Supporting Information

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SI Materials and Methods

Mice. WT C57BL/6 and BALB/c mice were purchased from Jackson Laboratories and Japan SLC, respectively. BALB/c Toll-like receptor 5 $(TLR5)^{-/-}$ mice, backcrossed to the BALB/c strain for seven generations, were previously reported (1). C57BL/6 *3d* mice, which were provided by Koichi Tabeta (Niigata University, Niigata, Japan), were introgressively backcrossed to the BALB/c strain for 12 generations, and used as BALB/c *3d* mice. UNC93B1-deficient mice in the C57BL/6 background were obtained from the Knockout Mouse Project Repository (University of California, Davis, CA). All animals were housed in specific pathogen-free facilities at Pohang University of Science and Technology or at the Institute of Medical Science at the University of Tokyo. All animal experiments were approved by the animal research committee of either Pohang University of Science and Technology or University of Tokyo.

Reagents. MALP-2, Pam_3CSK_4, R848, and loxoribine were purchased from Enzo Life Sciences. Ultrapure flagellin purified from *Salmonella typhimurium* was purchased from Life Sciences or InvivoGen. Lipid A purified from *Salmonella minnesota* (Re-595), LPS from *Escherichia coli* (026:B6), Brefeldin A, and anti-flag (M2) antibody were from Sigma-Aldrich. CpG ODN1826 and ODN1668 were synthesized by TIB Molbiol and FASMAC, respectively. Mouse anti–GFP (B-2) and rat monoclonal anti-HA (3F10) antibodies were purchased from Santa Cruz and Roche, respectively. Mouse monoclonal anti-myc (9B11), rabbit anti-IκB, and mouse anti–phospho-IκB (5A5) antibodies were from Cell Signaling. Rabbit anti-actin antibody was from Bethyl Laboratories. Rabbit anti-GFP and anti-myc antibodies were made in our laboratory by injecting rabbits with purified GFP protein and peptides containing the EQKLISEEDL sequence, respectively. Protein A-agarose bead was purchased from Repligen. APC-conjugated anti-mouse TNF (clone MP6-XT22) was from BD Biosciences.

Cell Lines. Murine RAW 264.7 macrophages and HEK 293T cells were cultured in DMEM media supplemented with 5% FBS. RAW stable cell lines expressing epitope-tagged TLR or UNC93B1 proteins were generated by retroviral transduction and selected with 100 μg/mL G418 for UNC93B1-transduced cells and 5 μg/mL puromycin for TLR-transduced cells. Retrovirus preparation and transduction were performed as previously described (2). Ba/F3 cells were cultured in RPMI 1640 media supplemented with 10% FBS, penicillin–streptomycin–glutamine, 50 μM 2-mercaptoethanol, and IL-3 produced by CHO cells overexpressing murine IL-3. J774 cells were cultured in DMEM supplemented with 10% FBS, penicillin–streptomycin–glutamine, and 50 μM 2-mercaptoethanol. Ba/F3 stable cell line with mouse TLR5-flag and NF-κB–GFP reporter were previously described (1). To suppress the endogenous UNC93B1 expression in Ba/F3 and J774 cells, each cell line was transduced with retrovirus generated by transient transfection of Plat-E packaging cells with the retroviral vector pSSCH encoding UNC93B1-specific shRNA (5′-ggaacttcctccaaggagata-3′) and selected with hygromycin B. Cells transduced with retrovirus made of the empty vector were used as a control.

DNA Constructs. pMSCVpuro-TLR9-myc, pMSCVpuro-TLR4-myc, pMSCVneo-UNC93B1-HA, pMSCVneo-UNC93B1-cherry, and pMSCVneo-cherry-KDEL (ER-cherry) were previously described (3). pMSCVpuro-TLR5-GFP and pMSCVpuro-TLR5-myc were cloned by PCR by using pcDNA3-TLR5-CFP (Addgene) as a template. The linker nucleotide sequence between TLR5 and EGFP is 5′-atgtcgacggtaccgcgggcccgggatccaccggtcgccacc-3′ (encoding the amino acid sequence MSTVPRARDPPVAT). The myc epitope tag (EQKLISEEDL) was directly fused to the carboxyl end of TLR5 without an intervening linker sequence. pMSCVpuro-TLR5-GFP mutant (DE/ST), in which the DEEE sequence (amino acid residues 630∼633) of TLR5 was substituted with STTT, were generated by PCR by using the following primers: 5′-tgtagtcgtactacaaccttccgtggaaagag-3′ and 5′-tgtagtacgactacagtcttaaagtccctaaagttc-3′. Underlined bases represent the sense or antisense nucleotide sequence for STTT.

Preparation of Splenic Dendritic Cells. Spleens were extracted from indicated mice, minced with scissors into small pieces, and incubated with RPMI media supplemented with 3% FBS, 20 mM Hepes, 1 mM sodium pyruvate, 1 mM nonessential amino acid, penicillin/streptomycin, 100 μg/mL DNase I (Roche), and 1 mg/mL collagenase D (Roche) for 45 min at 37 °C with vigorous shaking. The enzyme digestion was stopped by adding EDTA to the final concentration of 10 mM. The spleen homogenate was filtered with a cell strainer (100 μm); washed once with PBS buffer containing 3% FBS, 10 mM EDTA, 20 mM Hepes, 1 mM sodium pyruvate, penicillin/streptomycin; loaded onto 17.5% (wt/vol) Accudenz solution (Gentaur Molecular Products); and centrifuged at 800 × *g* for 20 min. After centrifugation, a cloudy cell layer in the middle of the gradient solution was collected as the splenic dendritic cell (DC) fraction, purity of which was analyzed by flow cytometry with staining of CD11c and MHC class II.

Preparation of Lamina Propria Dendritic Cells. The preparation of small intestinal lamina propria DCs (LP-DCs) has been described (4). In brief, small intestines were extracted, and the fatty tissues and Peyer patches were removed. The intestines were opened longitudinally, washed extensively in PBS solution, and cut into 1–2-cm lengths. The intestinal fragments were incubated in PBS solution containing 3% FBS, 20 mM Hepes, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 10 mM EDTA for 20 min at 37 °C with vigorous stirring. After washing with PBS solution, the fragments were further incubated at 37 °C for 45 min in RPMI 1640 media containing 3% FBS, 20 mM Hepes, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acid, 400 U/mL collagenase D, and 100 μg/mL DNase I with vigorous stirring. The cell suspension was filtered through nylon mesh and spun over a Percoll gradient [75/40% (wt/vol)]. The cells in the inter phase were collected and sorted on the basis of their expression of CD11c and CD11b using MoFlo XDP (Beckman Coulter). For cytokine production, lamina propria DCs (1×10^5 per well) were cultured in RPMI 1640 media supplemented with 10% FBS, 10 mM Hepes, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin, 1 mM sodium pyruvate, 55 μM 2-mercaptoethanol, 1 mM nonessential amino acid, 2 mM L-glutamine, and 10 ng/mL GM-CSF, and stimulated with 1 μg/mL of flagellin for 24 h.

Cell Staining and Flow Cytometry Analysis. Splenocyte single cell suspension was prepared as previously described (1). The splenocyte suspension and blood were cleared of red blood cells by lysis with 1× BD Pharm Lyse buffer (BD Biosciences) and filtering through nylon mesh. Blood leukocytes and splenocytes were stained as previously described (1) with the following

fluorophore-conjugated antibodies purchased from eBiosciences: CD4 (clone GK1.5), CD8α (clone 53–6.7), CD11b (clone M1/70), CD11c (clone HL3), CD317 (PDCA-1, BST2; clone 927), Ly6C (clone HK1.4), and Ly6G (clone 1A8). Biotinylated anti-TLR5 (clone ACT5), anti-TLR2 (clone CB225), anti-TLR4/MD-2 (MTS510), and anti-CD14 (clone Sa2-8) monoclonal antibodies were previously established in our laboratory (1, 5). Cells labeled with biotinylated antibodies were further stained with fluorophore-conjugated streptavidin and analyzed by FACSAria or LSR Fortessa flow cytometers (BD Biosciences).

ELISA. Levels of IL-6, IL-12p40, G-CSF, and TNF-α in the cell supernatants or serum were measured with ELISA kits (R&D Systems) according to the manufacturer's protocol.

Quantitative Real-Time PCR. One microgram of total RNA purified by Nucleospin RNA XS kit (Macherey-Nagel) was reverse-transcribed into cDNA by using a ReverTra Ace qPCR RT Kit (TOYOBO). Quantitative PCR analyses were performed with a 7300 Fast Real Time PCR System (Applied Biosystems) using the TaqMan Gene Expression Assay set for mouse unc93b1 (Mm00457643). Each sample was normalized with mouse β-actin (Mm00607939).

Immunoprecipitation and Deglycosylation. Ba/F3:TLR5-flag cells were lysed in the lysis buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin for 30 min on ice. The cell lysates were incubated with anti-flag (M2)–agarose beads for 4 h at 4 °C. After washing with the lysis buffer three times, the beads were treated with endoglycosidase H or PNGase F according to the manufacturer's instruction (New England Biolabs). The immunoprecipitated proteins were eluted from the beads by boiling in the SDS sample buffer and subjected to SDS/PAGE. TLR5-flag was detected by immunoblotting with anti-flag (M2) antibody.

Confocal Imaging and Analysis. Live bone marrow-derived DCs (BMDCs) or HEK 293T cells expressing various fluorescent fusion proteins were imaged with a spinning-disk confocal microscope as previously described (2). For costaining of the plasma membrane, BMDCs were incubated with 1 μg/mL of Alexa Fluor 555-conjugated cholera toxin B (Invitrogen) for 20 min on ice, fixed with 2% paraformaldehyde for 10 min, and imaged. To quantify the degrees of colocalization of two proteins, pixel intensities of two different fluorophores were measured by using a linescan tool in MetaMorph software and the Pearson correlation coefficient (ρ) was determined by using Microsoft Excel.

Bimolecular Fluorescence Complementation Assay. Vectors encoding the aminoterminal (V_N; aa 1–177) or the carboxyterminal (V_C; aa 155–238) fragment of Venus fluorescent protein were provided by Michael Frohman (Stony Brook University, Stony Brook, NY) (6). By standard molecular cloning protocols, the V_N or V_C fragment of Venus was fused to the carboxyl terminal end of TLR4, TLR5, and TLR9. UNC93B1 was fused with the V_C fragment of Venus at the aminoterminal or carboxyterminus. Combination of the split Venus-tagged TLRs and UNC93B1 was expressed in HEK 293T cells, and live cells were imaged by spinning disk confocal microscopy.

***Pseudomonas aeruginosa* Challenge and IL-12p40 Assay.** *P. aeruginosa* PAO1 and *fliC*-deficient (Δ*fliC*) strains were cultured in kanamycin-containing Luria broth. The cfu counts were calculated by counting colonies after overnight incubation of serially diluted bacteria culture on Luria broth/kanamycin agar plates and bacteria were heat-killed by incubation at 60 °C for 1 h. Splenic DCs were stimulated with *P. aeruginosa* (1,000 cfus per cell) in the presence of 10 μg/mL Brefeldin A for 4 h, and IL-12p40 production was measured by intracellular cytokine staining by using anti–IL-12p40 antibody (clone C15.6; BioLegend).

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Fig. S1. UNC93B1 is required for the cell surface expression of TLR5. J774 cells (*A*) and Ba/F3 cells stably expressing TLR5-flag (Ba/F3:TLR5-flag) (*B*–*D*) were retrovirally transduced with the control or Unc93B1 shRNA-encoding vector. (*A* and *B*) The expression level of Unc93B1 mRNA was evaluated by real-time RT-PCR and normalized to that of β-actin. Data represent mean ± SD of triplicate samples. (*C*) The levels of cell surface or total TLR5 and cell surface TLR2 were analyzed by flow cytometry by using biotinylated anti-TLR5 (ACT5) and anti-TLR2 (CB225) antibodies, respectively (open histograms). Shaded histograms represent control staining with streptavidin-PE alone. (*D*) Ba/F3:TLR5-flag cells were left untreated (control) or retrovirally transduced with Unc93B1-GFP (WT or H412R)-encoding vectors. The levels of GFP expression as well as cell surface TLR5 and TLR2 were analyzed by flow cytometry as in *C*. (*E*) TLR5-flag was immunoprecipitated from the Ba/F3:TLR5-flag cell lysates with a mouse anti-flag antibody, subjected to deglycosylation with endoglycosidase H (marked as "H") or PNGase F (marked as "F"), and detected by immunoblotting with an anti-flag antibody. Ba/F3 cells that do not express TLR5-GFP were used as a control (first lane).

Fig. S2. Generation and characterization of UNC93B1-deficient mice. (*A*) Strategy for *Unc93B1* gene deletion. Mouse *Unc93B1* gene consists of 10 exons in chromosome 19. A part of the first and the last exon and all intervening exons and introns were replaced by a targeting cassette encoding β-gal and neomycin resistance gene by homologous recombination. (*B*) Expression of endogenous UNC93B1 in BMDCs from WT, Unc93B1-deficient (KO), and *3d* mice was detected by immunoblotting with anti-UNC93B1 antibody (*Upper*). Actin is shown as a control for equal protein loading (*Lower*). (*C*) BMDCs from WT, KO or *3d* mice were stimulated with various doses of indicated TLR agonists for 4 h. The levels of TNF-α in the culture supernatants were measured by ELISA (mean ± SEM). Data shown are representative of two independent experiments.

Fig. S3. WT but not H412R mutant UNC93B1 promotes cell surface localization of TLR5. (*A*) TLR5-GFP was expressed in BMDCs from WT, *3d*, or UNC93B1-deficient (KO) mice, and the cells were imaged after surface staining with Alexa Fluor 555-conjugated cholera toxin B. Four representative images from each group are shown, and the histograms display fluorescent intensity plots of pixels on the white dashed line. The colocalization of TLR5-GFP with cholera toxin B was quantified by measuring the Pearson correlation coefficient ρ of signal intensities of two proteins. (*B*) TLR5-GFP and CD63-cherry were coexpressed in BMDCs from WT, KO, or *3d* mice and imaged. The histograms show fluorescent intensity plots for pixels on the white dashed line, and the colocalization of TLR5-GFP with CD63-cherry was quantified by measuring the Pearson correlation coefficient ρ of signal intensities of two proteins. (*C*) TLR5-GFP and ER-cherry with or without UNC93B1-HA (WT or H412R mutant) were transiently coexpressed in HEK 293T cells and imaged. The colocalization of TLR5-GFP with ER-cherry was quantified by measuring the Pearson correlation coefficient as in *A*. (Scale bar, 10 μm.)

Fig. S4. UNC93B1 colocalizes with TLR5 at the plasma membrane. (*A*) TLR5-GFP and UNC93B1-cherry were coexpressed in HEK 293T cells or BMDCs and imaged. The histograms display fluorescent intensity plots for pixels on the white dashed line in the magnified area (white box). The signals for TLR5-GFP and UNC93B1-cherry overlap at the plasma membrane (indicated by arrows in the histograms). (*B*) Bimolecular fluorescence complementation assay using a yellow fluorescent protein Venus. The N- or C-terminal fragment of Venus (V_N or V_C) was conjugated to the C terminus of TLR9, TLR4, or TLR5 and the N- or C terminus of UNC93B1. The Venus fragment-conjugated TLRs or UNC93B1 were transiently expressed in HEK 293T cells as indicated, and live cells were imaged. (Scale bar, 10 μm.)

Fig. S5. TLR5 signaling is independent of endosomal acidification. RAW cells stably expressing TLR5-GFP were stimulated with indicated TLR agonists in the presence of 10 μg/mL Brefeldin A for 6 h. LPS (100 ng/mL), flagellin (100 ng/mL), R848 (500 nM), or CpG (ODN1826; 100 nM) were used for stimulation. During the TLR stimulation, 200 nM of bafilomycin A was cotreated to increase the endosomal pH. Cells were then fixed and permeabilized and intracellular TNF-α was detected by flow cytometry with APC-conjugated anti–TNF-α antibody (open histograms). Shaded histograms represent the levels of TNF-α in unstimulated cells.

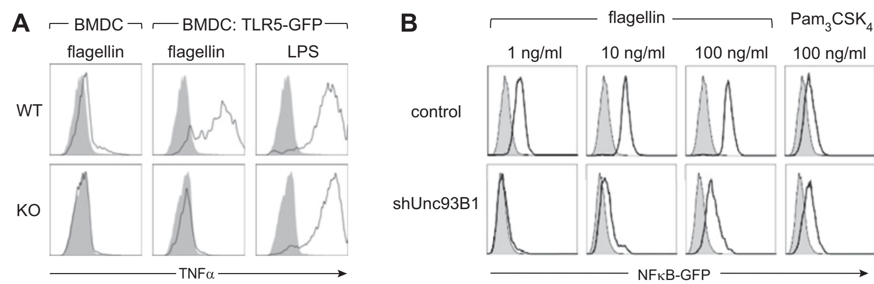

Fig. S6. UNC93B1 is required for TLR5-mediated signaling. (*A*) BMDCs from WT or UNC93B1-deficient mice were retrovirally transduced with TLR5-GFP (BMDC: TLR5-GFP) or not (BMDC). Cells were stimulated with flagellin (50 ng/mL) or LPS (100 ng/mL) in the presence of 10 μg/mL Brefeldin A for 6 h, and intracellular TNF-α was measured by flow cytometry (open histograms). Shaded histograms represent the levels of TNF-α in unstimulated cells. Data shown are representative of three independent experiments. (*B*) NF-κB–GFP reporter gene was stably introduced into Ba/F3:TLR5-flag cells and the cells were further transduced with a control or *Unc93B1* shRNA-encoding vector. After stimulation with flagellin (1, 10, 100 ng/mL) or Pam_3CSK_4 (100 ng/mL) for 24 h, induction of GFP expression was analyzed by flow cytometry. Shaded and open histograms show the GFP expression levels before and after stimulation, respectively. Data shown are representative of three independent experiments.

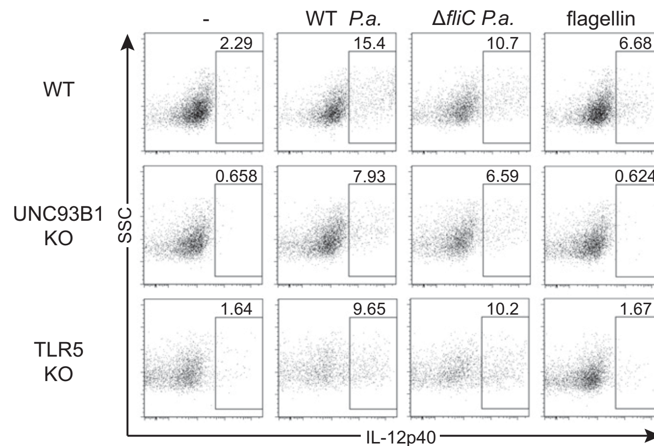

Fig. S7. Production of IL-12p40 in response to *P. aeruginosa* flagellin is dependent on UNC93B1. Splenic DCs enriched from WT, UNC93B1-deficient or TLR5-deficient mice were stimulated with heat-killed *P. aeruginosa* (WT and Δ*fliC*) or purified flagellin for 4 h, and the levels of IL-12p40 in splenic DCs (MHC class II^{+} $CD11b^{high}CD11c^{high}$) were measured by intracellular staining.