Generation of TNLG8A-deficient mice

Two ES TNLG8A clones ($CD70^{(KOMP)Vlcg}$, AC7 & AH3) were purchased from the Knockout Mouse Project Repository (KOMP, Davis, CA). Chimeras were generated and mated to AlbinoB6 females for germ line transmission and later crossed to generate homozygous mice in the Rutgers Transgenic Core. $TNLG8A^{ko}$ mice were co-housed with WT littermates to prevent potential intestinal microbiota differences.

Generation of GFP bone marrow chimera mice

Bone marrow cells were isolated from $B6.GFP^{tg}$ donor mice. B6 (WT) and $B6.Lt\alpha^{ko}$ recipient mice were sub-lethally irradiated with 500 rad, and then injected intravenously with ($5x10^6$/mouse) $GFP^{+/+}$ bone marrow cells. Mice were kept for 8 weeks, and then euthanized to harvest cells from different tissues.

Isolation of hematopoietic cells from mucosal tissues

Peyer's patches (PP) and cecal patches (CP) were excised from the small intestine and colon, respectively, disrupted through a 100 μm mesh, incubated in complete RPMI supplemented with collagen IV, washed, and reserved for analysis. The intestine was flushed with PBS and cut longitudinally along its length before being cut into smaller pieces. After three washes, intestinal pieces were transferred to a 50 mL tube in 20 mL Hank's balanced salt solution (HBSS) containing 2% FBS and 50 μM EDTA and incubated under gentle shaking at 37°C for 20 min. Intraepithelial lymphocytes (IEL) were isolated from the pooled supernatants. Intestinal pieces were then digested once with 100U/ml collagenase IV (Sigma) with shaking at 37°C for 20 min., and digested a

second time with 200U/ml collagenase IV with shaking at 37°C for 20 min. Lamina propria (LP) cells were isolated from the pooled supernatants. IEL and LP cells were used alone or pooled together. This protocol was also used to isolate hematopoietic-derived cells from the mouse tongue, esophagus, stomach, lung, and vagina.

Isolation of hematopoietic cells from human intestines

Seventeen human intestinal samples, small intestine (7) and colon (10), were obtained from the Cancer Institute of New Jersey's Tissue Retrieval Service (New Brunswick, NJ). Tissues were incubated with 50 μM EDTA under gentle shaking at 37°C for 4 hours. IELs were isolated from the supernatant. Intestinal pieces were then digested with 28 U/mL collagenase IV under shaking at 37°C for 6 hours to isolate LPs. Human IELs and LPs were then analyzed by flow cytometry. Human samples were used under the approval of the Institutional Review Board (IRB) of Rutgers University.

Isolation of hematopoietic cells from other tissues

Spleens (SPL), mesenteric lymph nodes (MLN), thymi (Thy), and peripheral lymph nodes (PLN) were disrupted through a 100 μm mesh, incubated in collagen IV-supplemented RPMI, washed in PBS, and reserved for further processing. To isolate hematopoietic cells from the mouse brain, heart and kidney, and liver, we employed purification techniques described by [49-51], respectively.

Flow cytometry

Antibodies to mouse SLAMF4 (CD244), CD48, CD45.1, CD45.2, B220, CD3, CD4, CD8α, CD8β, CD11b, CD11c, CD19, CD45, NK1.1, β-TCR, RORγt, NKp46, I-A/I-E, TNLGA8 (CD70), and γδ-TCR were obtained from BD Bioscience. Antibodies to mouse F4/80, human SLAMF4, and human CD45 were obtained from eBioscience. Anti-mouse PDCA1 was obtained from Miltenyi Biotech. Immunostaining and flow cytometry were performed using an LSRII flow cytometer (BD Biosciences). Briefly, cells were first stained with anti-mouse CD16/CD32 (2.4G2, eBioscience) to block Fc receptors, and then stained with fluorochrome-conjugated surface antibodies in the presence of Propidium Iodide (PI). For intracellular cytokine staining, cells were incubated overnight at 37˚C in the presence of protein transporter inhibitor Golgi stop (BD Bioscience). Subsequently, cells were surface stained, fixed and permeabilized using Cytofix/Cytoperm Kit (BD Biosciences), then stained for intracellular cytokines and transcriptional factors. Before conducting the analysis, the flow cytometer settings were checked using Cytometer Setup and Tracking beads (CS&T beads, BD) according to the manufacturer's instructions. Compensation beads were used with single stain of each antibody to determine the compensation settings, and these were applied in the FlowJo software (version 10.0.6, Tree Star, Ashland, OR, USA) after data collection. In addition, doublets were excluded by using a forward scatter (FSC)-H vs. FSC-A plot. A side scatter (SSC) threshold level was set at 5,000 units to eliminate debris, and at least 5,000 cells were counted. The same compensation matrix was applied to all samples. Recorded data were compensated post-hoc and analyzed using FACSDIVA, CellQuest (BD Biosciences), and FlowJo (Tree Star) software. Statistical analysis was performed using GraphPad Prism version 5.0a (GraphPad Software).

Gating strategy

Leukocyte types were identified based on the methods of Faucher et al**.** by using similar orientating and specific gates as previously described [52]. The first orientating gate was selected using a SSC vs. CD45 plot, where the cutoff for CD45+ cells was set using an FMO control. Subsequently, PI plot of CD45+ cells separated events into a major gate of PI^- events, which represents viable CD45 cells. This gating strategy was used to identify gut leukocyte types as well as cytokine-producing CD45+ cells.

Quantitative gene expression

Total RNA was extracted from homogenized intestines (qPCR for cytokines and transcriptional factors) or purified CD45+ intestinal cells (qPCR for SLAMF4 isoforms). Complementary DNA was prepared from 0.5 µg total RNA using SuperScript II reverse transcriptase (Invitrogen). QPCR reaction for the long and short splice variants of SLAMF4 was performed as described in [38]. The quantitative gene expression analysis of cytokines and transcriptional factors (primer sequences are listed in Table 2) was conducted using SYBR Green PCR Master Mix and performed on the 7500 Fast Real-Time PCR System (Applied Biosystems). Data were analyzed using a $2^{-\Delta\Delta Ct}$ (cycle threshold) method.

Oral infection with *Listeria monocytogenes* and *Cytobacter rodentium*

Eight hours prior to oral infection, access to sterile food and water was removed from co-housed $SLAMF4^{ko}$ mice and WT littermates. A viable inoculum ($4.6\text{-}12x10^9$ CFU in 0.2 ml/mouse) of *Listeria monocytogenes* or ($2x10^{10}$ CFU in 0.2 ml/mouse) of *Cytobacter rodentium* was introduced orally. For controls, mice were inoculated with

sterile bacterial broth. At indicated times post-infection, mice were euthanized and tissues were harvested. Moribund mice were euthanized in compliance with IACUC protocols at Rutgers University and the University of Michigan.

Survival assays

We used time to death as well as moribund for all survival assays. We identified moribund mice as those animals that are unable to move when gently touched or not able to self correct when placed on their side (ataxia). Moribund mice were euthanized in compliance with IACUC protocols at Rutgers University and the University of Michigan.

Direct intracellular staining

We employed the in vivo approach as described by [53], which circumvents the need for *ex vivo* Ag stimulation and thus, allowing the assessment of cytokines that are actively synthesized *in vivo* during infection. Briefly, 250 µg brefeldin A (BFA; Sigma, St. Louis, MO) was injected i.v. into control and infected mice at 4.5 d post Lm-inoculation. Mice were euthanized 6 hours post injection. Pooled IEL and LP cells were immediately processed to purify CD45+ cells by magnetic cell isolation, which were then homogenized with M-PER Mammalian Protein Extraction Reagent (Thermo Sci) supplemented with a cocktail of Protease Inhibitors (Sigma), and centrifuged at 11,000 x g for 10 min. at 4^{o}C. Supernatants were used to assess cytokine production using BD multiplex bead-based immunoassays (BD Bioscience).

Antibiotic treatment

Mice were treated with ampicillin (1 mg/mL), metronidazole (1 mg/mL), neomycin (1 mg/mL), and vancomycin (0.5 mg/mL) in the drinking water either as a cocktail of 4 antibiotics, or individually, for 4 weeks [29]. All antibiotics were obtained through Fisher Scientific (Waltham, MA).

Conventionalization of mice with normal gut microflora

Suspensions of intestinal luminal contents of conventionally-raised BALB/c mice were made in PBS and introduced intragastrically into germ-free BALB/c mice by oral gavage. Mice were allowed to rear in normal conditions with conventional animals, and 4 weeks later, they were euthanized and cell preparations were made from organs.

DNA isolation from mouse fecal material and QPCR

Bacterial pellets were resuspended in 567 µl TE, 30 µl 10% SDS, and 3 µl 20 ng/mL Proteinase K (Ambion) and incubated at 37^{o}C. One hour later, 100 µl 5M NaCl, 80 µl Cetyltrimethylammonium bromide (CTAB)/NaCl solution was added and samples incubated at 65^{o}C for 10 min. One volume of chloroform/isoamyl alcohol was then added, samples centrifuged, and aqueous phase removed. This process was repeated and aqueous phase added to 0.6 volumes of isopropanol to pellet DNA. DNA pellets were washed with 70% ethanol and resuspended in TE before measuring DNA concentrations by nanodrop (Thermo Scientific). DNA was isolated from fecal material using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. QPCR primers and conditions for *Firmicutes*, *Bacteroidetes*, and All

Bacteria were described previously [30]. Briefly, 10 µl reactions were set up using the SYBR GreenER master mix (Invitrogen) with 100 nM forward and reverse primers and 1 ng DNA. The thermocycling conditions used for amplification were 50°C for 2 min., 95°C for 10 min. and 40 cycles of 95°C for 15 seconds and 60°C for 1 min. followed by a dissociation curve. Fluorescence was detected during the 60°C step. QPCR reactions were run on an Mx3000P thermofluorocycler (Agilent) and analyzed using MxPro software (Agilent). Threshold cycle (CT) values were obtained from each reaction and the relative percentage of the total bacterial population was calculated by the following formula % = 100/ (2(CT group – CT 4 all bacteria)). Statistical analysis was performed using GraphPad Prism version 5.0a (GraphPad Software).

Culture of gut bacteria

Microbial supernatants were prepared as previously described [54]. Briefly, small intestine and colon were flushed with brain heart infusion broth (BHI, Fluka Analytical) supplemented with or without the oxygen scavenger OxyRase AnaSelect (OxyRase^{TM} Inc, Ohio) for anaerobic and aerobic cultures, respectively. OxyRase is a bacterial respiratory O2-binding enzyme that when added to media turns the tube or petri dish into an anaerobic chamber [55-57]. Luminal content suspensions were diluted in BHI. Five mL cultures were incubated shaking (aerobic) or standing (anaerobic) at 37°C until they reached an OD600 of approximately 0.7. Cultures were then centrifuged and the supernatant was filtered twice through a 0.22 µm filter and used to stimulate splenocytes.

Isolation of APCs and stimulation of splenocytes

To purify $_{gut}APC^{TNLG8A}$ [31], CD45.2+ lamina propria cells were first stained with CD3- and CD19-coated microbeads to deplete T and B lymphocytes using a Miltenyi miniMACS system. The remaining cells were stained with B220- and then with MHCII-beads to positively select $B220^{+}$MHCII+$TNLG8A^{+}$ cells which were >90% pure as assessed by staining with TNLG8A antibody. As control APCs, CD45.2+ splenocytes were stained with CD11c-coated microbeads to positively select splenic DCs. Stimulation with gut bacterial supernatants was carried out as described in [54]. Briefly, EasySep^{TM} magnetically purified CD3+, CD19+, NKp46+, and MHCII+CD19- cells from CD45.1+ splenocytes were cultured in complete RPMI supplemented with antimicrobial-antimycotic (Invitrogen), plated at $1x10^{6}$/ml in 24-well tissue culture plates, and stimulated with 8 U (normalized value of OD600 x µl) of bacterial supernatants in the presence or absence of ($1x10^{4}$/ml) $_{gut}APC^{TNLG8A}$ or $_{spl}$CD11c+. Eighteen hours later, the expression of SLAMF4 on CD45.1+ cell types was measured by flow cytometry.

In vitro stimulation of lamina propria cells

Lamina propria cells (1×10^{6}/ ml) were cultured in complete RPMI 1640 supplemented with or without 10 ng/ml mouse IL-23 (eBioscience) in the presence of the protein transporter inhibitor Golgi-Stop (BD Bioscience). Four hours later, cultured cells were collected and IL-22-producing cells were analyzed by intracellular cytokine staining.