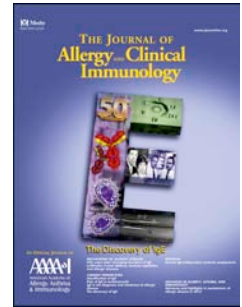


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**LRRC8A-dependent volume regulated anion channel activity is dispensable for T cell
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ABSTRACT

Background. Leucine-rich repeat containing 8A (LRRC8A) is a ubiquitously expressed transmembrane protein with 17 LRRs at its C-terminal end, and is an essential component of the volume regulated anion channel (VRAC) which controls cellular volume. A heterozygous mutation in LRRC8A that truncates the two terminal LRRs was reported in a patient with agammaglobulinemia and absent B cells, and was demonstrated to exert a dominant negative effect on T and B cell development in mice. *Lrrc8a*^{-/-} mice have severely defective T cell development and function. It is not known whether the T and B cell defects caused by LRRC8A deficiency are caused by loss of VRAC activity.

Objective. To determine whether VRAC activity is required for normal T cell development and function.

Methods. VRAC activity was examined by patch clamp analysis. Flow cytometry was used to examine T cell development. T cell proliferation, cytokine secretion and antibody titers were measured by standard techniques.

Results. We demonstrate that the spontaneous mouse mutant *ébouiffé* (*ebo/ebo*) harbors a homozygous 2 bp frameshift mutation in *Lrrc8a* that truncates the fifteen terminal LRRs of LRRC8A. The LRRC8A^{*ebo*} mutation does not impact protein expression but drastically diminishes VRAC activity in T cells. *ebo/ebo* mice share features with *Lrrc8a*^{-/-} mice that include curly hair, infertility, reduced longevity and kidney abnormalities. However, in contrast to *Lrrc8a*^{-/-} mice, *ebo/ebo* mice have normal T cell development and function and intact antibody response to T-dependent antigen.

Conclusion. LRRC8A-dependent VRAC activity is dispensable for T cell development and function.

Key messages:

LRRC8A is an essential component of the VRAC, which regulates cellular volume, and is important for T cell development and function.

ébouriffé (*ebo/ebo*) mutant mice harbor a homozygous mutation that truncates the fifteen terminal LRRs of LRRC8A and severely impairs VRAC activity but spares T cell development and function.

LRRC8A-dependent VRAC activity is dispensable for T cell development and function.

Capsule Summary: The role that LRRC8A plays in T cell development is independent of its role in regulating cellular volume.

Keywords:

LRRC8A, VRAC, thymocyte development

Abbreviations:

LRR: leucine rich repeat

VRAC: volume-regulated anion channel

ebo: *ébouriffé*

mTEC: medullary thymic epithelial cell

cTEC: cortical thymic epithelial cell

CMJ: corticomedullary junction

INTRODUCTION

LRRC8A encodes a ubiquitously expressed 810 amino acid cell surface protein that spans the plasma membrane four times and contains 17 leucine rich repeats (LRRs) in its C-terminal domain (1, 2). *LRRC8A* has at least two biologic functions: it is critical for T cell development (3) and also is an essential component of the volume-regulated anion channel (VRAC), important for preserving constant cell volume in response to osmotic stress (4, 5). VRAC has been implicated in a wide range of biologic processes, including proliferation and cell survival (6). T cells were among the first cell types in which VRAC was identified (7) though it is unknown whether the VRAC activity of *LRRC8A* is necessary for T cell development and survival.

LRRC8A was first identified through studies of a female patient with agammaglobulinemia and absent circulating B cells who was found to have a heterozygous mutation in *LRRC8A*. This mutation resulted in the expression of a mutant protein, designated *LRRC8A*^{Δ91/+35}, in which the C-terminal two and a half terminal LRRs were replaced by 35 novel aa encoded by intronic nucleotides (1). The number and function of T cells in this patient were not reported. Irradiated wild type mice reconstituted with syngeneic CD34⁺ cells transduced with the *LRRC8A* mutant had impaired T and B cell development (1). We subsequently demonstrated that *Lrrc8a*^{-/-} mice had a striking phenotype characterized by significantly increased prenatal mortality, markedly reduced longevity, growth retardation, abnormal hair, sterility and kidney abnormalities (3). Importantly, *LRRC8A* deficiency resulted in a severe block in early thymocyte differentiation and increased thymocyte apoptosis, resulting in markedly reduced thymic cellularity and severely impaired peripheral T cell proliferation in response to TCR ligation (3). *Lrrc8a*^{-/-}→*Rag2*^{-/-} bone marrow chimeras recapitulated the defects in thymocyte development and peripheral T cell function, demonstrating that the T cell defect in

Lrrc8a^{-/-} mice is cell autonomous. Ligation of LRRC8A activates AKT, a kinase important for thymocyte survival, proliferation, and metabolism, through a LCK–ZAP-70–GAB2–PI3K pathway (3, 8). AKT phosphorylation was markedly reduced in the thymus of *Lrrc8a*^{-/-} mice (3). These findings established a critical role for LRRC8A in T cell development.

VRAC is activated by conditions of low intracellular ionic strength, thereby permitting an efflux of anions that normalizes the osmotic gradient and restores cell volume (9, 10). Knockdown of LRRC8A virtually abolishes VRAC activity in multiple cell types, including human T lymphocytes (4, 5) demonstrating that LRRC8A, also known as SWELL1, is a necessary component of VRAC. Recent studies established that the pore-forming subunits of VRAC are composed of hexamers of LRRC8A and up to four LRRC8 family homologs (LRRC8B-E) (10). Since studies demonstrating the VRAC activity of LRRC8A were performed in transfected cell lines, the contribution of LRRC8A-driven VRAC activity to physiologic processes remains undetermined.

We report here that the spontaneous mouse mutation designated *ébouriffé* (*ebo*) is caused by a 2 bp deletion in *Lrrc8a* that results in truncation of the 15 C-terminal LRRs of LRRC8A and a dramatic reduction of VRAC activity in T cells. Like *Lrrc8a*^{-/-} mice, *ebo/ebo* mice have reduced survival, abnormal hair, infertility, and abnormal renal tubular morphology (3). In contrast to *Lrrc8a*^{-/-} mice, *ebo/ebo* mice have intact T cell development and function. Thus, the critical role LRRC8A plays in T cell development and function is independent of its VRAC activity.

METHODS

Mice. FVB/N.Cg-*ebo* (N10) congenic mice (11), *Lrrc8a*^{-/-} mice (3), CD4 Cre *Lrrc8a*^{f/f} mice, and their WT littermates were kept in microisolator cages under specific-pathogen free conditions. The *ebo* mutant allele was backcrossed into FVB/N background for 10 generations (partially by marker-assisted backcrossing). All procedures were performed within the guidelines of the Animal Care and Use Committee of Boston Children's Hospital.

Semi-quantitative RT-PCR. Total RNA was prepared from splenocytes of WT and *ebo/ebo* mice using the RNeasy kit (Qiagen). cDNA synthesis and amplification were performed from total RNA using the Superscript III One Step RT-PCR System with Platinum Taq DNA polymerase. Primers used for *Lrrc8a* were: forward primer 5'-TCACAGCCAATAGGATTGAAGC-3' (spans exon 3 and exon 4), reverse primer 5'-CCTAGCCCAGTGCCAATAAG-3' (exon 4). Primers for *18S* were used as a control: forward primer 5'-CGGCTACCACATCCAAGGAA-3', reverse primer 5'-GCTGGAATTACCGC GGCT-3'.

Preparation of cells and flow cytometry. Single-cell suspensions from thymi and spleens of 3–11 week-old mice were prepared by disrupting tissue and passing through a 70 µm cell strainer (Falcon). Cells were stained with the appropriate fluorochrome-labeled monoclonal antibodies and analyzed on a LSRFortessa (BD). Antibodies were purchased from eBioscience unless otherwise indicated. Splenocyte and thymocyte subsets were defined using fluorescently labeled antibodies against CD3 (clone 145-2C11), CD4 (clone RM4-4) CD8 (clone 53–6.7) and B220 (clone RA3-6B2). To distinguish thymocyte progenitors, lineage-negative cells were identified by excluding cells stained with single fluorochrome-labeled cocktail of PE-labeled CD19 (BD, clone 1D3), CD3 (clone 145-2C11), CD4 (clone GK1.5), CD8 (clone 53–6.7),

CD11b (clone M1/70), CD11c (clone N418), Ly6 (clone HK1.4), NK1.1 (clone PK136), TCR $\gamma\delta$ (BD, clone GL3) and Ter-119 (clone Ter119). Cells were subsequently stained with fluorescent-labeled monoclonal antibodies to CD44 (clone IM-7) and CD25 (Biolegend, clone PC61).

Immunoblotting and flow cytometry analysis of LRRC8A expression. A polyclonal antibody (C18) was raised in rabbits against an 18-aa sequence (aa 791–808) at the C-terminus of LRRC8A and affinity purified as previously described (3). Cell lysates were immunoblotted using C18 or anti-actin (clone ACTN05 (C4), Abcam) followed by HRP-conjugated goat anti-rabbit antibody or goat anti-mouse antibody conjugated to horseradish peroxidase–HRP. Intracellular flow cytometry staining was done as per the instructions provided with the Cytoperm/Cytofix cell permeabilization and staining kit (BD). Cells were stained with a rabbit antibody against the aa sequence 87-104 in the N-terminus of LRRC8A (anti-LRRC8A₈₇₋₁₀₄) prior to detection with FITC-labeled anti-rabbit Ab (BD).

Electrophysiology. Splenic T cells were purified by negative selection (Miltenyi Biotech). Peritoneal macrophages were obtained via peritoneal lavage with ice cold PBS. T cells and macrophages were cultured for 2 days. Current traces were acquired following initiation of 220 mOsm saline perfusion, normalized to membrane capacitance. Whole-cell patch clamp recordings were performed using an Axopatch 200B amplifier, Digidata 1320A analog-to-digital converter, and pClamp 9.2 software (Molecular Devices). Digital acquisition was performed at a 10 kHz sampling frequency; voltage-clamped data was low-pass filtered at 1 kHz. Whole-cell patch clamp was performed using thick-walled, borosilicate glass micropipettes pulled to a pipette resistance of 3-4 M Ω . Series resistance was compensated to 80%. Cells were held at -70 mV with voltage ramps from -100 mV to +100 mV over 400 ms at 5 s intervals. Currents were measured at -80 mV and +80 mV to plot time courses. Voltage-steps were from -70 mV to +140

mV at +10 mV intervals; step duration, 40 ms. Voltage-steps were recorded at 5 minutes after shift to hypotonic solution for T cells, and 12 minutes for macrophages. All recordings were performed at ~ 22°C. Internal pipette solution 120 mM CsASP, 10 mM Cs₄BAPTA, 4 mM MgATP, 2 mM MgCl₂, 8 mM NaCl, and 10 mM HEPES (pH 7.2 with CsOH). External bath solution (220 mOsm): 95 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM CsCl, 10 mM HEPES, and 10 mM Glucose (pH 7.4 with NaOH), with 100 mM mannitol added to yield 320 mOsm solutions.

Histopathology. Thymic morphology and expression of specific markers was assayed by both routine H&E staining and immunohistochemistry using 2 µm thick formalin fixed paraffin embedded thymic sections as described previously (3).

***In vitro* proliferation and activation of splenic T cells.** Purified splenic T cells were labeled with CellTrace Violet (Life Technologies) prior to culture in medium alone or in wells with plate-bound anti-CD3 mAb (clone KT3; Abcam) with or without anti-CD28 mAb (clone L293; BD) or 40 ng/ml IL-2 (PeproTech). PMA (Sigma-Aldrich) was used at 50 ng/ml, and ionomycin (Sigma-Aldrich) at 0.5 µM. Harvested cells were labeled with anti-CD4-PE (clone RM4-4, eBioscience) and proliferation was assessed by dilution of CellTrace violet by flow cytometry using an LSRFortessa (BD). Concentrations of IL-2 and IFN-γ in supernatants were measured by ELISA (Ready-Set-Go, eBioscience)

Immunization and assessment of T cell and B cell responses. Two month-old mice were immunized i.p. with 10 µg of TNP-KLH (Biosearch Technologies) and Imject Alum (Thermo Fisher Scientific). Mice were subsequently boosted on day 14 with 2.5 µg of TNP-KLH and Imject Alum. They were bled on day 0 and day 21 prior to sacrifice. Splenocytes were labeled with CellTrace Violet (Life Technologies) and cultured at a concentration of 3x10⁵ cells

in the presence of media alone, or KLH at the indicated concentration. On day 3, supernatants were harvested and IL-2 and IFN- γ were measured by ELISA (Ready-Set-Go, eBioscience). Levels of antigen-specific antibody responses from serum obtained on day 0 and day 21 were analyzed by TNP-specific ELISA using 96-well plates coated with TNP-conjugated BSA (Biosearch Technologies) at 10 μ g/ml in PBS. Plates were coated with TNP:BSA conjugated at a molar ratio of 2:1 to detect high-affinity antibody and TNP:BSA with a molar ratio of 32:1 to detect low-affinity antibody. Sera were diluted 1:300, 1:1000, 1:3000, 1:9000, 1:27,000 and 1:81,000. KLH-specific antibodies were detected with anti-IgG-AP. PNPP substrate (Sigma) was added and OD 405 was determined using a Microplate reader (Molecular Devices).

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

RESULTS

***ebo/ebo* mice have a homozygous frameshift mutation in *Lrrc8a* and share phenotypic features with *Lrrc8a*^{-/-} mice.** The *ebo* autosomal recessive mutation arose spontaneously in a substrain of BALB/c (11). Positional cloning localized the mutation to a region in mouse chromosome 2 (between D2Mit153 and D2Mit64, 29.27-31.20 Mb) homologous with human chromosome 9q34, which includes *LRRC8A*. Next-generation DNA sequencing of this interval revealed a homozygous 2 bp deletion in exon 3 of *Lrrc8a* in *ebo/ebo* mice (Fig. 1A). No other mutations were detected. The *ebo* frameshift mutation in *Lrrc8a* (c.1325_1326delTA;p.F443*) is predicted to result in a truncated LRRC8A₁₋₄₄₂ protein (LRRC8A^{ebo}) that lacks the fifteen terminal LRRs (LRR3–LRR17), but retains all four transmembrane (TM) domains and the first two LRRs. RT-PCR analysis of splenocytes revealed that *Lrrc8a* mRNA expression levels were comparable in *ebo/ebo* mice and WT littermates (Fig. 1B), indicating that the *ebo* mutation does not alter *Lrrc8a* mRNA stability.

We next examined the effect of the mutation on the expression of the LRRC8A mutant protein. As expected, immunoblotting using an antibody raised against a sequence at the C-terminal end of LRRC8A (anti-LRRC8A₇₉₁₋₈₀₈) revealed robust expression of LRRC8A in WT splenocytes, but no detectable expression of LRRC8A in cells from *ebo/ebo* mice (Fig. 1C). The N-terminal region of LRRC8A shows a high degree of homology with other LRRC8 family members (LRRC8B, LRRC8C, LRRC8D, and LRRC8E). The two available commercial antibodies against the N-terminus of LRRC8A, anti-LRRC8A₁₋₅₀ (Sigma) and anti-LRRC8A₅₀₋₁₀₀ (Bethyl) showed no selectivity for LRRC8A, as they stained T cells from WT and *Lrrc8a*^{-/-} mice equally well (data not shown). Subsequently, we raised a rabbit antibody against the aa sequence 87-104 located between TM1 and TM2 in the N-terminus of LRRC8A, as this sequence showed

the least homology with the corresponding aa sequences in other LRRC8 family members among regions with suitable antigenicity (Fig. 1D). Nevertheless, this homology ranged from 22% for LRRC8E to 39% for LRRC8B. Intracellular staining with anti-LRRC8A₈₇₋₁₀₄ antibody yielded a significantly lower MFI in T cells from *Lrrc8a*^{-/-} mice compared to WT controls (474.9±10.73 vs 631.2±28.3, n=6, p<0.001, Fig. 1E left panel). This incomplete loss of signal in LRRC8A KO cells is likely due to cross-reactivity with other LRRC8 family members. In contrast, there was no significant shift in the MFI of T cells from *ebo/ebo* mice and WT controls (318.7±42.1 vs 330.1±79.2, n=5 p=0.89), Fig. 1E right panel). These results suggest that the LRRC8A^{ebo} mutant is expressed in T cells from *ebo/ebo* mice. Taken together, these results indicate that the *ebo* mutation leads to deletion of the C-terminus of LRRC8A, but does not appear to affect the expression level of the truncated protein.

As previously reported (11), *ebo/ebo* mice have wavy hair and curled vibrissae, features also shared by *Lrrc8a*^{-/-} mice (Fig. 2A) (3). The frequency of live *ebo/ebo* pups among 124 pups obtained from matings of *+ebo* heterozygotes was 22%, close to the expected Mendelian frequency of 25%. This is in contrast to the 5.5% frequency of live *Lrrc8a*^{-/-} pups obtained from mating *Lrrc8a*^{+/-} heterozygotes (3). *ebo/ebo* mice were sterile, like *Lrrc8a*^{-/-} mice. In contrast to *Lrrc8a*^{-/-} mice, which have severe growth impairment (3), the weight of *ebo/ebo* mice was comparable to that of WT littermates (Fig. 2B). Nevertheless, *ebo/ebo* mice had significantly decreased survival compared to WT littermates, with only 60% reaching the age of 100 days (Fig. 2C). This survival is significantly longer than that of *Lrrc8a*^{-/-} mice, all of which die by 100 days of age (3). Histologic examination of organs from *ebo/ebo* mice revealed hepatocellular swelling in the liver, atrophy of the secretory convoluted ducts in the submandibular gland, and tubular degeneration in the kidneys, which is also observed in *Lrrc8a*^{-/-} mice (Fig. 2D) (3). The histology

of the skin and gonads in *ebo/ebo* mice was also similar to that of *Lrrc8a*^{-/-} mice (3), and will be detailed in a separate publication. *ebo/ebo* mice, unlike *Lrrc8a*^{-/-} mice, had normal striated muscle histology (data not shown). Heterozygous *+ebo* mice, like heterozygous *Lrrc8a*^{+/-} mice, appear phenotypically identical to WT littermates (Fig. 2A). The phenotypic characteristics shared by the *ebo/ebo* and *Lrrc8a*^{-/-} mice provide strong evidence that the 2 bp deletion in *Lrrc8a* is causative of the *ebo/ebo* phenotype.

The *ebo* mutation severely impairs VRAC activity in T cells. VRAC activity was measured using whole-cell voltage clamp recordings of cells subjected to 220 mOsm hypoosmotic stress using the solution scheme outlined in Fig 3A. Voltage step analysis of currents in splenic T cells from C57BL/6 and FVB/N mice showed delayed activation, outward rectification, and desensitization during strong depolarization, features characteristic of VRAC (Fig. 3B, left panel). Currents were severely diminished in *ebo/ebo* T cells (Fig. 3B, right panel). Current density (current normalized to capacitance to account for differences in cell size), which is representative of VRAC activity in response to hypoosmotic stress, was readily detectable in T cells from C57BL/6 and FVB/N mice with no significant difference between the two strains (Fig 3C). In contrast, current density was drastically diminished in T cells from both *Lrrc8a*^{-/-} mice (C57BL/6 background), and *ebo/ebo* mice (FVB/N background), demonstrating that full-length LRRC8A, including the fifteen terminal LRRs, is essential for normal VRAC activity in T cells (Fig. 3C). The virtually absent current density in *Lrrc8a*^{-/-} T cells is consistent with the critical role of LRRC8A in VRAC activity (4, 5). Importantly, there was no significant difference between *Lrrc8a*^{-/-} and *ebo/ebo* mice in the residual current density measured at -80 mV, which closely approximates the -70 mV resting membrane potential under physiologic conditions. When analyzed at +80 mV, the residual VRAC activity was slightly higher in *ebo/ebo* T cells

than in *Lrrc8a*^{-/-} T cells, suggesting that LRRC8A^{ebo} may contribute to the residual VRAC activity induced under such non-physiologic conditions in T cells.

Peritoneal macrophages from *Lrrc8a*^{-/-} and *ebo/ebo* mice also had drastically reduced VRAC activity, indicating that the *ebo* mutation impacts VRAC activity in cell types other than T cells (Fig. 3D, E). There was no significant difference in residual VRAC activity between *ebo/ebo* and *Lrrc8a*^{-/-} macrophages at -80 mV or +80 mV. T cells and peritoneal macrophages from +/-*ebo* heterozygous mice exhibited normal VRAC activity with kinetics comparable to that of WT cells (data not shown).

Normal T cell development and function in *ebo/ebo* mice. *Lrrc8a*^{-/-} mice exhibit a severe cell-intrinsic defect in thymocyte development, with a ten-fold reduction in thymic cellularity, a block in thymocyte development at the double negative 2 (DN2) stage and decreased p-AKT content (3). Thymi from *Lrrc8a*^{-/-} mice exhibit effacement of the corticomedullary junction (CMJ), numerous pyknotic nuclei, abnormal distribution of cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC) (3). We therefore examined T cell development in *ebo/ebo* mice to determine whether this was affected by dramatically reduced VRAC activity. Thymic size and cellularity were comparable in *ebo/ebo* mice and WT littermates (Fig. 4A, B). Furthermore, thymi from *ebo/ebo* mice had a normal CMJ with no increase in the numbers of pyknotic nuclei (Fig. 4C) and normal numbers and distribution of TdT⁺ immature thymocytes, and CD3⁺ maturing thymocytes (Supplementary Fig. 1). The thymi of *ebo/ebo* mice had normal numbers and distribution of cytokeratin-8⁺ cTECs and cytokeratin-5⁺ mTECs, and their mTECs showed normal expression of *Ulex europaeus* agglutinin and the autoimmune regulator protein (Supplementary Fig. 1). The percentages of DN, DP, and SP thymocytes as well as DN1-DN4 thymocytes were comparable in *ebo/ebo* mice

and WT littermates (Fig 4D, E). Furthermore, phospho-AKT in the thymus of *ebo/ebo* mice was comparable to that of WT littermates (Fig. 4F). Splenic cellularity, T and B cell numbers, and CD4⁺:CD8⁺ T cell subset distribution were comparable between *ebo/ebo* mice and WT littermates (Fig 4G-I). Overall, there was no detectable defect in thymocyte maturation.

We next assessed T cell function, as we previously demonstrated that *Lrrc8a*^{-/-} mice and *Lrrc8a*^{-/-}→*Rag2*^{-/-} chimeras have significantly impaired splenic T cell proliferation and IL-2 secretion in response to immobilized anti-CD3 (3). In contrast, the proliferation of splenic T cells in response to anti-CD3, anti-CD3+anti-CD28 and anti-CD3+IL-2 was comparable between *ebo/ebo* mice and WT littermates (Fig. 5A). Furthermore, IL-2 and IFN-γ production by splenic T cells following stimulation with immobilized anti-CD3 mAb was comparable in the two strains (Fig 5B, C). We next assessed the T cell response to immunization with the T-dependent (TD) antigen TNP-KLH. Splenic T cell proliferation and cytokine secretion in response to *in vitro* re-stimulation with KLH were comparable in *ebo/ebo* mice and WT littermates (Fig. 5D-F). Furthermore, the titers of both high and low affinity anti-TNP IgG antibodies were comparable in the two strains (Fig. 5G, H). The normal TD antibody response of *ebo/ebo* mice, together with the previously demonstrated normal TI antibody response in *Lrrc8a*^{-/-} mice, suggest that VRAC activity is not essential for B cell function.

DISCUSSION:

We demonstrate that *ebo/ebo* mice, which express a truncated LRRC8A mutant that virtually lacks VRAC activity have normal T cell development and function, but retain some of the phenotypic features of LRRC8A^{null} mice. These findings demonstrate that LRRC8A-dependent VRAC activity is dispensable for T cell development and function.

It is important to note that when the VRAC activity of T lymphocytes and peritoneal macrophages were evaluated in response to osmotic stress at -80 mV, which approximates resting membrane potential, there was dramatic reduction in VRAC activity, similar to *Lrrc8a*^{-/-} mice. This finding strongly suggests that under physiologic conditions, the 15 terminal LRRs that are deleted in the LRRC8A^{ebo} mutant are necessary for VRAC activity. In a previous study, the LRRC8A^{Δ91/+35} mutant in which the two and a half terminal LRRs are replaced by 35 novel aa encoded by intronic nucleotides, failed to generate a VRAC current (4). The observation that in contrast to LRRC8A^{ebo}, LRRC8A^{Δ91/+35} perturbed T and B cell development, suggests that the added 35 amino acids rather than loss of VRAC activity may have played an important role in the effect the LRRC8A^{Δ91/+35} mutant exerted on T and B cells.

Given the dramatic defects in T cell development and function in *Lrrc8a*^{-/-} mice, it was surprising to find that *ebo/ebo* mice, which virtually lacked VRAC activity, had normal thymocyte development, normal thymus morphology and normal *in vitro* and *in vivo* T cell function. This suggests a role for LRRC8A in T cell development function that is distinct from its role in the regulation of cellular volume. We previously found that ligation of LRRC8A results in AKT phosphorylation in thymocytes (3). Furthermore a GST-LRRC8A₃₄₃₋₈₁₀ fusion protein containing all 468 C-terminal aa that follow the TM4 region bound to TECs and to the OP9-DL1 cell line and inhibited OP9-DL1 driven maturation of DN thymocytes into DP

thymocytes (3), suggesting that aa 343-810 of LRRC8A are important for T cell development. However, since LRRC8A may multimerize via its LRR repeats (12), we cannot rule out the possibility that another region of LRRC8A binds to the putative ligand and that the inhibition by GST-LRRC8A₃₄₃₋₈₁₀ is due to steric effects. Further experiments are needed to precisely map the region of LRRC8A that interacts with its putative ligand.

Epitope tagging studies suggest that both N- and C- termini may be intracellular (4, 5). However, studies using antibodies directed at the C-terminus of LRRC8A and the loop between the TM2 and TM3 regions, clearly indicate that a fraction of LRRC8A is inserted in the cell membrane in the opposite orientation, in which both the N- and C- termini are extracellular (3). Dual membrane topology has been described for a number of other proteins that span the plasma membrane four times (13) and the available evidence (3–5) supports such a model for LRRC8A (Fig. 6). We suggest that when the N and C termini of LRRC8A are extracellular (Orientation A, Fig. 6A) the LRR-containing C-terminal region is extracellular and is available to interact with the putative LRRC8A ligand, resulting in GRB2-LCK-ZAP-70 mediated activation of AKT and thymocyte survival. In contrast, when the N and C termini are intracellular (Orientation B, Fig. 6B) the LRR-containing C-terminal region of LRRC8A is intracellular and may mediate VRAC activity (Fig 6B).

In summary, we have identified a hypomorphic mutation in LRRC8A, LRRC8A^{ebo}, that dramatically reduces VRAC activity, but spares T cell development and function. Although under physiologic voltage conditions VRAC activity was virtually negligible in T cells from both *ebo/ebo* and *Lrrc8a*^{-/-} mice, unlike *Lrrc8a*^{-/-} mice which demonstrate a severe defect in T cell development, *ebo/ebo* mice had intact T cell development. This leads us to conclude that the role LRRC8A plays in T cells is independent of its role in regulating cellular volume.

FIGURE LEGENDS

Figure 1. *ebo* mutation leads to truncation of LRRC8A c-terminus while preserving protein expression. (A) Sanger sequencing showing a 2 bp deletion (del TG) in exon 3 of *Lrrc8a* in *ebo/ebo* mice. (B) Semi-quantitative RT-PCR of *Lrrc8a* mRNA from splenocytes of WT and *ebo/ebo* mice. (C) Immunoblot analysis of LRRC8A expression in splenocytes of WT and *ebo/ebo* mice using anti-LRRC8A₇₉₁₋₈₀₈ Ab (D). Comparison of the aa sequence 87-104 in LRRC8A and the corresponding sequences in LRRC8 family member homologues. Amino acids shared between LRRC8 family members and LRRC8A are highlighted in yellow. (E) Flow cytometric analysis of intracellular LRRC8A expression in CD3⁺ T cells of CD4 Cre *Lrrc8a*^{f/f} mice and C57BL/6 WT controls (left panel) and *ebo/ebo* mice and FVB/N WT controls (right panel) using anti-LRRC8A₈₇₋₁₀₄ Ab. Columns and bars represent means and SEM. ns = not significant. *** $p < 0.001$ (Student's *t* test).

Figure 2. Effect of the *ebo* mutation on mouse phenotype. (A) Gross appearance at 8 weeks of age of *ebo/ebo*, *+ebo* and WT littermates. (B) Weight at 11 weeks of age of *ebo/ebo* (n=14) and WT (n=15) littermates. (C) Kaplan-Meier analysis of survival of *ebo/ebo* mice and WT littermates (n=10 from each group). (D) Representative H&E-stained tissue sections from salivary gland, liver, and kidney of *ebo/ebo* mice and WT littermates (n=3 per group), 10x magnification, bars = 200 μ m. Arrow shows tubular degeneration. Results in C and D are representative of 3 independent experiments, each using one mouse per strain. Columns and bars represent means and SEM. ns = not significant. * $p < 0.05$ (log-rank test).

Figure 3. *ebo* dramatically reduces VRAC activity. (A) Solution scheme used for patch clamp analysis. (B, D) Current traces induced in splenic T cells (B) and peritoneal macrophages (D) from WT mice (left panels) and *ebo/ebo* mice (right lower panels) by the indicated voltage

step protocol shown in the right upper panels. Dashed line indicates 0 current. (C, E) Current densities induced in splenic T cells (C) and peritoneal macrophages (E) from *ebo/ebo* mice, *Lrrc8a*^{-/-} mice and WT controls by a 220 mOsm hypotonic stimulus at -80 mV (left panels) or +80 mV (right panels). Results are representative of 8-12 cells pooled from 3 or more independent experiments using one mouse per group in each experiment.

Figure 4. Normal T cell development and function in *ebo/ebo* mice. (A) Gross appearance of thymus and (B) number of thymocytes in 3 week old WT and *ebo/ebo* mice. (C) H&E staining of thymus from WT and *ebo/ebo* mice (left panels: 10x, C=cortex, M=medulla, bars=200 μ m, right panels: medullary area 60x, bars=30 μ m). (D) Percentages of DN, DP and SP thymocytes in 3 week old WT and *ebo/ebo* mice. (E) Percentages of DN1, DN2, DN3 and DN4 thymocytes in WT and *ebo/ebo* mice. (F) AKT and pAKT immunohistochemistry of thymus from WT and *ebo/ebo* mice. 40x, bars=50 μ m). (G) Number of splenocytes, (H) percentages of CD3⁺ and B220⁺ cells, and (I) percentages of CD4⁺ and CD8⁺ cells in spleens from 11 week-old WT and *ebo/ebo* mice. Results in B represent 4 mice per group pooled from 2 independent experiments. Results in D and E are of one representative experiment with 3 mice per group. Similar results were obtained in 2 additional experiments with 1 mouse per group each. Results in G represent 9 mice per group pooled from 3 independent experiments. Columns and bars represent mean and SEM. Results in H and I represent 4 mice per group pooled from 2 independent experiments.

Figure 5. Normal T cell function in *ebo/ebo* mice. (A-C) Proliferation (A) and secretion of IL-2 (B) and IFN- γ (C) by splenic T cells from WT and *ebo/ebo* mice in response to stimulation with immobilized anti-CD3 mAb, anti-CD3+anti-CD28 mAbs or , anti-CD3 mAb + IL-2. (D-F) Proliferation (D) and secretion of IL-2 (E) and IFN- γ (F) by splenic T cells from WT

and *ebo/ebo* mice in response to stimulation with KLH. High and low affinity TNP-specific serum antibody titers in KLH-TNP immunized WT and *ebo/ebo* mice. Results in A-C are pooled from one experiment with 3 mice per group. Similar results were obtained in 3 other experiments with 1 mouse per group each. Results in D-H are pooled from three experiments with 4 mice per group. Columns/lines and bars represent means and SEM.

Figure 6. Schematic representation of the membrane topology of LRRC8A and interacting molecules. The two opposite orientations that LRRC8A potentially assumes (A and B) and their distinct roles in T cell signaling (orientation A) and VRAC activity (orientation B) are shown. The LRRC8A region lacking in the *ebo* mutant is shown in light blue.

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References

1. Sawada A et al. A congenital mutation of the novel gene LRRC8 causes agammaglobulinemia in humans. *J. Clin. Invest.* 2003;112(11):1707–1713.
2. Smits G, Kajava A V. LRRC8 extracellular domain is composed of 17 leucine-rich repeats. *Mol. Immunol.* 2004;41(5):561–562.
3. Kumar L et al. Leucine-rich repeat containing 8A (LRRC8A) is essential for T lymphocyte development and function. *J. Exp. Med.* 2014;211(5):929–942.
4. Qiu Z et al. SWELL1, a Plasma Membrane Protein, Is an Essential Component of Volume-Regulated Anion Channel. *Cell* 2014;157(2):447–458.
5. Voss FK et al. Identification of LRRC8 heteromers as an essential component of the volume-regulated anion channel VRAC. *Science* 2014;344(6184):634–638.
6. Pedersen SF, Klausen TK, Nilius B. The identification of a volume-regulated anion channel: an amazing Odyssey. *Acta Physiol.* 2015;213(4):868–81.
7. Cahalan MD, Lewis RS. Role of potassium and chloride channels in volume regulation by T lymphocytes. *Soc Gen Physiol Ser.* 1988;43:281–301.
8. Juntilla MM, Koretzky GA. Critical roles of the PI3K/Akt signaling pathway in T cell development. *Immunol. Lett.* 2008;116(2):104–110.
9. Hoffmann EK, Lambert IH, Pedersen SF. Physiology of cell volume regulation in vertebrates.. *Physiol. Rev.* 2009;89(1):193–277.
10. Syeda R et al. LRRC8 Proteins Form Volume-Regulated Anion Channels that Sense Ionic Strength. *Cell* 2016;164(3):499–511.
11. Lalouette A, Lablack A, Guenet JL, Montagutelli X, Segretain D. Male sterility caused by sperm cell-specific structural abnormalities in ebouriffé, a new mutation of the house mouse.

- 426 *Biol. Reprod.* 1996;55(2):355–363.
- 427 12. Bella J, Hindle KL, McEwan PA, Lovell SC. The leucine-rich repeat structure. *Cell. Mol.*
- 428 *Life Sci.* 2008;65(15):2307–2333.
- 429 13. von Heijne G. Membrane-protein topology. *Nat. Rev. Mol. Cell Biol.* 2006;7(12):909–918.
- 430

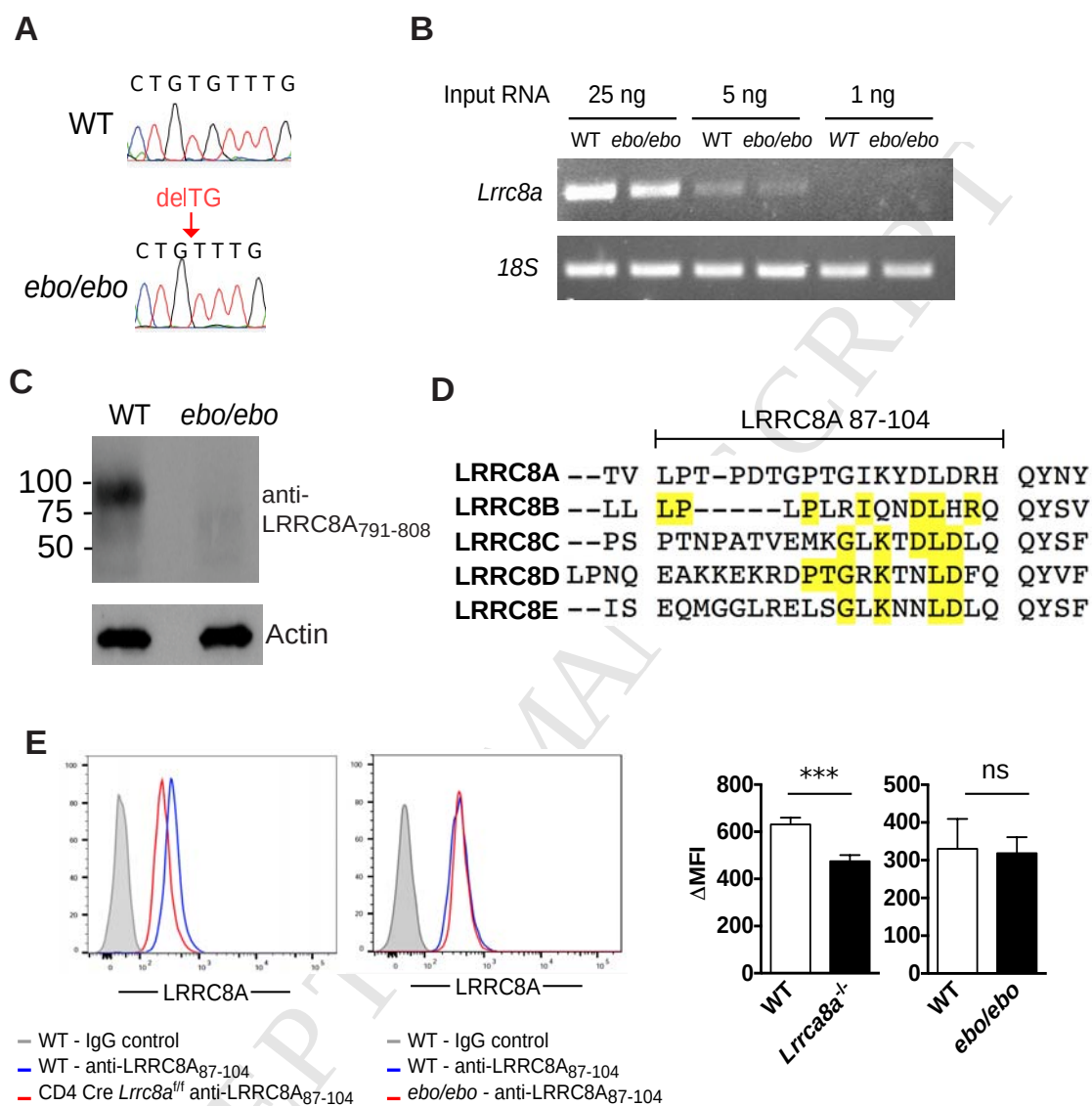
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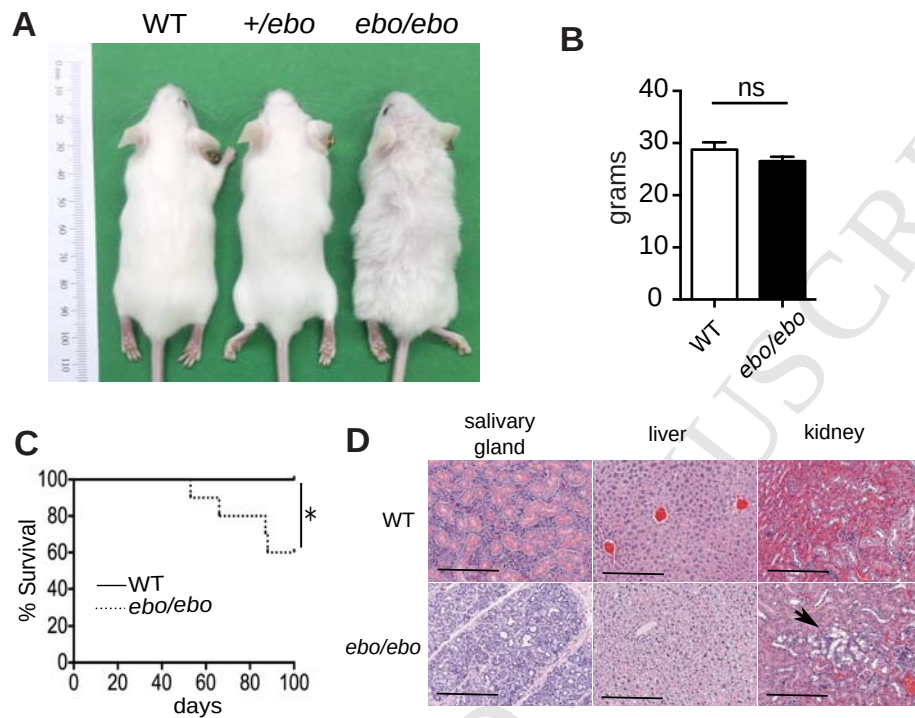
Figure 2

Figure 3

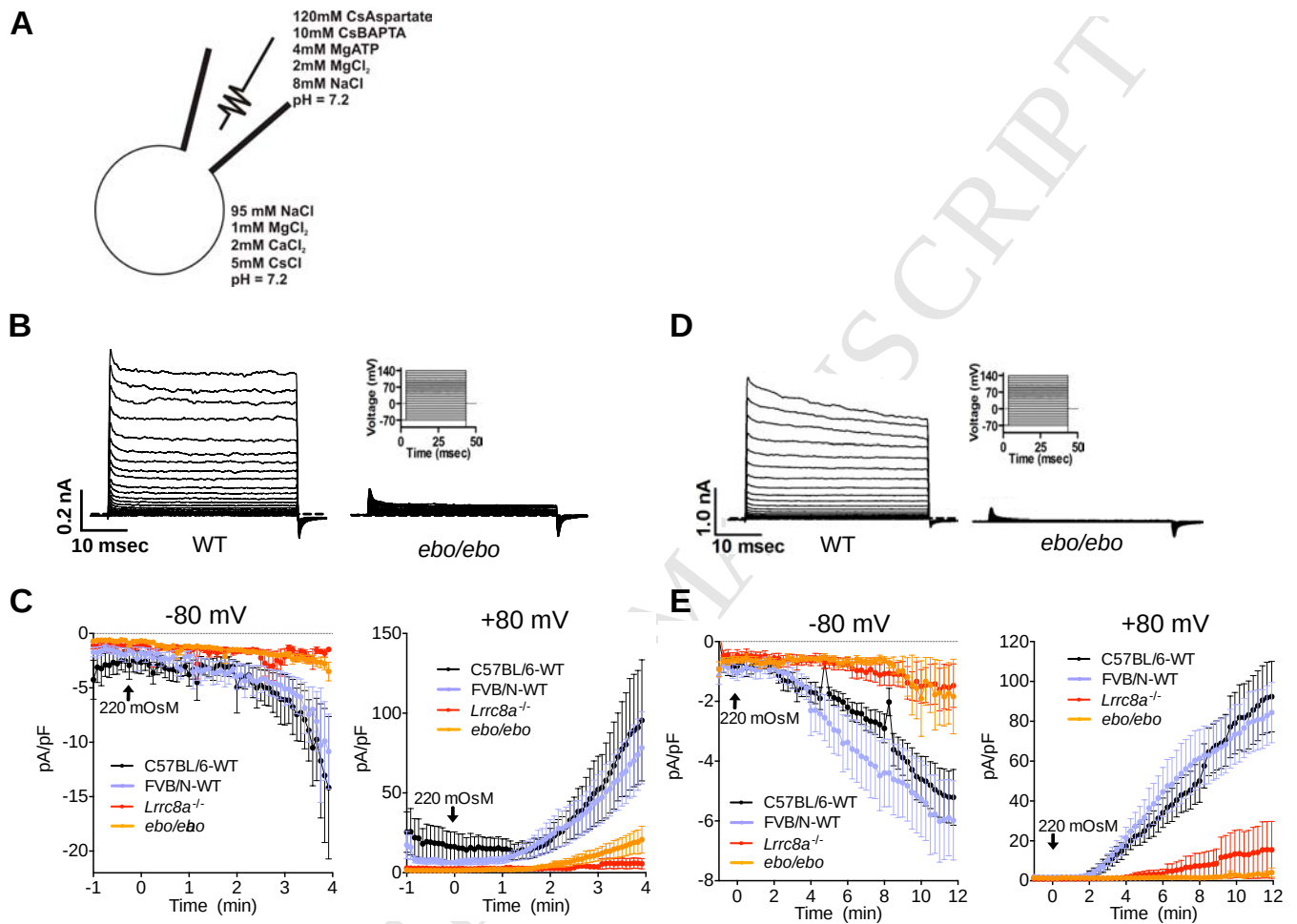


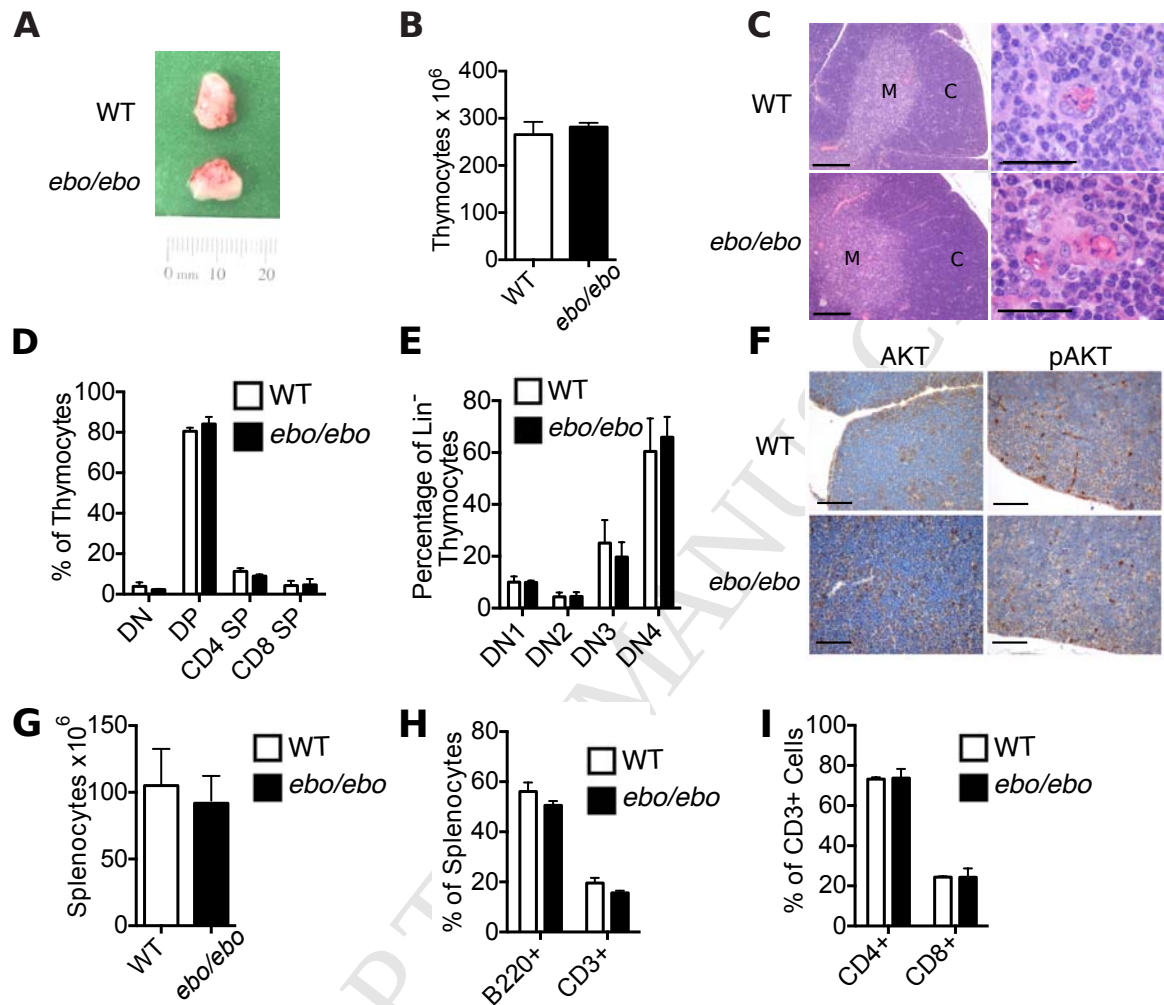
Figure 4

Figure 5

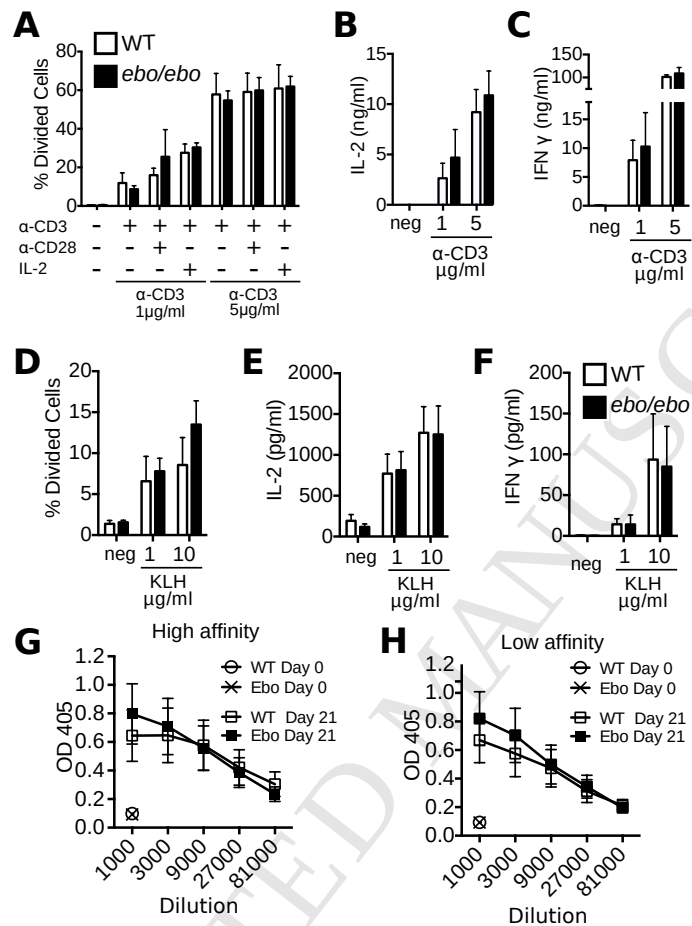
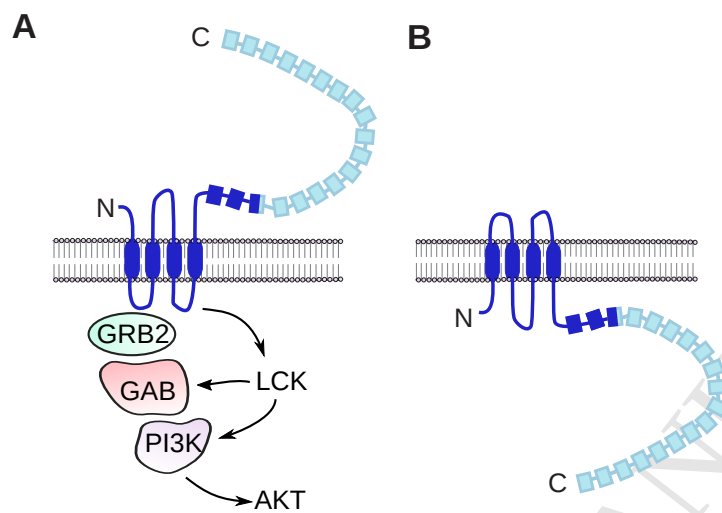
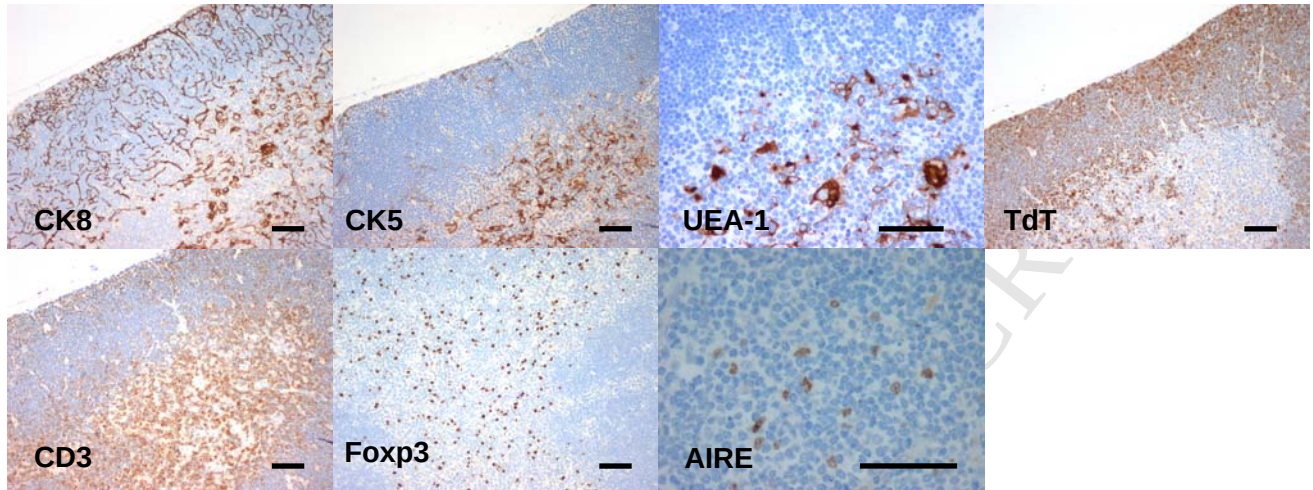


Figure 6

Supplementary Figure 1

WT



ebo/ebo

