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Tyrosine Phosphorylation-independent Regulation of LPS-mediated Response by the Transmembrane Adaptor Protein LAB

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Abstract

LAB (linker for activation of B cells)/NTAL (non-T cell activation linker) is a transmembrane adaptor protein that functions in immunoreceptor-mediated signaling. Published studies have shown that LAB has both positive and negative roles in regulating T cell receptor and high-affinity Fc receptor-mediated signaling and cellular function. In this study, we showed that LAB was also expressed in dendritic cells and that LAB deficiency affected LPS-mediated signaling and cytokine production. LPS-mediated MAPK activation was enhanced in $LAB^{-/-}$ bone marrow-derived dendritic cells (BMDCs). These BMDCs also produced more TNF-α, IL-6, and IL-10 than WT cells. Moreover, $LAB^{-/-}$ mice were hyper responsive to LPS-induced septic shock. These data indicated that LAB has a negative role in LPS-mediated responses. By using LAB knock-in mice, which harbor mutations at five membrane-distal tyrosines, we further showed that, in contrast to its role in immunoreceptor-mediated signaling, LAB function in LPS-mediated signaling pathway did not depend on its tyrosine phosphorylation. Our study suggested a novel mechanism by which LAB functions in the regulation of innate immunity.

Keywords

dendritic cells; cell activation; tyrosine phosphorylation; signal transduction

INTRODUCTION

Upon the engagement of immunoreceptors, transmembrane adaptor proteins (TRAPs) are phosphorylated on multiple tyrosine residues and serve as scaffolds that recruit SH2-containing proteins to the plasma membrane and ultimately cause the activation of downstream signaling cascades(1–3). One of the most studied TRAPs is LAT (linker for activation of T cells). Studies using LAT-deficient Jurkat cells and LAT knockout mice indicate that LAT is essential during T cell activation and thymocyte development(4, 5). In addition, LAT is critical for FcεRI-mediated signaling and mast cell function(6).

LAB (linker for activation of B cells), also called NTAL (non-T cell activation linker), is the other member of the LAT family. Like LAT, it is palmitoylated and has multiple tyrosine residues in its cytoplasmic domain. Upon engagement of the BCR and FcεRI, LAB is phosphorylated and interacts with Grb2 and other signaling proteins(7, 8). In contrast to LAT, LAB does not contain a PLC-γ1 site, therefore, fails to bind to PLC-γ1. Functionally, LAB is able to partially restore thymocyte development in $LAT^{-/-}$ mice(9). Among the nine

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conserved tyrosine residues in LAB, the three membrane-distal tyrosines are the most heavily phosphorylated following receptor engagement. Mutation of these tyrosines abolishes LAB phosphorylation and function(10). While LAT clearly plays an indispensable role in T cells and mast cells, LAB has been shown to be important to fine-tune the activation and function of these cells. LAB deficiency leads to enhanced TCR-mediated LAT, PLC-γ1, AKT, and ERK phosphorylation, as well as increased calcium flux. Conversely, overexpression of LAB suppresses these signaling events(11). Similarly, $LAB^{-/-}$ mast cells display increased FcεR-Imediated PLC-γ phosphorylation, calcium mobilization, ERK activation, and degranulation, implying that LAB has a negative role in mast cell signaling. However, mast cells lacking both LAT and LAB have a more dramatic block in FcεRI signaling than $LAT^{-/-}$ mast cells, indicating that LAB may also exert a positive effect on FcεRI-mediated signaling(12, 13). Together, these data suggest that LAB has both positive and negative roles in TCR and FcεRI-mediated signaling.

While LAB is highly expressed in B cells, mast cells, and NK cells, it is also expressed in macrophages. Recent studies have suggested that LAB functions in the regulation of TREM(triggering receptor expressed on myeloid cells)-mediated signaling in macrophages(14, 15). TREM-1 is a glycoprotein that is mainly expressed on myeloid cells. It interacts with DAP12 (DNAX activation protein of 12kDa), an adaptor protein containing an ITAM motif. Upon cross-linking of TREM-1, DAP12 is tyrosine phosphorylated, leading to the activation of PLC-γ and ERK. TREM-1 cross-linking also leads to LAB phosphorylation. Knocking down LAB protein expression enhances ERK activation, calcium flux, and production of TNF-α and IL-8(14), suggesting that LAB negatively regulates TREM-1-mediated signaling. Interestingly, another study indicates that LAB has a positive role in TREM-2 signaling. LAB is also phosphorylated after crosslinking of TREM-2; however, TREM-2-mediated ERK activation is reduced in $LAB^{-/-}$ macrophages(15). Together, these studies reveal that LAB may have an additional role in innate immune responses, although it is still not clear how LAB regulates TREM-mediated signaling and macrophage function.

In this study, we showed that LAB is expressed in dendritic cells (DCs). LPS-mediated MAPK activation and cytokine production were enhanced in $LAB^{-/-}$ DCs, suggesting that LAB has a negative role in LPS-mediated responses. This negative role of LAB was supported by our *in vivo* data indicating that $LAB^{-/-}$ mice were hyper responsive to LPS-induced septic shock. By using LAB knock in mice, which contain mutations at the five membrane distal tyrosines, we further demonstrated that LAB function in LPS-mediated signaling in DCs was not dependent on its tyrosine phosphorylation. Our data suggested a novel mechanism by which LAB functions to regulate innate immunity.

MATERIALS AND METHODS

Mice

$LAB^{-/-}$ mice were described previously(12). $LAB^{m/m}$ knock-in mice with mutations at five tyrosines (Y^{98}, Y^{121}, Y^{139}, Y^{160}, and Y^{192})were generated by using the targeting construct illustrated in Fig. 4A. This construct was used to transfect ES cells. Two positive clones were used to generate chimeric mice, which were then crossed with β-actin-Cre transgenic mice to delete the PGK-Neo cassette. Mice expressing the LAB 5YF mutant ($LAB^{m/+}$) were backcrossed with C57Bl/6 mice for at least 10 generations. $LAB^{m/+}$ mice were intercrossed to generate $LAB^{m/m}$ mice. Mice used in this study were bred and maintained in specific pathogen-free conditions at the Duke University animal facility. All mice were used in accordance with the National Institutes of Health guidelines. The experiments described in this study were reviewed and approved by the Duke University Animal Care Committee.

Differentiation of BMDC, BMMC, and BMMφ *in vitro*

Bone marrow cells were extracted from the femurs of $LAB^{-/-}$, $LAB^{m/m}$, and $LAB^{+/+}$ mice. For *in vitro* differentiation of BMDCs, $1x10^7$ BM cells were cultured in 10ml RPMI 1640, 10% FBS, 1% non-essential amino acid, and 10% GM-CSF supernatant from X63 cell culture. Non-adherent cells were harvested at day 6–7. To induce the maturation of BMDCs, cells were stimulated with 1μg/ml LPS for 16–24 hours. BMMCs were generated by culturing BM cells in the presence of IL-3 for 3–4 weeks. BMMφs were derived by culturing BM cells in L929 conditioned media for 6–7 days.

Cell purification and FACS analysis

Single-cell suspensions were prepared, incubated with the 2.4G2 antibody, and stained with fluorescence-conjugated antibodies. Fluorescence-conjugated antibodies, such as CD4, CD8, CD11c, B220, CD11b, MHC class II, CD40, CD86, and CD80 were purchased from Biolegend, Inc. For purification of $CD4^+$, $CD8^+$, $B220^+$, and $CD11c^+$ cells from spleens, single-cell suspensions were incubated with biotin-conjugated CD4, CD8, B220, and CD11c antibodies. Cells were further purified using anti-biotin EasySep kit (StemCell Technology). Purified cells were then subjected to FACS analysis to monitor the purity. Live cells were identified using 7AAD or Live/Dead staining kit (Invitrogen). FACS data were acquired using the FACS-Canto (BD Biosciences) and analyzed with the FlowJo software.

For intracellular staining of cytokines, cells were stimulated as indicated in the figures in the presence of Monensin (BioLegend). Cells were first stained with surface markers, fixed, and then stained intracellularly using cytokine staining sets (BD Biosciences). Cytokine levels in the sera or culture supernatants were measured by ELISA following the manufacturer's protocol (eBiosciences).

LPS-induced septic shock

Age- (5–7 weeks old) and sex-matched $LAB^{-/-}$, $LAB^{m/m}$, and $LAB^{+/+}$ mice were challenged with LPS (E. coli 0111:B4, from Sigma-Aldrich) by i.p. injection (20mg/kg body weight). The survival of mice was monitored for up to 30 hours. For cytokine production *in vivo*, mice were injected with either LPS (5mg/kg body weight) or PBS. Blood was collected from tail veins at 1.5 and 4 hours after injection.

Immunoprecipitation and Western blotting

Anti-LAB sera were described previously(7). Antibodies used in Western blotting were anti-pTyr (4G10), anti-pERK, pp38, pJNK, pNFκB, NFκB, pIκB, IκB, pSyk/Zap, Syk (Cell Signaling), ERK2, and JNK1 (Santa Cruz). Cells were lysed in RIPA lysis buffer (1% Triton, 0.5% sodium deoxycholic acid, 0.1% SDS, 25mM Tris-Cl pH7.6, 150mM NaCl, 5mM EDTA, 1mM Na_3VO_4). Lysates were used in immunoprecipitation and Western blotting analysis. Immunoprecipitated proteins or total lysates were resolved on SDS-PAGE and transferred to nitrocellulose membranes. After incubation with primary antibodies, membranes were blotted with either anti-mouse or rabbit Ig conjugated to AlexaFluor 680 (Molecular Probes) or IRDye 800 (Rockland). The signal on the membranes was then detected using the LI-COR Bioscience Odyssey system (LI-COR).

Calcium Flux

To prepare activated T cells, splenocytes were cultured in anti-CD3 antibody (2C11)-coated plates in the presence of murine IL-2 (10ng/ml) for 2 days and then were moved into new plates to expand for three more days in the presence of IL-2. Activated T cells were rested for 4–6 hours and were then loaded with 4μM Fluo-4 (Molecular Probe). Calcium mobilization was initiated by the addition of biotin-anti-CD3 and biotin-anti-CD8, followed

by cross-linking with 25 μg/ml streptavidin (Sigma), and was monitored using FACS-Canto. BMMCs were first sensitized with anti-DNP IgE for 4–6 hours, and then loaded with 4μM Fluo-4. Calcium mobilization was initiated by the addition of 100ng/ml DNP-HSA.

Real-Time PCR

Total RNAs from purified cell populations were extracted with the Trizol reagents. cDNAs were synthesized with the Super-Script reverse transcriptase (Invitrogen) using oligo-dT as the primer. Quantification of LAB and cytokine RNAs was performed by real-time PCR with SYBR Green Super mix (Bio-Rad). The primers used to amplify LAB were described previously(11).

RESULTS

Expression of LAB in dendritic cells

Our published data show that LAB is expressed in B cells, mast cells, and activated T cells(7, 11, 12). LAB is also expressed in NK cells and macrophages(15, 16). To explore the potential function of LAB in DCs, we first examined the expression of LAB in these cells. We purified $CD4^+$, $CD8^+$, $B220^+$, and $CD11c^+$ cells from the spleens of wild type (WT) mice by FACS sorting. We also derived dendritic cells (BMDC), mast cells (BMMC), and macrophages (BMMφ) from the bone marrow of WT mice. Total RNAs were made from these cells and used in real-time PCR. As shown in Fig. 1A, the amount of LAB RNA in splenic $CD11c^+$ cells was similar to that in $B220^+$ cells. In addition, LAB transcript was detected in BMDCs, although at a level lower than that in BMMCs or BMMφs. We next determined whether LAB protein was present in those cells. BMDCs, together with B cells, activated T cells, BMMCs, and BMMφs, were lysed and postnuclear lysates were subjected to immunoprecipitation with anti-LAB sera followed by anti-LAB Western blotting. The lysate of B cells from $LAB^{-/-}$ mice was used as a negative control. As indicated in Fig. 1B, LAB protein could clearly be detected in BMDCs.

Since LAB is expressed in dendritic cells, we next examined whether LAB deficiency affects dendritic cell differentiation and maturation *in vitro*. Bone marrow cells from $LAB^{+/+}$(WT) and $LAB^{-/-}$ mice were cultured in media with GM-CSF for 6–7 days to differentiate into DCs. FACS analysis showed that similar percentages of $CD11c^+$ cells were derived (data not shown), suggesting that DC differentiation *in vitro* is not affected by LAB deficiency. We further examined whether $LAB^{-/-}$ DCs could undergo maturation normally upon LPS stimulation. Analysis of different activation markers showed that upon LPS stimulation, $LAB^{-/-}$ DCs were able to upregulate MHC class II ($I\text{-}A^b$), CD40, CD80, and CD86; however, the percentage of $LAB^{-/-}$ DCs that expressed high levels of these markers was lower than that of $LAB^{+/+}$ DCs (Fig. 1C). Additionally, there was a substantial percentage of $LAB^{-/-}$ DCs with low levels of class II, CD80, and CD86, even after LPS stimulation. Interestingly, the basal level of class II expression on $LAB^{-/-}$ DCs was lower compared with WT DCs. These data indicated that LPS-mediated DC maturation *in vitro* was impaired by LAB deficiency.

Enhanced cytokine production by $LAB^{-/-}$ BMDC

Next, we examined the ability of $LAB^{-/-}$ BMDCs to produce cytokines. $LAB^{-/-}$ and $LAB^{+/+}$ BMDCs were stimulated with different concentrations (0 to 1000ng/ml) of LPS for 16 hrs. The supernatants were collected and used in ELISA to quantitate cytokine concentration. As shown in Fig. 2A, $LAB^{-/-}$ BMDCs produced increased levels of IL-10; however, they produced lower levels of IL-12. The production of IL-6 and TNF-α was similar between $LAB^{-/-}$ and $LAB^{+/+}$ cells (Fig. 2A and data not shown). Interestingly, $LAB^{-/-}$ BMDCs produced a detectable amount of IL-10 with low concentrations of LPS,

suggesting that these DCs were able to constitutively produce IL-10. Addition of high concentrations of LPS induced more production of IL-10. IL-10 is known to suppress the production of pro-inflammatory cytokines, such as IL-12, by dendritic cells and macrophages and to inhibit the ability of antigen-presenting cells (APCs) to induce Th1 differentiation(17). Thus, the reduced production of IL-12 by $LAB^{-/-}$ BMDCs is likely due to increased production of IL-10. We also quantified the transcription levels of these cytokines by real-time PCR. The transcript levels of these cytokines correlated with their concentrations in the supernatants (data not shown).

Enhanced MAPK activation in LPS-stimulated $LAB^{-/-}$ BMDCs

Since LPS-induced cytokine production by $LAB^{-/-}$ BMDCs was enhanced, we further examined whether LAB deficiency affected LPS-mediated activation of signaling pathways. LPS stimulation activates two main pathways, MAPK (ERK, JNK, and p38) and NFκB. $LAB^{-/-}$ and $LAB^{+/+}$ BMDCs were stimulated with LPS at 100ng/ml and lysed at different time points. Activation of the MAPK and NFκB pathways was analyzed by Western blotting with different phospho-specific and pan-antibodies, followed by quantification of each band. As shown in Fig. 2B, activation of ERK, JNK, and p38 was enhanced in $LAB^{-/-}$ BMDCs at 0.5 and 1 hour after LPS stimulation. Among the three MAPKs, p38 and JNK showed enhanced and sustained activation in $LAB^{-/-}$ BMDCs. However, activation of the NFκB pathway, as indicated by phosphorylation of NFκB and IκBα and degradation of IκBα, was not affected. These results indicated that LAB negatively regulates LPS-mediated MAPK activation.

$LAB^{-/-}$ mice are hyper responsive to LPS-induced septic shock

We next examined whether LAB regulates innate immune responses *in vivo* using a mouse model of LPS-induced septic shock. $LAB^{+/+}$and $LAB^{-/-}$ mice (5 per group) were injected with LPS (20mg/kg body weight) and their survival was monitored. At this dose, all $LAB^{+/+}$mice survived; however, $LAB^{-/-}$ mice died within 30 hrs (Fig. 3A), indicating that $LAB^{-/-}$ mice are more susceptible to LPS-induced septic shock.

LPS-induced death is caused by the excessive production of cytokines, such as TNF-α. To determine whether LAB deficiency also affects cytokine production *in vivo*, WT and $LAB^{-/-}$ mice were injected with a low dose of LPS (5mg/kg body weight). Sera collected from these mice at different time points were used to determine the concentrations of IL-6, TNFα, IL-12, and IL-10 by ELISA. As shown in Fig. 3B, compared with WT controls, $LAB^{-/-}$ mice produced significantly higher levels of TNFα, IL-6, and IL-10, but similar amounts of IL-12. Therefore, LAB plays a role in the negative regulation of LPS-induced cytokine production *in vivo*.

LAB phosphorylation in activated T cells and BMMCs

Our previous data showed that the membrane-distal tyrosine residues in the cytoplasmic domain of LAB are critical for LAB function(10). To further determine the importance of LAB phosphorylation in the activation of different signaling pathways, we generated LAB5YF knock-in mice ($LAB^{m/m}$) with mutations at the five critical tyrosines that are within a Grb2-binding motif (Y^{98}, Y^{121}, Y^{139}, Y^{160}, and Y^{192}). Our published data indicate that at least three of these tyrosines in human LAB are phosphorylated and necessary to rescue LAT deficiency during thymocyte development(10). We made a targeting construct in which exons 7–12 were replaced with a cDNA fragment carrying Tyr to Phe mutations at these residues (Fig. 4A). After transfection of ES cells, nine positive clones were identified and two of them were used to generate chimeric mice, which were then crossed with β-actin-Cre transgenic mice to delete the PGK-Neo cassette. Mice carrying Tyr to Phe mutations ($LAB^{m/+}$) were backcrossed with C57Bl/6 mice for at least 10 generations to eventually

produce $LAB^{m/m}$ miceon the B6 background. To confirm expression of the mutant LAB protein, we analyzed whole lysates of bone marrow-derived macrophages from $LAB^{-/-}$, $LAB^{m/-}$, and $LAB^{+/-}$ mice by anti-LAB Western blotting. As shown in Fig. 4B, mutant LAB in $LAB^{m/-}$ cells was expressed at a similar level as endogenous LAB in $LAB^{+/-}$ cells.

To determine the role of tyrosine phosphorylation in LAB function, we first examined whether LAB function in TCR-mediated signaling is affected by these mutations. T cells from WT, $LAB^{-/-}$, and $LAB^{m/m}$ mice were expanded *in vitro* by anti-CD3 stimulation in the presence of IL-2 for 2–3 days. After resting for 6 hours, these T cells were restimulated with anti-CD3 and anti-CD8 for the indicated time points (minutes) before lysis and analyzed by immunoblotting with different antibodies. As shown in Fig. 5A, although TCR-mediated protein phosphorylation was similar in these cells, LAT phosphorylation was increased in $LAB^{-/-}$ and $LAB^{m/m}$ cells. We also measured TCR-mediated calcium flux in these T cells. Similar to $LAB^{-/-}$ cells, $LAB^{m/m}$ cells showed enhanced calcium flux. In addition, $LAB^{-/-}$ and $LAB^{m/m}$ $CD8^+$ T cells also produced more IFN-γ. More importantly, like $LAB^{-/-}$ mice(11), aged $LAB^{m/m}$ mice developed splenomegaly and had a higher percentage of activated/memory T cells ($CD44^+CD62L^-$) (data not shown).

Published data indicate that LAB also negatively regulates FcεRI-mediated signaling and mast cell function(12, 13). We derived mast cells from the bone marrow of WT, $LAB^{-/-}$, and $LAB^{m/m}$ mice. These cells were sensitized with anti-DNP IgE and activated by crosslinking with DNP-HSA. As shown in Fig. 5D, FcεRI-mediated degranulation by both $LAB^{-/-}$ and $LAB^{m/m}$ mast cells was enhanced. Similar to TCR-mediated calcium flux, FcεRI-mediated calcium flux was also augmented in $LAB^{-/-}$ and $LAB^{m/m}$ mast cells. Together, our data indicated that these tyrosine residuesare necessary for LAB function in the negative regulation of TCR- or FcεRI-mediated signaling.

LAB phosphorylation in LPS signaling and response

We next examined whether the function of LAB in dendritic cells is affected by Tyr to Phe mutations. We derived dendritic cells from the bone marrow of WT, $LAB^{-/-}$, and $LAB^{m/m}$ mice to examine LPS-mediated production of IL-10 and IL-12, two cytokines that were affected by LAB deficiency (Fig. 2A). Similar to the data in Fig. 2A, LPS-induced IL-10 production by $LAB^{-/-}$ DCs was enhanced, while IL-12 production was reduced. Interestingly, $LAB^{m/m}$ DCs produced similar amounts of IL-10 and IL-12 as WT controls (Fig. 6A). These data suggested that, in dendritic cells, the five membrane-distal tyrosine residues in LAB were not required for its negative role in LPS-mediated cytokine production *in vitro*.

Our data demonstrated that the five membrane-distal tyrosine residues are important in the regulation of TCR- and FcεRI-mediated signaling; however, they are dispensable for LPS-mediated cytokine production. Despite the fact that LAB deficiency affected LPS-mediated MAPK activation (Fig. 2B), we failed to detect LPS-mediated LAB phosphorylation in WT BMDCs (Fig. 6B). As a positive control, we stimulated DCs for 5 minutes with pervanadate, which strongly induces protein phosphorylation through the inhibition of phosphatase activity. Pervanadate induced strong LAB phosphorylation in $LAB^{+/+}$ DCs (Fig. 6B). The phosphorylation of LAB in $LAB^{m/m}$ DCs was greatly reduced, indicating that these tyrosines were successfully mutated.

To further determine how LAB negatively regulates LPS-mediated signaling, we examined total phosphorylation of proteins after LPS stimulation of WT, $LAB^{-/-}$, and $LAB^{m/m}$ DCs. As shown in Fig. 6C, LPS-induced tyrosine phosphorylation of proteins in $LAB^{-/-}$ cells, especially a protein with molecular weight around 72kDa, was enhanced compared with that in WT and $LAB^{m/m}$ cells. Based on its molecular weight, we speculated that this protein is

likely the Syk kinase. Further Western blotting analysis using an anti-pSyk (Tyr352) antibody showed that Syk phosphorylation was indeed enhanced in $LAB^{-/-}$ DCs. Notably, Syk phosphorylation in WT and $LAB^{m/m}$ cells was similar. These data suggest that LAB likely regulates Syk activation through a novel mechanism that is independent of its five membrane-distal tyrosines.

Since our data in Fig. 2 showed that LAB deficiency enhanced LPS-mediated MAPK activation in dendritic cells, we next examined whether mutation of these tyrosine residues also affects MAPK activation. As shown in Fig. 6D, phosphorylation of ERK, JNK, and p38 in $LAB^{m/m}$ BMDCs was similar to that in WT cells after LPS stimulation, indicating that the negative role of LAB in LPS-mediated MAPK activation is independent of phosphorylation of its five membrane-distal tyrosines.

As shown in Fig. 3A, $LAB^{-/-}$ mice were hyper responsive to LPS-induced septic shock, which was likely due to enhanced cytokine production by DCs and other cell types. Next, we examined $LAB^{m/m}$ mice were also hyper responsive to LPS stimulation *in vivo*. $LAB^{m/m}$, $LAB^{-/-}$, and WT mice (8 in each group) were injected with LPS (20mg/kg body weight) and their survival was monitored. As shown in Fig. 7A, $LAB^{m/m}$ mice appeared to have an intermediate response to LPS stimulation (Fig. 7A). Further statistical analysis using the Kaplan-Meyer method showed that the differences in the survival of $LAB^{m/m}$ and $LAB^{-/-}$ mice after LPS treatment were significant, while the differences between $LAB^{m/m}$ and WT mice were not. These data suggested that LAB function in LPS-mediated responses *in vivo* is independent of these tyrosine residues. We also measured cytokine levels in the sera collected from these mice after LPS stimulation (Fig. 7B). Interestingly, while $LAB^{m/m}$ and WT mice produced similar amounts of IL-6, IL-10, and IL-12, $LAB^{m/m}$ mice produced more TNF-α than WT mice. These data suggested that LAB phosphorylation is important for TNF-α production *in vivo*, but not for IL-6, IL-10, and IL-12 production.

DISCUSSION

In this study, we demonstrated a novel role for LAB in the regulation of LPS-mediated responses. LPS, with help from other accessory molecules, such as CD14, is known to activate innate responses through binding toll-like receptor 4 (TLR4). Our data here showed that LPS-mediated activation of MAPKs was enhanced in $LAB^{-/-}$ DCs; consequently, cytokine production by these DCs was also increased. These *in vitro* data were supported by *in vivo* observations showing that $LAB^{-/-}$ mice were hyper responsive to LPS-mediated septic shock. We further demonstrated that the regulation of LPS signaling in DCs by LAB is likely independent of LAB tyrosine phosphorylation. Our data suggested that LAB regulates immunoreceptor-mediated signaling and LPS-mediated signaling through distinct mechanisms.

Previous data indicate that LAB is a transmembrane adaptor protein involved in immunoreceptor-mediated signaling. It is tyrosine phosphorylated by Syk kinase and LAB phosphorylation is important for its function. Our biochemical analysis here showed that LPS-mediated activation of ERK, JNK, and p38 MAPKs was enhanced in $LAB^{-/-}$ DCs, indicating that LAB negatively regulates MAPK activation in TLR4-mediated signaling. Although LAB deficiency clearly has an effect on LPS-mediated signaling, cytokine production, and septic shock, LAB phosphorylation was not detected upon LPS stimulation of DCs; however, Syk phosphorylation was increased in $LAB^{-/-}$ DCs. Thus, while LAB is a substrate of Syk in B cells and mast cells, it is possible that LAB can function to down-regulate Syk activity in DCs.

Syk functions downstream of a variety of receptors and regulates the activation of different cell types, either positively or negatively. While it is widely recognized that Syk is essential in BCR-mediated signaling, it is also important in innate immune responses. Recent data show that through binding to DAP12, Syk negatively regulates TLR-mediated responses, such as cytokine production and clearance of bacteria(18). TLR-mediated production of cytokines, such as IL-12, TNF, and IL-6, is increased in dendritic cells lacking SykorDAP12(19). Despite these interesting findings, how Syk downregulates TLR-mediated signaling is still unclear. Similar to $DAP12^{-/-}$ cells, $LAB^{-/-}$ DCs had increased TLR-mediated ERK, JNK, and p38 activation. If Syk indeed negatively controls LPS-mediated signaling and responses, we would expect to observe reduced MAPK activation and cytokine production by $LAB^{-/-}$ cells due to increased Syk phosphorylation and activation. Thus, in our experimental system, Syk is likely to function positively in the regulation of LPS-mediated signaling and cytokine production, as it does in Dectin-1-mediated signaling pathway(20).

Recent studies suggest that TREM-1 on monocytes and neutrophils can amplify septic response to help clearpathogens(21), while TREM-2 on macrophages suppresses inflammatory responses to protect hosts from septic shock(22). Two published studies have implicated a potential role of LAB in TREM-mediated signaling. The reduced expression of LAB causes enhanced TREM-1-mediated ERK activation, calcium flux, and production of TNF-α and IL-8(14). In TREM-1 signaling, phosphorylated LAB may interact with Grb2 and c-Cbl, which may in turn downregulate Syk activity. In contrast, TREM-2-mediated ERK activation is reduced in $LAB^{-/-}$ macrophages(15). It is possible that LAB modulates Erk activation through the recruitment of the Grb2-Sos complex after TREM-2 engagement. It has been suggested that low affinity interaction of TREM receptors with as yet unidentified ligands induces tonic signals that regulate TLR4-mediated responses(18, 22). One study shows that TLR4 and TREM-1 can co-localize upon LPS stimulation(23). Based on these studies, it is reasonable to speculate that LAB negatively regulates LPS-mediated responses through its role in TREM-1 mediated signaling; however, as LAB was not phosphorylated during LPS stimulation, this is not a likely explanation.

Our previous studies show that the rescue of LAT deficiency by LAB requires its membrane-distal tyrosine residues, indicating that tyrosine phosphorylation of LAB is important for its positive role in immunoreceptor-mediated signaling. Our data here showed that the negative role of LAB in TCR and FcεRI-mediated signaling also required its five membrane-distal tyrosine residues. When these tyrosines were mutated, LAB was no longer able to suppress the signaling events activated through these receptors. Despite the critical role of these tyrosine residues in immunoreceptor-mediated signaling, LAB was not phosphorylated in DCs upon stimulation by LPS. Further analysis of DCs expressing the mutant form of LAB indicated that the mutation of the five membrane-distal tyrosine residues had no effect on the role of LAB in LPS-mediated signaling and cytokine production. LPS-mediated ERK, JNK, and p38 activation in $LAB^{m/m}$ and WT DCs was similar. These data clearly indicated that LAB phosphorylation is not required in LPS-mediated signaling. Because LAB is a protein that is constitutively localized in lipid rafts, it is possible that LAB deficiency may increase recruitment of TLR4 into lipid rafts after LPS stimulation, thus enhancing activation of downstream signaling and cytokine production. Similar to immunoreceptors, TLR receptors are also recruited to lipid rafts upon engagement with pathogen-associated molecules(24). While this possibility exists, it seems more likely that LAB may recruit other negative regulators, such as phosphatases, to downregulate Syk activation through a mechanism independent of its tyrosine phosphorylation. One published study supports this possibility. This study found that a region in the membrane proximal half of the LAB cytoplasmic domain has an inhibitory effect on pre-B cell differentiation(25), although it is still not clear how this region plays such an inhibitory role. We have attempted

to look for such proteins that interact with LAB by co-immunoprecipitation, but we have not had much success. Thus, how LAB regulates LPS-mediated signaling remains to be investigated.

The engagement of TLRs in dendritic cells activates the MAPK, PI3K, and NFκB pathways, leading to cytokine production and the up-regulation of MHC class II and co-stimulatory molecule expression(26). Stimulation through TLRs induces the production of cytokines, both pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines, such as IL-12, TNFα, and IL-6, are important for protective responses to infection; on the other hand, anti-inflammatory cytokines, such as IL-10, suppress the production of pro-inflammatory cytokines by DCs and macrophages to dampen the immune response following infection. Our data *in vitro* using DCs derived from WT, $LAB^{-/-}$, and $LAB^{m/m}$ mice indicate that LAB deficiency enhanced LPS-mediated production of cytokines, which included both inflammatory (TNFα and IL-6) and anti-inflammatory (IL-10) cytokines. IL-12 production and the expression of MHC class II, CD40, CD80, and CD86 in $LAB^{-/-}$ cells was also reduced, a likely consequence of increased IL-10 production. Even though LPS-induced IL-10 production was enhanced *in vivo* and *in vitro*, $LAB^{-/-}$ mice were more sensitive to LPS-induced septic shock, indicating that increased IL-10 in those mice was not sufficient to suppress the production and effect of inflammatory cytokines. In contrast, $LAB^{m/m}$ mice were not as sensitive as $LAB^{-/-}$ mice in response to LPS stimulation, suggesting that LAB function in LPS-mediated response does not depend on phosphorylation of these tyrosine residues. This result was supported by the fact that $LAB^{m/m}$ DCs produced similar amounts of cytokines as WT DCs *in vitro*. However, $LAB^{m/m}$ mice showed enhanced production of TNF-α *in vivo*, but not IL-6 and IL-10. This result is likely due to the contribution of other cell types during LPS stimulation. In addition to DCs, LAB is expressed in B cells, T cells, macrophages, and NK cells. These cells may also participate in LPS-mediated response and LAB may function through a phosphorylation-dependent mechanism in these cells. Together, our data here demonstrated that, in addition to its important role in immunoreceptor-mediated signaling and activation, LAB plays a critical role in the regulation of innate immune responses through a novel mechanism. The molecular mechanism by which LAB regulates innate immunity remains to be investigated.

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References

1. Samelson LE. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. Annu Rev Immunol. 2002; 20:371–394. [PubMed: 11861607]
2. Jordan MS, Singer AL, Koretzky GA. Adaptors as central mediators of signal transduction in immune cells. Nat Immunol. 2003; 4:110–116. [PubMed: 12555096]
3. Fuller DM, Zhang W. Regulation of lymphocyte development and activation by the LAT family of adapter proteins. Immunol Rev. 2009; 232:72–83. [PubMed: 19909357]
4. Finco TS, Kadlecek T, Zhang W, Samelson LE, Weiss A. LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. Immunity. 1998; 9:617–626. [PubMed: 9846483]
5. Zhang W, Sommers CL, Burshtyn DN, Stebbins CC, DeJarnette JB, Trible RP, Grinberg A, Tsay HC, Jacobs HM, Kessler CM, Long EO, Love PE, Samelson LE. Essential role of LAT in T cell development. Immunity. 1999; 10:323–332. [PubMed: 10204488]

6. Saitoh S, Arudchandran R, Manetz TS, Zhang W, Sommers CL, Love PE, Rivera J, Samelson LE. LAT is essential for Fc(epsilon)RI-mediated mast cell activation. Immunity. 2000; 12:525–535. [PubMed: 10843385]
7. Janssen E, Zhu M, Zhang W, Koonpaew S, Zhang W. LAB: A new membrane-associated adaptor molecule in B cell activation. Nat Immunol. 2003; 4:117–123. [PubMed: 12514734]
8. Brdicka T, Imrich M, Angelisova P, Brdickova N, Horvath O, Spicka J, Hilgert I, Luskova P, Draber P, Novak P, Engels N, Wienands J, Simeoni L, Osterreicher J, Aguado E, Malissen M, Schraven B, Horejsi V. Non-T cell activation linker (NTAL): a transmembrane adaptor protein involved in immunoreceptor signaling. J Exp Med. 2002; 196:1617–1626. [PubMed: 12486104]
9. Janssen E, Zhu M, Craven B, Zhang W. Linker for activation of B cells: a functional equivalent of a mutant linker for activation of T cells deficient in phospholipase C-gamma1 binding. J Immunol. 2004; 172:6810–6819. [PubMed: 15153499]
10. Koonpaew S, Janssen E, Zhu M, Zhang W. The importance of three membrane-distal tyrosines in the adaptor protein NTAL/LAB. J Biol Chem. 2004; 279:11229–11235. [PubMed: 14722116]
11. Zhu M, Koonpaew S, Liu Y, Shen S, Denning T, Dzhagalov I, Rhee I, Zhang W. Negative regulation of T cell activation and autoimmunity by the transmembrane adaptor protein LAB. Immunity. 2006; 25:757–768. [PubMed: 17081783]
12. Zhu M, Liu Y, Koonpaew S, Granillo O, Zhang W. Positive and negative regulation of FcepsilonRI-mediated signaling by the adaptor protein LAB/NTAL. J Exp Med. 2004; 200:991–1000. [PubMed: 15477350]
13. Volna P, Lebduska P, Draberova L, Simova S, Heneberg P, Boubelik M, Bugajev V, Malissen B, Wilson BS, Horejsi V, Malissen M, Draber P. Negative regulation of mast cell signaling and function by the adaptor LAB/NTAL. J Exp Med. 2004; 200:1001–1013. [PubMed: 15477348]
14. Tessarz AS, Weiler S, Zanzinger K, Angelisova P, Horejsi V, Cerwenka A. Non-T Cell Activation Linker (NTAL) Negatively Regulates TREM-1/DAP12-Induced Inflammatory Cytokine Production in Myeloid Cells. J Immunol. 2007; 178:1991–1999. [PubMed: 17277102]
15. Whittaker GC, Orr SJ, Quigley L, Hughes L, Francischetti IM, Zhang W, McVicar DW. The linker for activation of B cells (LAB)/non-T cell activation linker (NTAL) regulates triggering receptor expressed on myeloid cells (TREM)-2 signaling and macrophage inflammatory responses independently of the linker for activation of T cells. J Biol chem. 2010; 285:2976–2985. [PubMed: 19948717]
16. Whittaker GC, Burshtyn DN, Orr SJ, Quigley L, Hodge DL, Pascal V, Zhang W, McVicar DW. Analysis of the linker for activation of T cells and the linker for activation of B cells in natural killer cells reveals a novel signaling cassette, dual usage in ITAM signaling, and influence on development of the Ly49 repertoire. Blood. 2008; 112:2869–2877. [PubMed: 18645037]
17. Maynard CL, Weaver CT. Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. Immunol Rev. 2008; 226:219–233. [PubMed: 19161427]
18. Hamerman JA, Tchao NK, Lowell CA, Lanier LL. Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. Nat Immunol. 2005; 6:579–586. [PubMed: 15895090]
19. Chu CL, Yu YL, Shen KY, Lowell CA, Lanier LL, Hamerman JA. Increased TLR responses in dendritic cells lacking the ITAM-containing adapters DAP12 and FcRgamma. Eur J Immunol. 2008; 38:166–173. [PubMed: 18081038]
20. Mocsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. Nature reviews Immunology. 2010; 10:387–402.
21. Turnbull IR, McDunn JE, Takai T, Townsend RR, Cobb JP, Colonna M. DAP12 (KARAP) amplifies inflammation and increases mortality from endotoxemia and septic peritonitis. J Exp Med. 2005; 202:363–369. [PubMed: 16061725]
22. Turnbull IR, Gilfillan S, Cella M, Aoshi T, Miller M, Piccio L, Hernandez M, Colonna M. Cutting edge: TREM-2 attenuates macrophage activation. J Immunol. 2006; 177:3520–3524. [PubMed: 16951310]
23. Fortin CF, Lesur O, Fulop T Jr. Effects of TREM-1 activation in human neutrophils: activation of signaling pathways, recruitment into lipid rafts and association with TLR4. Int Immunol. 2007; 19:41–50. [PubMed: 17098818]

24. Motshwene PG, Moncrieffe MC, Grossmann JG, Kao C, Ayaluru M, Sandercock AM, Robinson CV, Latz E, Gay NJ. An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4. J Biol Chem. 2009; 284:25404–25411. [PubMed: 19592493]

25. Herzog S, Jumaa H. The N terminus of the non-T cell activation linker (NTAL) confers inhibitory effects on pre-B cell differentiation. J Immunol. 2007; 178:2336–2343. [PubMed: 17277139]

26. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004; 4:499–511. [PubMed: 15229469]

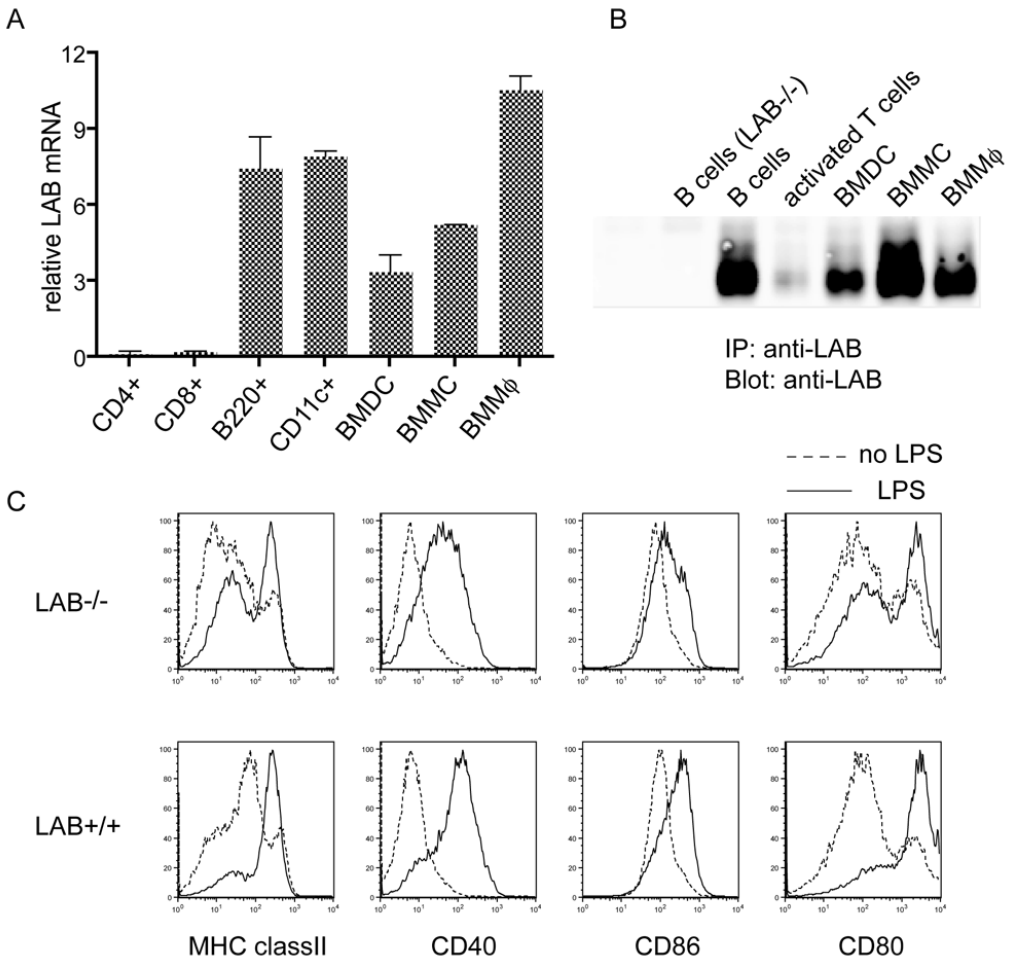

Figure 1. LAB expression and function in dendritic cells

(A) Levels of LAB transcripts in myeloid lineages. $CD4^+$, $CD8^+$, $B220^+$, and $CD11c^+$ cells were isolated from WT mice by FACS sorting. Dendritic cells, macrophages, and mast cells were derived from bone marrow. The relative expression of LAB was detected by real-time PCR and was normalized by GAPDH expression. Data shown are representative of three independent experiments performed in triplicates. Bars indicate mean ± SD. **(B) LAB protein expression.** Cells were lysed in RIPA buffer and lysates were immunoprecipitated with anti-LAB serum followed by immunoblotting with an anti-LAB monoclonal antibody. **(C) The effect of LAB deficiency on DC maturation *in vitro***. $LAB^{-/-}$ and $LAB^{+/+}$ BMMCs were either stimulated with LPS for 24 hrs or left untreated. The surface expression of MHC class II, CD40, CD86, and CD80 on $CD11c^+$ cells was analyzed by FACS. Dashed lines: untreated; solid lines: LPS stimulated. Data shown are representative of three independent experiments.

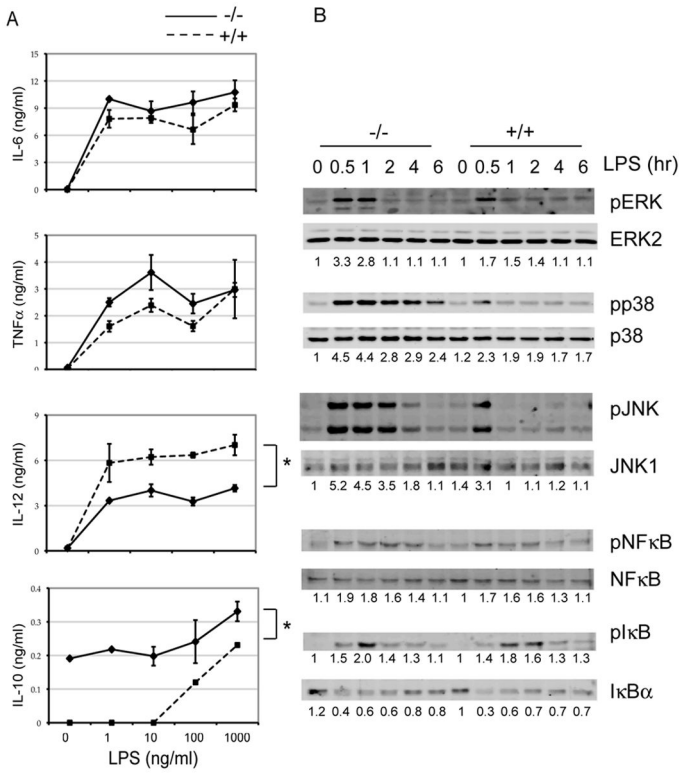

Figure 2. The effect of LAB deficiency on LPS-mediated cytokine production and signaling

(A) Cytokine secretion. CD11c^{+} cells from LAB$^{-/-}$ and LAB$^{+/+}$ BMDC culture were purified and seeded in a 96-well plate in triplicate in the presence of different concentrations of LPS for 16 hrs. The concentrations of different cytokines in the supernatants were measured by ELISA. Error bars are the standard deviation of triplicates of the same samples. Data with significant differences ($p<0.05$ for two-tailed t-test) are indicated by *. Data shown are representative of three independent experiments. **(B) The effect of LAB deficiency on LPS-mediated signaling.** BMDCs from LAB$^{-/-}$ and LAB$^{+/+}$ mice were stimulated with LPS for the indicated time points before lysis in RIPA buffer. The lysates were analyzed by immunoblotting with ***phospho-specific or pan-antibodies against ERK, p38, JNK, NFκB, and IκB. Data shown are representative of three experiments. The numbers shown are relative intensities for the phosphorylated ERK, p38, JNK1, and NFκB normalized to their ***pan-forms. For pIκBα and IκBα, the numbers shown are relative intensities without normalization.

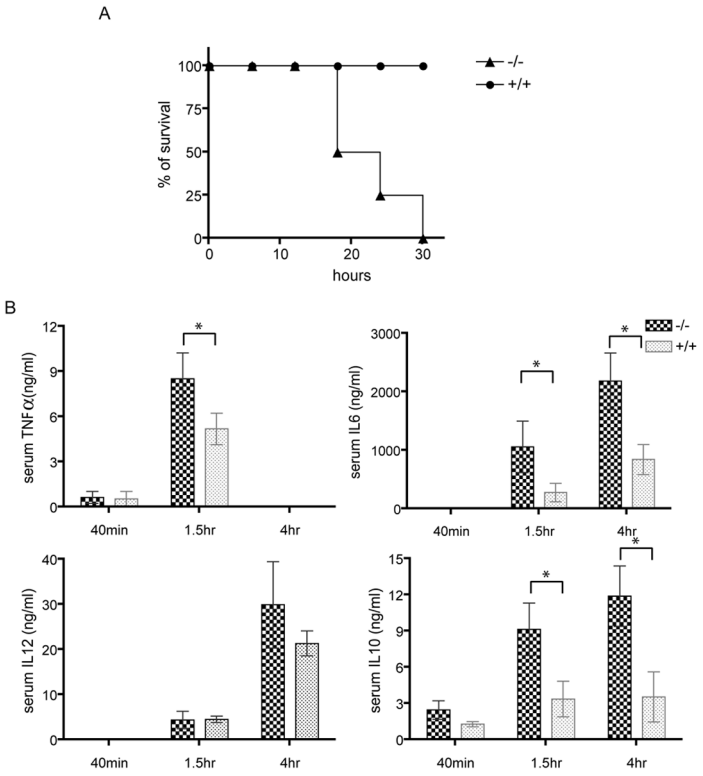

Figure 3. LAB$^{-/-}$ mice are hyper responsive to LPS-induced septic shock.(A)Septic shock After injection of LPS (20mg/kg body weight), the survival of LAB$^{-/-}$ and LAB$^{+/+}$ mice (5 in each group) was monitored for up to 30 hrs. **(B) LPS-induced cytokine production *in vivo***. LAB$^{-/-}$ and LAB$^{+/+}$ mice (5 each) were injected with LPS (5mg/kg body weight). Sera were collected at the indicated time points and cytokine concentrations were determined by ELISA. Each value is the mean and standard deviation of data from five mice. Data with significant differences ($p<0.05$ for two-tailed t-test) are indicated by *. Data shown are representative of three experiments.

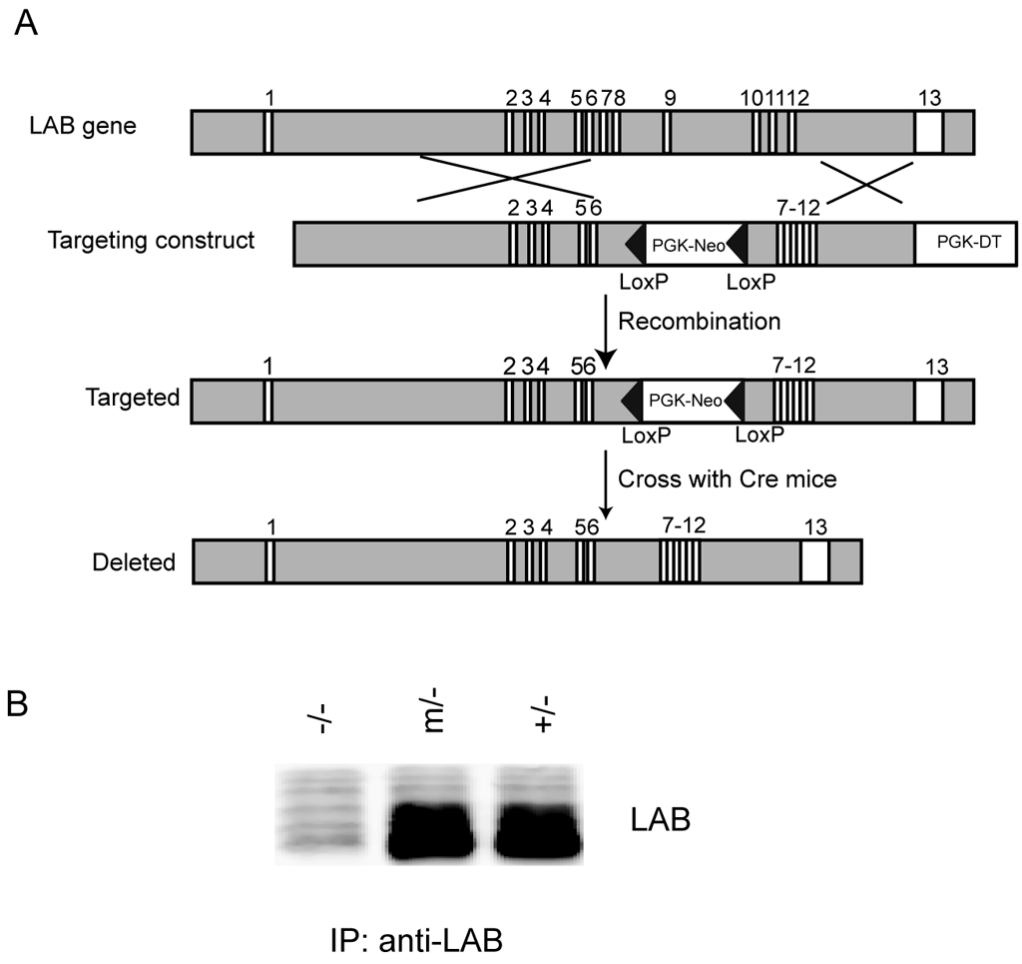

Figure 4. Generation of LAB knock-in mice with Y to F mutations

(A) The LAB5YF knock-in strategy. Exons 7–12 were replaced with a cDNA fragment carrying Tyr to Phe mutations at residues 98, 121, 139, 160, and 192. **(B) Expression of mutant LAB protein.** $LAB^{-/-}$, $LAB^{m/-}$, and $LAB^{+/-}$ BMMφs were stimulated with pervandate for 5 minutes and then lysed. Lysates were subjected to immunoprecipitation with anti-LAB antisera. The immunoprecipitates were then analyzed by Western blotting with anti-LAB.

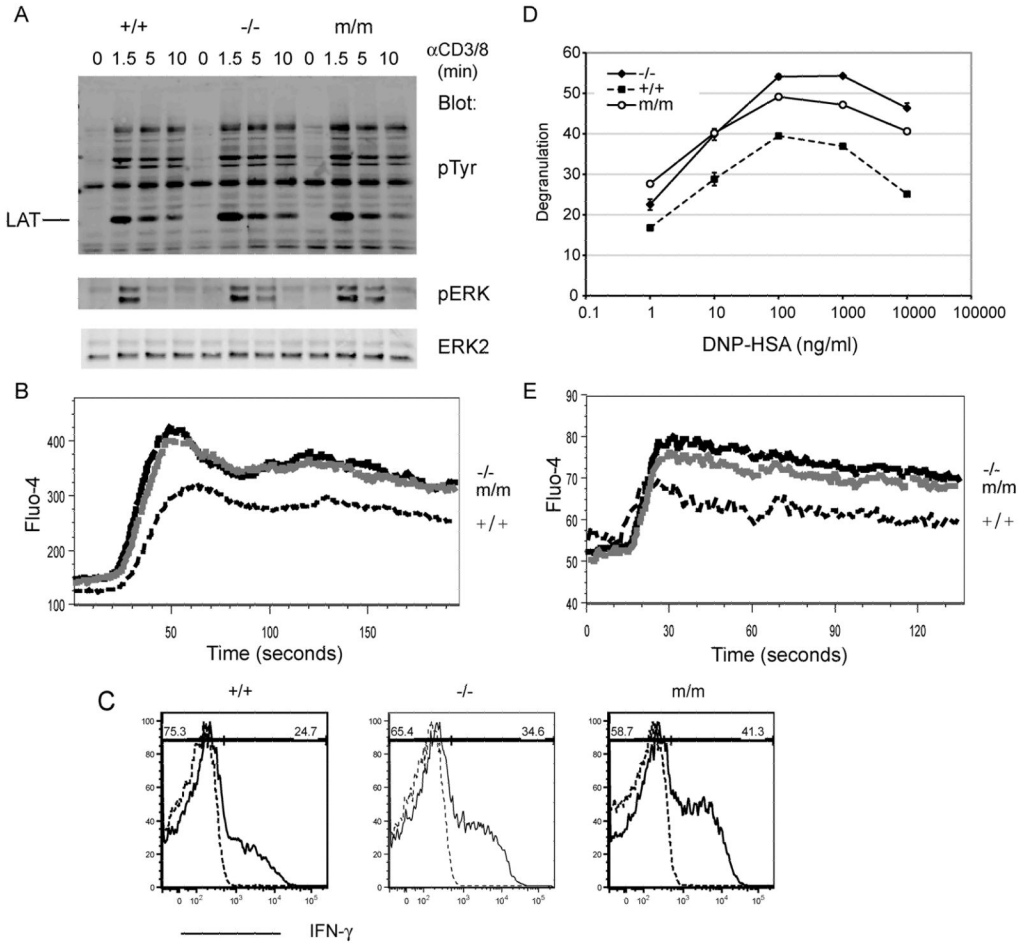

Figure 5. LAB phosphorylation is required for its inhibitory function in TCR and FcεRImediated signaling

(A) TCR-mediated phosphorylation of proteins. Activated $CD8^+$ T cells from $LAB^{+/+}$, $LAB^{-/-}$, and $LAB^{m/m}$ mice were restimulated with anti-CD3 and anti-CD8 for the indicated time points and analyzed by immunoblotting with anti-pTyr, pERK, and ERK2 antibodies. **(B)TCR-mediated Ca^{2+} mobilization.** $CD8^+$ T cells were loaded with fluo-4. Ca^{2+} flux was initiated by the addition of biotin-conjugated anti-CD3 and anti-CD8, followed by cross-linking with streptavidin. Data shown are representative of three experiments.**(C)IFN-γ production by $CD8^+$ T cells.** Activated T cells were rested for 4–6 hours, followed by stimulation with anti-CD3 in the presence of Monensin. Cells were analyzed by staining with anti-CD8 followed by intracellular staining with antibodies against IFN-γ. Dashed line: unstimulated. Solid line: anti-CD3 stimulated. **(D)BMMC degranulation.** $LAB^{+/+}$, $LAB^{-/-}$, and $LAB^{m/m}$ BMMCs were first sensitized with anti-DNP IgE and then stimulated with various concentrations of DNP-HSA for 10 minutes. Degranulation data are expressed as the percentage of released versus total β-hexosaminidase activity and reflect three independent experiments.**(E)FcεRI-mediated Ca^{2+} mobilization.** BMMCs were sensitized with anti-DNP IgE before being loaded with fluo-4. Ca^{2+} mobilization was initiated by the addition of DNPHSA.

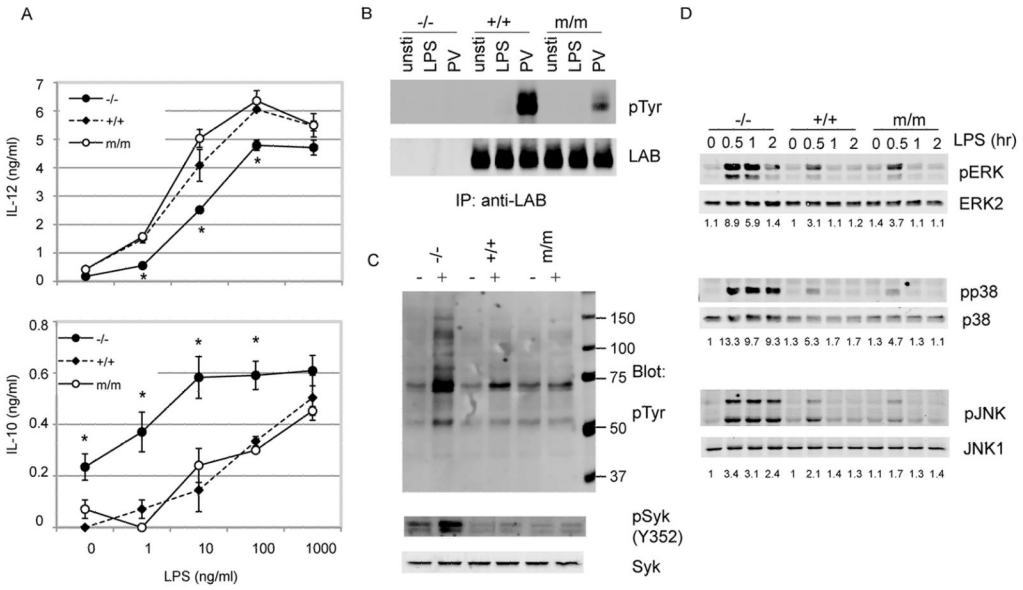

Figure 6. Phosphorylation of LAB is not required for its function in BMDCs

(A) Cytokine production. $LAB^{+/+}$, $LAB^{-/-}$, and $LAB^{m/m}$ BMDCs were purified and seeded in a 96-well plate and were stimulated with different concentrations of LPS for 16 hrs before ELISA. Each value is the mean and standard deviation of data from triplicate samples. Data with significant differences ($p<0.05$ for two-tailed t-test) are indicated by *. Data shown are representative of three experiments.**(B) The absence of LAB phosphorylation.** $LAB^{+/+}$, $LAB^{-/-}$, and $LAB^{m/m}$ BMDCs were stimulated with LPS for 10 mins or pervanadate for 2 mins before lysis. LAB was immunoprecipitated and blotted with anti-pTyr and anti-LAB antibodies. **(C) Hyperphosphorylation of Syk in $LAB^{-/-}$ DCs.** BMDCs were stimulated with 100ng/ml LPS for 30 minutes before lysis in RIPA buffer. The lysates were analyzed by immunoblotting with anti-pTyr, phospho-Syk (pY352), and Syk antibodies. Data shown are representative of three experiments.**(D) The effect of LAB deficiency and LAB 5YF mutation on LPS-mediated MAPK activation.** BMDCs from $LAB^{-/-}$, $LAB^{+/+}$ and $LAB^{m/m}$ mice were stimulated with LPS before lysis at the indicated time points. The lysates were analyzed by blotting with phospho-specific or pan-antibodies against ERK, p38, and JNK. Data shown are representative of three experiments. Numbers shown are relative intensities for phosphorylated ERK, p38, and JNK1 normalized to their pan forms.

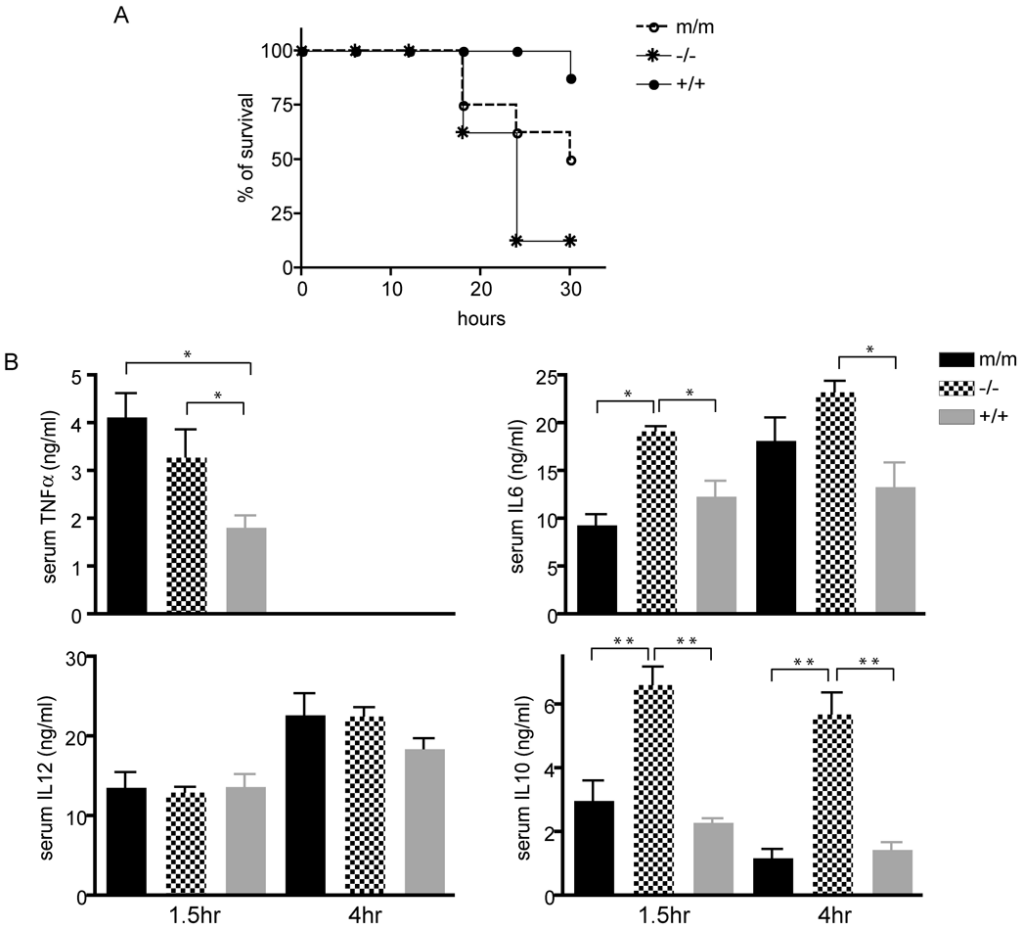

Figure 7. LPS-induced septic shock in LAB$^{m/m}$ mice

(A)Septic shock. After injection of LPS, the survival of LAB$^{m/m}$, LAB$^{-/-}$ and LAB$^{+/+}$ mice(eight mice for each genotype) was monitored for up to 30 hrs. The survival of these mice after LPS treatment was analyzed statistically by the Kaplan-Meyer method (log-rank test: LAB$^{-/-}$ vs. LAB$^{+/+}$, $p<0.0001$; LAB$^{-/-}$ vs. LAB$^{m/m}$, $p=0.04$; LAB$^{m/m}$ vs. LAB$^{+/+}$, $p=0.07$).**(B) LPS-induced cytokine production *in vivo***. LAB$^{m/m}$, LAB$^{-/-}$, and LAB$^{+/+}$ mice (4 each) were injected as above. Sera were collected at the indicated time points and cytokine concentrations were determined by ELISA. Each value is the mean and standard deviation of data from four mice. Data with significant difference ($p<0.05$ and $p<0.01$ for two-tailed t-test) are indicated by * and **. Data shown are representative of three experiments.