WT and Lrrc8a<sup>−/−</sup> embryos were transplanted under the kidney capsule of anesthetized recipient mice. For staining cryosections, thymi were harvested, fixed in phosphate-buffered 1% paraformaldehyde/polyethylene (PLP), and then incubated with primary antiserum for 24 h at 4°C. Sections were then washed, dehydrated in 30% sucrose in PBS, and then frozen in OCT freezing medium (Sakura Finetek USA). Cryosections were mounted on slides and stained with the following antibodies: anti–AIRE (provided by P. Peterson, University of Tartu, Tartu, Estonia; 1:2,000), anti-β2-microglobulin (clone 11-26C), IgM (clone eB121-15F9), Sca-1 (clone D7), 6C3), CD3<sup>+</sup> (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 for pAkt specificity.
in TBS–tissue-freezing liquid (Triological 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), Volocity (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). Purified 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 F(ab’)2, 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-β repertoire analysis. TCR-β repertoire clonality determination on splenic T cells was performed using spectratyping by BioMed ImmunoJtech, Inc.

Generation of LRRCA8 fusion proteins and analysis of binding of LRRCA8 fusion protein to cells. LRRCA8 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-LRRCA8 and MBP-LRRCA8 fusion proteins were purified as per the manufacturer’s instructions. Target cells were incubated with GST/GST-LRRCA8 or MBP/MBP-LRRCA8 on ice for 30 min. The binding of LRRCA8 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-Pflücker, University of Toronto, Toronto, Canada) in recombinant IL-7 and Flt3L (Pepro-Tech) containing medium in the presence of either GST/GST-LRRCA8 or MBP/MBP-LRRCA8 for 4–6 d as described earlier (Schmitt and Zúñiga-Pflücker, 2002), after which cells were harvested and analyzed by FACS.

LRRCA8 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-AKT (clone DE9, 1:1,000; Cell Signaling Technology), anti-AKT (clone 11E7, 1:1,000; Cell Signaling Technology), anti–pGAB2 (Y32, 1:1,000; Cell Signaling Technology), anti–ZAP-70 (Y92, 1:1,000; Cell Signaling Technology), anti–ZAP-70 (clone D1C10E, 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 R-406 (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. LRRCA8 and LCK immunoprecipitations were performed as described previously (de la Fuente et al., 2006) using anti-LRRCA8 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 level 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 LRRCA8 in untransfected and LRRCA8–transfected 293T cells by FACS using LRRCA8 and FLAG tag–specific antibodies and the strategy to generate Lrrca8™/™ mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131379/DC1.

The work was supported by the National Institutes of Health (AI-39796, AI–79679, and AI–106529) and a grant from Fondazione Cariplo (P.L. Poliani), and AI–097769 (U.H. van Andriën). The authors declare no competing financial interests.

Submitted: 1 July 2013
Accepted: 21 March 2014

REFERENCES


Submitter: 1 July 2013
Accepted: 21 March 2014

REFERENCES


Role of LRRC8A in the immune system | Kumar et al.