ARTICLE

NKR-P1B expression in gut-associated innate lymphoid cells is required for the control of gastrointestinal tract infections

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Helper-type innate lymphoid cells (ILC) play an important role in intestinal homeostasis. Members of the NKR-P1 gene family are expressed in various innate immune cells, including natural killer (NK) cells, and their cognate Clr ligand family members are expressed in various specialized tissues, including the intestinal epithelium, where they may play an important role in mucosal-associated innate immune responses. In this study, we show that the inhibitory NKR-P1B receptor, but not the Ly49 receptor, is expressed in gut-resident NK cells, ILC, and a subset of γδT cells in a tissue-specific manner. ILC3 cells constitute the predominant cell subset expressing NKR-P1B in the gut lamina propria. The known NKR-P1B ligand Clr-b is broadly expressed in gut-associated cells of hematopoietic origin. The genetic deletion of NKR-P1B results in a higher frequency and number of ILC3 and γδT cells in the gut lamina propria. However, the function of gut-resident ILC3, NK, and γδT cells in NKR-P1B-deficient mice is impaired during gastrointestinal tract infection by *Citrobacter rodentium* or *Salmonella typhimurium*, resulting in increased systemic bacterial dissemination in NKR-P1B-deficient mice. Our findings highlight the role of the NKR-P1B:Clr-b recognition system in the modulation of intestinal innate immune cell functions.

Key words: Innate lymphoid cells; Natural killer cells; Gut-associated immune cells; NKR-P1B receptor; Innate immunity

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INTRODUCTION

In addition to its role in digestion and nutrient uptake, the mammalian gastrointestinal (GI) tract hosts a vast population of commensal and pathogenic microorganisms, collectively referred to as the gut microbiota. This mixture of commensal and pathogenic bacteria creates a daunting task for the host mucosal immune system as follows: it must respond to harmful, invasive pathogens while tolerating benign organisms. Tilting this equilibrium in either direction can lead to GI pathologies, including dysbiosis and inflammatory bowel disease (IBD). Mucosal lesions associated with IBD, such as ulcerative colitis and Crohn's disease, can be caused by a hyperactive immune response to commensal bacteria,[1,2] demonstrating the need for immunological tolerance. However, satisfactory immune responses are still paramount for host survival as reduced or impaired immune responsiveness can render the gut susceptible to invading pathogens.[3] Multiple cellular components of the innate immune system, including innate lymphoid cells (ILC), natural killer (NK) cells, γδT cells, macrophages, and dendritic cells (DC), are involved in mediating GI homeostasis.

Helper-type ILC are recently discovered immune cells that play important roles in mucosal immunity. ILC originate from lymphoid lineage progenitors and act in a broad manner.[4] Thus far, three ILC subtypes have been described; however, group-3 ILC (ILC3) are critical for intestinal immunity. ILC3 cells reside primarily in the intestinal tract and include NKp46$^+$ (*Ncr1*-expressing) ILC3, NKp46$^-$ ILC3, and lymphoid tissue inducer (LTi) cells. LTi cells are essential for the developmental formation of lymph nodes during embryogenesis and are present in the postnatal gut, where they play an important role in the structural regulation of gut cryptopatches and intestinal lymphoid follicles.[5] However, NKp46$^+$ ILC3 are essential producers of cytokines, such as interleukin-22 (IL-22) and, to a lesser extent, IL-17, that regulate the function of other immune cells and ensure the integrity of the intestinal barrier.[4] The disruption of this regulation can lead to increased pathogen susceptibility in mice and autoimmune diseases.[6] Similar to IL-22-producing ILC3 cells, γδT cells have been described to modulate intestinal epithelial homeostasis through the release of keratinocyte growth factor-1 (KGF-1).[7] Notably, this cytokine is also protective against colitis in mice and is correlated with the increased expression of IL-15 in intestinal epithelial cells and decreased production of inflammatory mediators, such as interferon gamma (IFNγ) and tumor necrosis factor-α (TNFα), by intraepithelial lymphocytes (IEL).[8] Additionally, γδT cells are capable of lysing stressed or transformed epithelial cells by activating NK lineage receptors, such as NKG2D, and recognizing

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CMV-infected epithelial cells independent of antigen presentation on class I major histocompatibility complex (MHC-I)-like molecules.[9]

NK cells are classified as a cytotoxic subset of group-1 ILC, while the other subset includes noncytotoxic IFNγ-producing ILC1 cells. NK cells rapidly seek and destroy host cells that have become infected by a variety of microbes (viruses, bacteria, and fungi), transformed, or cancerous. NK cells are able to distinguish between normal healthy cells and abnormal cells using a sophisticated repertoire of activating and inhibitory cell surface receptors.[10] Two receptor families involved in the regulation of conventional NK cell functions are the Ly49 and NKR-P1 families of C-type lectin-like transmembrane proteins, which recognize classical MHC-I molecules or C-type lectin-related (Clr) proteins, respectively. Interestingly, the genes encoding the Nkrp1 receptors and Clr ligands are intermingled and coinherited en bloc, leading to their conserved retention as a family across multiple species with limited but notable intraspecific polymorphisms.[11] In contrast, the Ly49 family comprises a group of highly polymorphic receptors that recognize similarly polymorphic MHC-I ligands encoded on separate chromosomes. The Ly49 family of receptors is vital for conventional NK cell function as mice with low Ly49 expression show impaired MHC-I-dependent missing-self recognition.[12] The most well-studied NKR-P1:Clr family pair is the inhibitory NKR-P1B receptor and its ligand Clr-b (*Clec2d*). NK cells lacking the NKR-P1B receptor exhibit impaired Clr-b-dependent missing-self recognition and fail to reject Clr-b-deficient target cells.[13]

In humans, the closest NKR-P1B homolog is NKR-P1A (*KLRB1*/CD161), which displays inhibitory function upon binding to its cognate ligand lectin-like transcript-1 (LLT1).[14,15] NKR-P1A is also a type II transmembrane protein that belongs to the C-type lectin superfamily. Similar to the mouse NKR-P1:Clr system, the *CLEC2D* gene, which encodes LLT1, is genetically linked to the *NKR-P1A/KLRB1* gene. The engagement of NKR-P1A in NK cells by antibody cross-linking or LLT1 interaction in target cells results in the inhibition of NK cell activity.[14–16] NKR-P1A is also expressed on subsets of $CD4^+$ and $CD8^+$ T cells with a memory phenotype in peripheral blood, where these cells constitute 20–25% of the T cell population.[16,17] However, most T cells in the intestinal lamina propria and the liver express NKR-P1A.[17,18] In addition, NKR-P1A has been shown to be expressed in Th17, $FoxP3^+$ Treg, and subsets of ILC and LTi cells in humans.[19–22] The function of the NKR-P1A receptor in these cells is not fully known.

Recent in vitro studies have determined that ILC3-like (MNK-3) cells express certain receptors common to NK cells, and most of these receptors are induced in ILC1-like (MNK-1) culture conditions.[23] The expression of the members of the NKR-P1:Clr receptor-ligand family has been observed in subsets of intestinal IEL and intestinal epithelial cells.[24–26] Here, we explore the expression and function of the inhibitory NKR-P1B receptor in lymphoid and myeloid immune cells in the mouse intestine under steady-state conditions and during pathogenic bacterial GI infections. We show that NKR-P1B is expressed in gut-associated ILC3 cells, ILC2 cells, NK cells, γδT cells, DC, and macrophages in a tissue-specific manner. The genetic ablation of NKR-P1B (in B6.*Nkrp1b*$^{-/-}$ mice) results in increased numbers of intestinal ILC3 and γδT cells; furthermore, the functions of ILC3, NK, and γδT cells appear to be impaired during GI *Salmonella typhimurium* and *Citrobacter rodentium* infections. Finally, NKR-P1B deficiency results in greater systemic dissemination of *S. typhimurium* from the gut.

MATERIALS AND METHODS

Mice

The C57BL/6 (B6) and *Rag1*$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The NKR-P1B-deficient (*Nkrp1b*$^{-/-}$) mice have been previously described.[13] The Clr-b-deficient (B6.*Ocil*$^{-/-}$ or B6.*Clrb*$^{-/-}$) mice have been previously described[27] and were provided by Drs. Vicky Kartsogiannis and Matthew T. Gillespie (Monash Medical Centre, Clayton, VIC, Australia). The *Rag1*$^{-/-}$ mice were bred with the *Nkrp1b*$^{-/-}$ mice to generate the *Rag1*$^{-/-}$*Nkrp1b*$^{-/-}$ mice. Male and female littermate mice aged between 7 and 10 weeks were used in all experiments. All manipulations involving animals were performed in accordance with university guidelines and approved by the University of Ottawa Animal Ethics Committee.

Germ-free animals

Groups of age and gender-matched germ-free B6 mice were either kept untreated or colonized (ex-germ-free) with a complete specific-pathogen-free (SPF) B6 microbiome by fecal microbiome transplant through oral gavage. Fresh fecal pellets (200–400 mg) from SPF B6 mice were harvested and resuspended in 2 mL of ice-cold sterile phosphate-buffered saline (PBS). Debris was removed by filtering through a 40-μm cell strainer, and the germ-free mice subsequently received a gavage of 200 μl through an oral gavage needle. The ex-germ-free mice were analyzed 8 weeks post-colonization with the complete SPF B6 microbiome.

Cell isolation

Intraepithelium: Following the tissue collection and cleaning, Peyer's patches were visually quantified and excised from the intestine. Then, the intestines were opened longitudinally and cut into 0.5 cm pieces. The pieces were subjected to two rounds of 20 min incubation with Ca/Mg-free Hank's Balanced Salt Solution (HBSS) solution (Lonza, Chicago, IL) supplemented with 10 mM HEPES (AMRESCO, Solon, OH) and 5 mM EDTA (Invitrogen, Grand Island, NY) at 37 °C in a shaker at 100 RPM. The supernatants from each round were collected and combined, filtered through a 70-μm cell strainer (Fisher Scientific, Ottawa, ON), pelleted at 300 × *g* for 10 min, and resuspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), penicillin, and streptomycin (Lonza, Chicago, IL). The cells were layered on a discontinuous 40–70% Percoll gradient (GE Healthcare, Chicago, IL) and centrifuged at 600 × *g* for 30 min at 4 °C with minimal acceleration and deceleration. The intraepithelial lymphocytes were collected from the interphase of the gradients. Then, the cells were pelleted at 300 × *g* for 10 min, washed, resuspended in PBS, and used for the flow cytometry analysis.

Lamina propria: The remaining intestinal fragments from above were dissociated using Ca/Mg-free HBSS containing 0.75 mg/mL collagenase IV (Worthington, Lakewood, NJ) and 0.5 mg/mL DNase I (Roche, Mississauga, ON) at 37 °C with shaking at 100 RPM for 20 min. The cells were pelleted at 500 × *g* for 5 min, resuspended in 10 mL of 40% Percoll (GE Healthcare, Chicago, IL), and centrifuged at 900 × *g* for 20 min at 20 °C. Then, the leukocytes were collected from the pellet. Alternatively, a two-phase 40–70% Percoll gradient was used as described above. In both cases, the cells were washed and resuspended in PBS.

Lymph nodes, spleen, and liver: The tissues were mechanically dissociated to isolate the cells, which were washed and resuspended in PBS. The hepatocytes were processed through a 37.5% single-phase Percoll solution and centrifuged at 700 × *g* for 12 min. Then, the leukocytes were collected from the pellet, and the red blood cells (RBC) were lysed using ACK lysis buffer.

Flow cytometry

The following fluorophore-conjugated mAbs were purchased from commercial sources (eBioscience, Dallas, TX; BD Bioscience, San Jose, CA; or BioLegend, San Diego, CA): anti-TCRβ, anti-Ly49D, anti-MHC-II, anti-B220, anti-NK1.1, anti-NKp46, anti-CD19, anti-CD11c, anti-CD11b, anti-TCRγδ, anti-Ly49G, and anti-Ly49C/I/F/H. Fc block (anti-CD16/CD32, 2.4G2); Fixable Viability Dye was purchased from

eBioscience. The anti-Clr-b (4A6) mAb has been previously described.[28] Anti-NKR-P1B (2D12) was a kind gift from Dr. Koho Iizuka (University of Minnesota, Minneapolis, MN, USA). Approximately 1–2 × 10^6 cells were incubated with Fc block for 15 min at 4 °C, followed by a 20-min incubation in the dark with the appropriate fluorophore-conjugated antibodies and Fixable Viability Dye in FACS buffer containing 0.5% bovine serum albumin and 0.02% sodium azide in PBS. The staining of Clr-b and NKR-P1B was performed with biotin-conjugated 4A6 and 2D12 mAbs, respectively, followed by fluorophore-conjugated streptavidin. The intracellular staining was performed using an IC fixation/permeabilization buffer set according to the manufacturer's instructions (eBioscience, Dallas, TX) with anti-GATA-3 (BD Biosciences, San Jose, CA), anti-RORγt, anti-IL-22, and anti-IFNγ (eBioscience, Dallas, TX) mAbs. The flow cytometry acquisition was performed using CyAN-ADP (Beckman Coulter, Brea, CA) or LSR-Fortessa (BD Bioscience, San Jose, CA) instruments. The data were analyzed using Kaluza Version 1.2 software (Beckman Coulter, Brea, CA).

Bacterial infections

Citrobacter rodentium (DBS100) and *Salmonella typhimurium* were cultured in sterile brain heart infusion (BHI) or LB broth (BD Biosciences, San Jose, CA), respectively, and the colony forming units (CFU) were determined on BHI or LB-agar plates. For the *S. typhimurium* infection, the mice were treated with 20 mg streptomycin by oral gavage 24 h prior to infection. On the following day, the mice were inoculated by oral gavage with 10^8 CFU of bacteria diluted in 200 µL of saline (0.9% NaCl) solution. The control groups were only inoculated with saline solution. A similar infection method was performed for the *C. rodentium* infection without the antibiotic treatment group. The *S. typhimurium* growth was quantified by plating fecal and splenic homogenates on streptomycin-supplemented LB agar plates on day 5 postinfection.

Histology

The colon sections were washed, fixed in 4% paraformaldehyde, and embedded in paraffin, and the sections (4 µm) were stained with hematoxylin and eosin (H&E) at the Histology Core Facility, University of Ottawa, Ontario. The tissue sections were examined blindly by a gastrointestinal pathologist at the Ottawa Hospital Research Institute (OHRI) to assess the histological changes. The tissue sections were assessed for lamina propria and IEL infiltrates per 100 enterocytes at ×40 magnification.

In vitro cell stimulations

Colon lamina propria leukocytes were cultured in 96-well plates at a density of 10^6 cells/well in 200 µL RPMI 1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamycin, and 10% heat-inactivated FBS in the presence of 5 µg/mL brefeldin A (eBioscience, Dallas, TX) for 5 h at 37 °C. The stimulation of the NK, γδT, and ILC3 cells was performed with IL-12/IL-18 (5 ng/mL), IL-1β/IL-23, and IL-2/IL-1β/IL-23 (10 ng/mL), respectively. Subsequently, the cells were stained for surface markers, IFNγ and IL-22 as described above.

In situ hybridization

The in situ hybridization analysis of *Clr-b* gene expression in the small intestinal tissue was performed as previously described.[25] Briefly, digoxigenin-labeled antisense and sense RNA probes were transcribed using DIG RNA labeling mix (Roche, Mississauga, ON) and purified (RNeasy Mini Kit, Qiagen, Toronto, ON). Intestinal tissue sections from 8-week-old mice were prepared as described above. Following deparaffinization and proteinase K digestion, the sections were prehybridized with hybridization solution (50% (v/v) formamide/5× SSC (pH 4.5), 2% (w/v) blocking powder (Roche, Mississauga, ON), 0.05% (w/v) CHAPS, 5 mM EDTA, 50 µg/mL heparin, and 1 µg/mL yeast RNA) in an oven at 58 °C for 1.5 h and incubated with hybridization solution containing 500 ng/mL digoxigenin-labeled probe overnight at 58 °C. The slides were washed, stained with a sheep anti-digoxigenin alkaline phosphatase-conjugated antibody, and equilibrated in NTM buffer (0.1 M Tris-Cl pH 9.5, 0.1 M NaCl, and 0.05 M $MgCl_2$). The color development was performed by NBT/BCIP (Roche, Mississauga, ON). The slides were counterstained with 0.025% methyl green and visualized under a light microscope.

Statistical analysis

Statistical significance was determined by Student's *t*-test and log rank test as applicable with a *p*-value ≤0.05 as the cut-off value.

RESULTS

NKR-P1B is expressed in gut-associated lymphoid and myeloid immune cells

We determined the expression of NKR-P1B in immune cells in different intestinal compartments. All lymphoid cells were isolated from the lamina propria (LP) of the jejunum, colon, and cecum, small intestinal intraepithelium, mesenteric lymph nodes (MLN), liver, and spleen of WT and NKR-P1B-deficient mice. Then, the cells were stained with antibodies specific for NKR-P1B and various cell surface markers for the flow cytometric analysis. The immune cell gating strategies are shown in Supplemental Fig. 1a, b. We found that NKR-P1B was expressed in LP-associated γδT cells (TCRβ$^-$ B220$^-$ TCRγδ$^+$), NK/ILC1 cells (TCRβ$^-$ NKp46$^+$ RORγt$^-$), NKp46$^+$ ILC3 cells (TCRβ$^-$ NKp46$^+$ RORγt$^+$), NKp46$^-$ ILC3 cells, including LTi (TCRβ$^-$ NKp46$^-$ RORγt$^+$), and ILC2 cells (TCRβ$^-$ NKp46$^-$ RORγt$^-$ GATA3$^+$) but not in conventional T (TCRβ$^+$) or B (B220$^+$) cells (Fig. 1a). While most ILC3 cells in the small intestine expressed NKR-P1B, approximately 30–40% of the colon ILC3 cells did not express NKR-P1B (Fig. 1a). Only very low levels of NKR-P1B could be detected among the B220$^+$ cells in the colon and cecum (Fig. 1a). NK/ILC1 cells were the predominant NKR-P1B$^+$ cells in the MLN, liver, and spleen (Fig. 1a). Within the NK/ILC1 cell compartment, the percentage of cells expressing NKR-P1B in the gut was higher than that in the MLN, liver, and spleen (Fig. 1a). In contrast to the LP-associated γδT cells, the intraepithelial γδT cells did not express NKR-P1B (Suppl. Figure 1c and d). The expression of NKR-P1B was also detected on DC subsets in the colon but not in other LP compartments or lymphoid organs (Fig. 1b, c). Low levels of NKR-P1B were detected in a subset of macrophages in the colon (Fig. 1b). Considering the close proximity of the intestinal microbiome and its implications in the positive and negative regulation of ILC3 functions, we investigated the impact of the gut microbiome on NKR-P1B expression in ILC3 cells.[29–31] Therefore, we analyzed the NKR-P1B expression in ILC3 cells in germ-free (GF) and germ-free mice colonized with B6 microflora (ex-germ-free). All ILC3 cells from the germ-free mice, including those from the colon, expressed NKR-P1B (Suppl. Figure 2a). A decreased NKR-P1B expression profile was observed in the GF mice following the microflora recolonization only in the colon (Suppl. Figure 2a). Altogether, our data indicate that NKR-P1B expression is prevalent among the ILC subsets within the LP of the small intestine, whereas NK cells constitute most of the NKR-P1B$^+$ population in lymphoid organs.

Clr-b is broadly expressed in gut-resident leukocytes

Clr-b is the only known NKR-P1B ligand; therefore, we determined the tissue specificity of mucosal Clr-b expression to identify potential cellular interactions. The in situ hybridization using a Clr-b mRNA probe revealed that Clr-b was expressed in the intraepithelium, LP, and crypts of the small intestine (Fig. 2a). Our previous microarray analysis revealed that Clr-b transcripts were expressed in cells of hematopoietic origin in various mouse organs.[25] We confirmed this finding by performing a flow

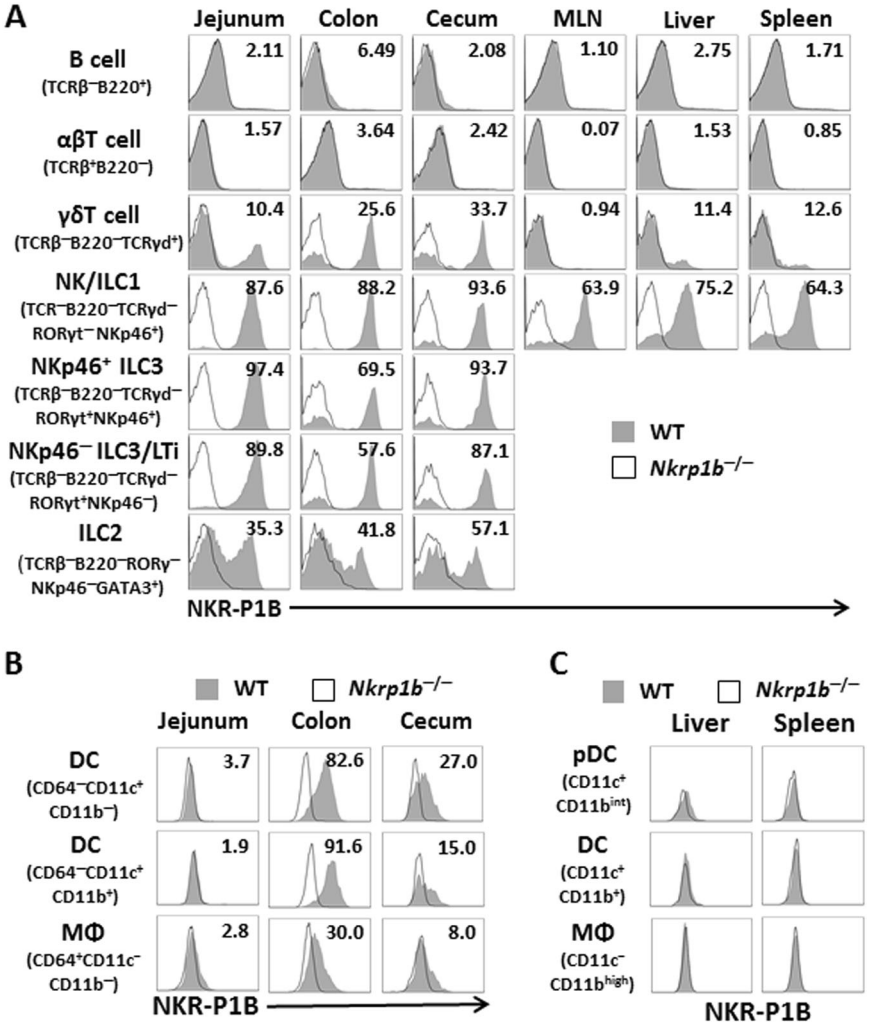

Fig. 1 NKR-P1B expression in immune cells in the small intestine and secondary lymphoid organs. **a** Flow cytometry analysis of NKR-P1B expression in lymphocytes in the lamina propria of the jejunum, colon and cecum, MLN, liver, and spleen of WT and NKR-P1B-deficient (*Nkrp1b*$^{-/-}$) mice. **b**, **c** Flow cytometry analysis of NKR-P1B expression in DC and macrophage subsets in different gut compartments (**b**), liver and spleen (**c**) of WT and NKR-P1B-deficient mice. Percentage of cells expressing NKR-P1B is indicated. The data are representative of multiple independent experiments ($n = 9$). Gating strategy used for the flow cytometry analysis is shown in Suppl. Figure 1a and b

cytometric analysis and detected Clr-b expression in T, B, NK, and γδT cells, myeloid and plasmacytoid DC, and macrophages in the small intestinal LP (Fig. 2b). Interestingly, based on the mean fluorescence intensity (MFI), we detected that the expression of the NKR-P1B receptor in the small intestinal NK/ILC1, ILC3, LTi, ILC2, and γδT cells in the Clr-b$^{-/-}$ mice was higher than that in the WT mice (Fig. 2c). These results are consistent with previous findings showing a higher NKR-P1B expression in Clr-b-deficient spleen and liver NK cells.[32] Altogether, these data demonstrate that Clr-b is broadly expressed in hematopoietic cells in the small intestine.

Ly49 receptors are not highly expressed in immune cells in the gut

Subsequently, we investigated whether other lectin-like receptors are also expressed in immune cells in the gut. Since the Ly49 family of receptors plays an important role in NK cell function, we assessed the overall expression of Ly49 receptors in all gut and spleen lymphocytes. The gut LP lymphocytes exhibited a lower Ly49 expression (0.80 ± 0.15%) than their splenic counterparts (4.46 ± 1.21%) (Fig. 3a). The percentage of gut-resident NK cells expressing Ly49 receptors (19.92 ± 2.20%) was significantly lower than that in their splenic counterparts (77.78 ± 1.77%) (Fig. 3b). However, the ILC2 and ILC3 cells completely lacked Ly49 receptor expression (Fig. 3b). These data are consistent with a previous study showing low levels of Ly49 expression in mouse intestinal LP NK cells.[33,34]

ILC subsets constitute most NKR-P1B^{+} cells in the intestine

To determine the tissue-specific composition of cells expressing NKR-P1B, we analyzed the frequency and distribution of NKR-P1B^{+} leukocytes isolated from the LP of the jejunum, colon and cecum, MLN, liver, and spleen of WT mice. NKR-P1B^{+} cells constituted a higher proportion of lymphocytes in the small intestine compared to those in the spleen (Fig. 4a). NKp46^{-} and NKp46^{+} ILC3 cells constitute the largest percentage of NKR-P1B^{+} lymphocytes in the jejunum (Fig. 4b). However, NK/ILC1 cells and NKp46^{-} ILC3 constitute the largest percentage of NKR-P1B^{+} cells in both the colon and cecum (Fig. 4b). Moreover, the ILC2 cell subset comprised 10–12% of all NKR-P1B^{+} cells in different parts of the gut LP. The NK/ILC1 cells constituted most cells expressing NKR-P1B in the MLN (69.88 ± 3.51%), liver (78.66 ± 2.42%), and spleen (83.29 ± 1.59%) (Fig. 4b). This finding partially reflects the higher abundance of NK cells in lymphoid organs, while ILCs are more abundant in the intestine. Altogether, these data show that a high number of ILCs constitute the NKR-P1B^{+} leukocyte subsets in the intestinal LP.

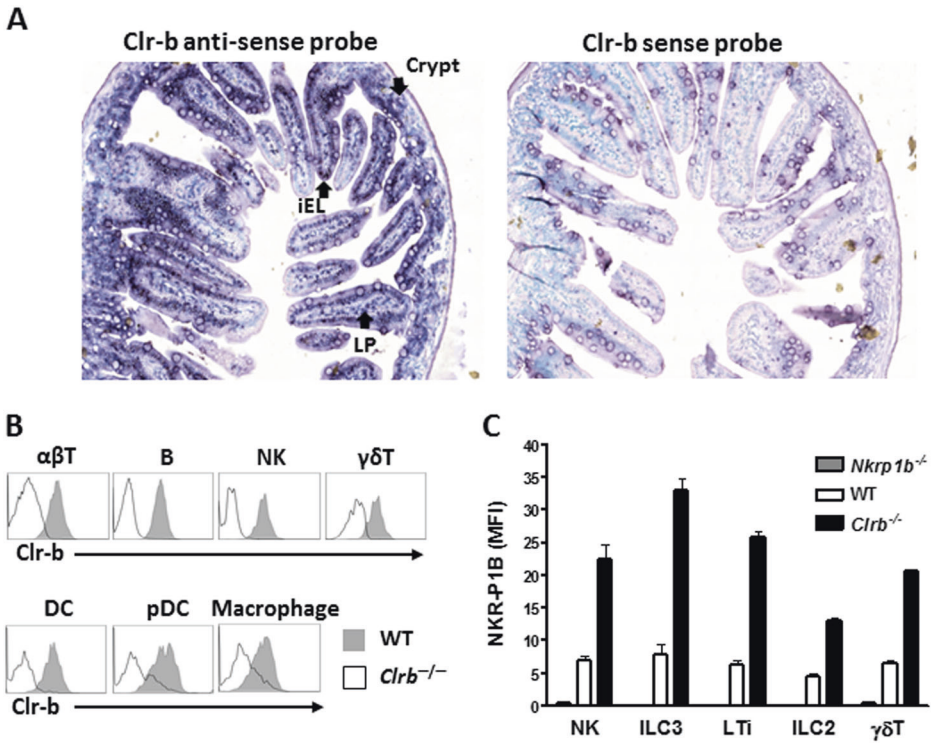

Fig. 2 Clr-b expression in immune cells in the small intestine. **a** In situ hybridization staining of the small intestinal section using Clr-b anti-sense and sense RNA probes. Staining with anti-sense probe in the lamina propria (LP), intraepithelial layer (iEL), and crypt is shown with arrows. Images are representative of at least three independent experiments. **b** Flow cytometry analysis of Clr-b expression in leukocytes in the small intestine of WT and Clr-b-deficient (*Clr-b*$^{-/-}$) mice ($n = 6$). Data from one representative experiment are shown. **c** Median fluorescence intensity (MFI) values of NKR-P1B expression in intestinal lymphocyte subsets in WT, NKR-P1B-deficient, and Clr-b-deficient mice. The data are representative of at least two independent experiments

Higher frequency and numbers of intestinal γδT cells and ILC in NKR-P1B-deficient mice

Next, we investigated whether NKR-P1B deficiency affected the frequency and numbers of immune cell populations, which could otherwise express this receptor in the intestinal LP and other organs. Compared to the WT mice, we detected an increase in both the percentage and numbers of γδT cells, NKp46$^-$ ILC3/LTi cells, and ILC2 cells in the jejunum (Fig. 5a, b) and a slight increase in the percentage, but not numbers, of NKp46$^+$ ILC3 cells in the colon of the NKR-P1B-deficient mice (Suppl. Figure 3a and b). Similarly, the ILC3 and LTi cells in the cecum were increased in frequency but not numbers in the NKR-P1B-deficient mice (Suppl. Figure 3c and d). However, no statistically significant differences in the numbers of DC subsets or macrophages were detected in distinct parts of the gut LP (Suppl. Figure 3k and l). In contrast to the small intestine, the MLN, liver, and spleen innate and adaptive immune cell types had similar percentages and numbers in the WT and NKR-P1B-deficient mice (Suppl. Figure 3e–j).

Notably, most innate immune cell types in different parts of the gut LP displayed a tendency towards an increased cell frequency and number in the NKR-P1B-deficient mice. This increase was associated with an increase in the small intestinal length in the NKR-P1B-deficient mice and fewer Peyer's patches along the length of the intestine (Fig. 5c, d). The NKR-P1B-deficient mice also exhibited a lower body weight than the WT mice (Fig. 5e). Altogether, these data show that the loss of the NKR-P1B inhibitory receptor results in increased numbers of innate immune cells in different intestinal compartments.

Defective ILC3, NK, and γδT cell responses to bacterial infections in NKR-P1B-deficient mice

Gut-resident NK, γδT, and ILC3 cells have been shown to protect the intestinal tract against pathogenic bacteria.[8,35,36] To determine whether NKR-P1B plays a role in the regulation of the function of these cells during bacterial infection, WT and NKR-P1B-deficient mice were orally infected with *Citrobacter rodentium* or *Salmonella typhimurium*. We also infected WT and NKR-P1B-deficient mice on a Rag-deficient background (*Rag1*$^{-/-}$) that lack adaptive T and B cells with *C. rodentium* to specifically determine the role of NKR-P1B in ILC subsets during infection.

ILC3 cells are the main source of IL-22 during *C. rodentium* infection in mice, and previous studies have shown that ILC3 cells play an important role in the control of *C. rodentium* infection in T and B cell-deficient Rag$^{-/-}$ mice.[37] Therefore, we investigated whether the absence of the NKR-P1B receptor could affect IL-22 production by ILC3 cells following oral *C. rodentium* infection. Following stimulation in vitro with various cytokines (IL-1β, IL-23, and IL-2), NKR-P1B-deficient NKp46$^+$ but not NKp46$^-$ ILC3/LTi cells produced significantly less IL-22 during both steady state and following infection with *C. rodentium* (Fig. 6a, b). Additionally, relative to the WT mice, the frequency of IL-22-producing ILC3 cells in the NKR-P1B-deficient mice was significantly reduced following infection (Fig. 6a).

In the *S. typhimurium*-infected mice, the intestinal NK and γδT cell functions were analyzed following isolation and in vitro stimulation with cytokines (IL-12 and IL-18) or PMA and ionomycin. Both the NK/ILC1 and γδT cells from the colon of the infected NKR-P1B-deficient mice had a lower percentage of IFNγ$^+$ cells than those in the WT mice (Fig. 6c, d). Interestingly, while the NK/ILC1 cells from the infected mice were more responsive to the in vitro stimulation than those from the uninfected mice, the γδT cells from the infected mice were less responsive than those from the uninfected mice. This deficiency was more pronounced in the NKR-P1B-deficient mice (Fig. 6d). Previous studies have demonstrated the role of IFNγ in recruiting inflammatory leukocytes to sites of infection and limiting systemic

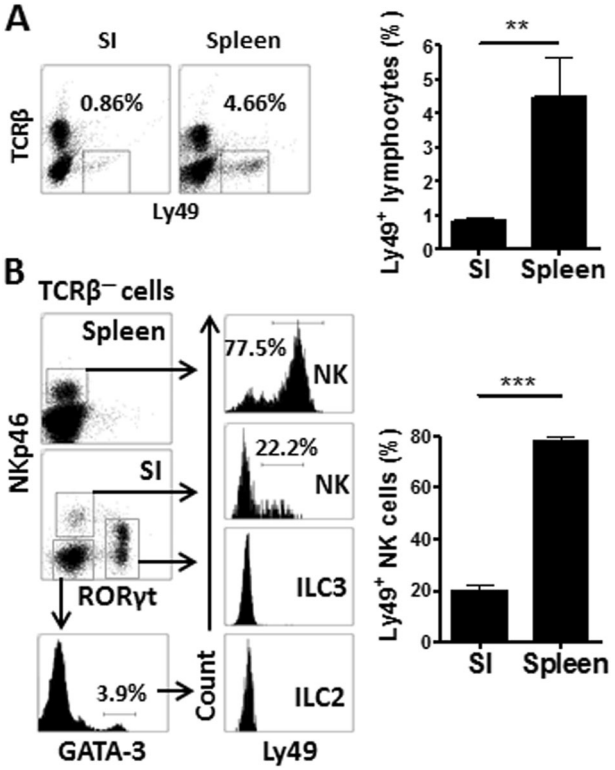

Fig. 3 Ly49 expression in immune cells in the small intestine and spleen. **a** Flow cytometry analysis of Ly49 expression in lymphocytes in the small intestinal (SI) lamina propria and the spleen of WT mice. Bar graph represents the percentage (mean + SD) of Ly49$^+$ lymphocytes in SI ($n = 3$) and spleen ($n = 4$). **b** Flow cytometry analysis of Ly49 expression in NK cells from the spleen ($n = 4$) and NK, ILC2, and ILC3 cells from SI ($n = 3$) of WT mice. The Ly49 antibody cocktail used for the flow cytometry detection of Ly49 receptors comprised antibodies specific for Ly49C, I, F, H, D, and G. Percentage of Ly49$^+$ cells is indicated. Bar graph representing the percentage (mean + SD) of Ly49$^+$ NK cells in SI ($n = 5$) and spleen ($n = 4$). Statistical analysis was performed using a two-tailed unpaired *t*-test; $^{**}p < 0.01$, $^{***}p < 0.001$

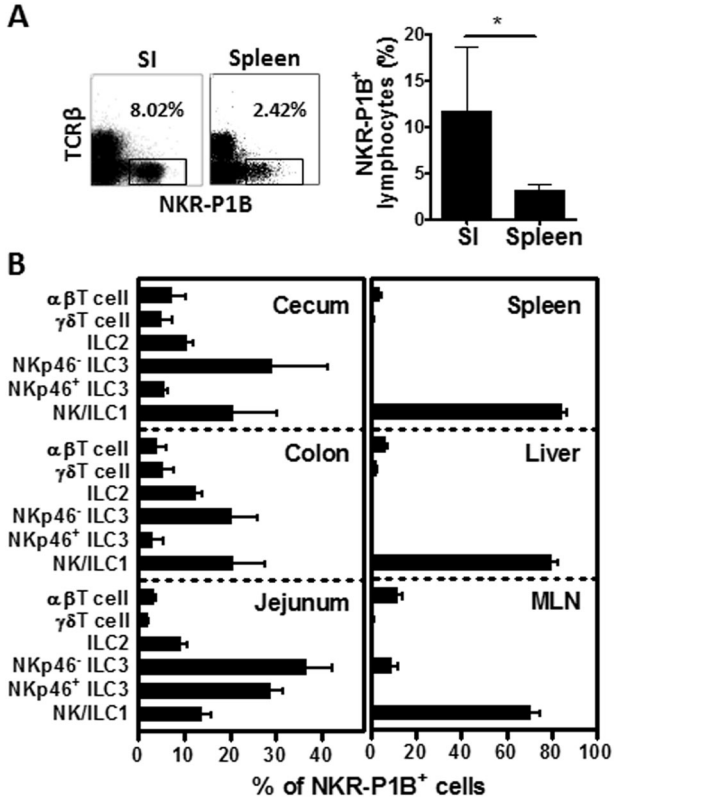

Fig. 4 Tissue-specific distribution of NKR-P1B$^+$ immune cells. **a** Flow cytometry analysis of NKR-P1B expression in all lymphocytes isolated from the SI and spleen. Percent of NKR-P1B$^+$ cells is indicated. Bar graph representing the percentage (mean + SD) of NKR-P1B$^+$ lymphocytes in the SI ($n = 6$) and spleen ($n = 5$). **b** Distribution of immune cell subsets and their percentage composition in NKR-P1B$^+$ immune cell subsets in the jejunum, colon, cecum, MLN, liver, and spleen. The data are pooled from at least three independent experiments ($n = 6$). Statistical analysis was performed using a two-tailed unpaired *t*-test; $^*p < 0.05$

bacterial dissemination.[38,39] Thus, we investigated whether the difference in IFNγ production between the WT and NKR-P1B-deficient NK and γδT cells could influence the inflammatory response and control of *S. typhimurium* infection. Indeed, the IEL infiltration detected in the colon of the WT mice was higher than that in the NKR-P1B-deficient mice (Fig. 6e). While the fecal bacterial load determined by serial dilution and growth on selective medium was similar between the WT and NKR-P1B-deficient mice, a significantly higher bacterial load was detected in the spleens of NKR-P1B-deficient mice on day 5 postinfection (Fig. 6f).

Despite the hyporesponsive nature of the NK, γδT, and ILC3 cells and higher systemic *S. typhimurium* dissemination in the NKR-P1B-deficient mice, the WT and NKR-P1B-deficient mice showed similar body weight loss and survival kinetics following infection with *C. rodentium* and *S. typhimurium* (Suppl. Figure 4). Altogether, these data suggest that the NKR-P1B receptor is required for optimal innate immune function in the gut during bacterial infection but is not essential for mouse survival.

DISCUSSION

NK cell-associated receptors have been shown to be expressed in several non-NK cells. Previous studies have demonstrated that NKp46 and NKp44 are expressed in mouse and human ILC3 cells, respectively.[40] Here, we report that the inhibitory NKR-P1B receptor is expressed in gut-resident NK/ILC1 cells, ILC3/LTi cells, ILC2 cells, γδT cells, DC, and macrophage subsets, demonstrating a broad tissue-specific distribution. NKR-P1B expression appears to be prevalent among ILC3 lymphocytes in the jejunum, colon, and cecum, whereas NK/ILC1 cells constitute most NKR-P1B$^+$ lymphocytes in the MLN, spleen, and liver. In contrast to NKR-P1B, Ly49 receptors are absent in ILC, and only a small proportion of NK/ILC1 cells in the gut express Ly49. Moreover, our data revealed that the expression of the NKR-P1B receptor among intestinal leukocytes is significantly higher than that among splenocytes. NK/ILC1 cells expressing high levels of NKR-P1B have been previously detected in gut-associated lymphoid tissue in rats.[41] Similarly, murine intestinal lamina propria NK cells have been shown to display low levels of Ly49 receptor expression compared to their spleen and liver counterparts.[33,34] These studies, along with ours, indicate that NK cell-associated receptors, including NKR-P1B, may play an important and tissue-specific immunoregulatory role in the intestine. Moreover, Clr-b has been shown to be broadly expressed in cells of hematopoietic origin,[25,28] and here, we confirmed this finding in lymphoid and myeloid immune cells in the small intestine. We also detected higher expression levels of NKR-P1B in NKR-P1B$^+$ intestinal lymphocytes from Clr-b-deficient mice, which could indicate active NKR-P1B:Clr-b interactions; the absence of such interactions could result in receptor upregulation (due to the loss of interactions in *trans*) or higher surface availability of NKR-P1B for antibody binding and detection

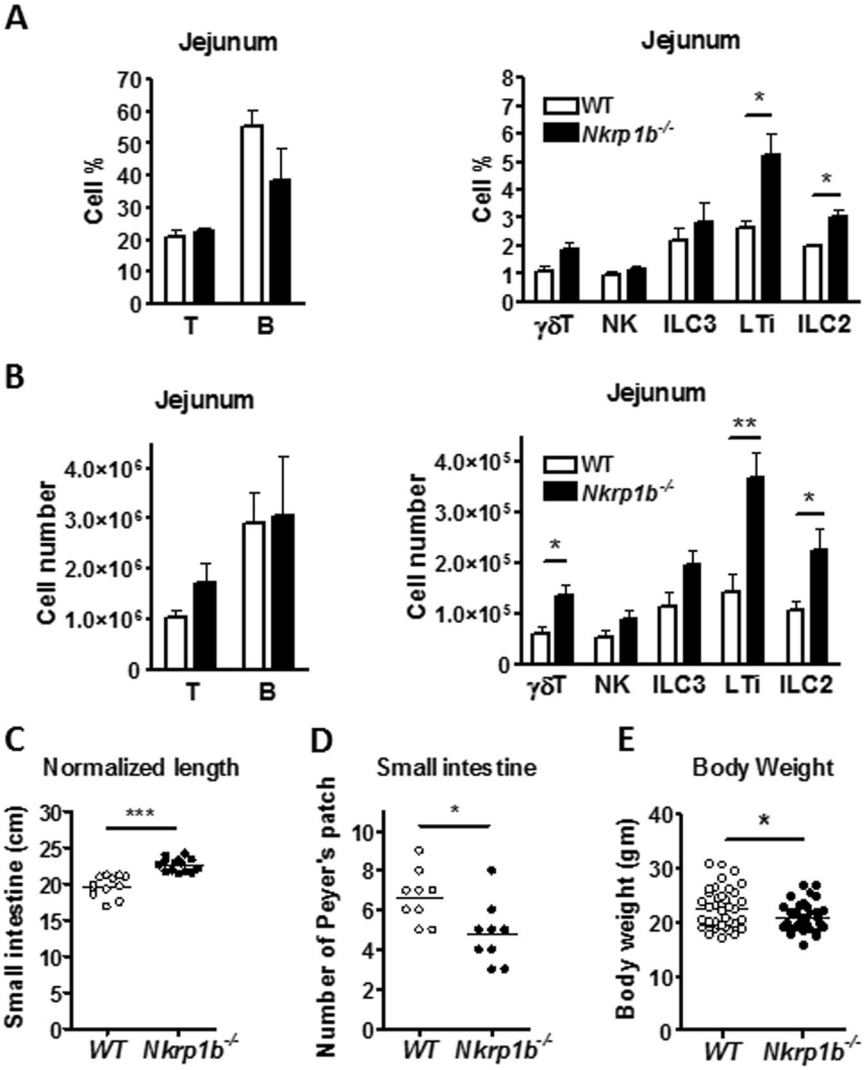

Fig. 5 Frequency and numbers of lymphoid cells in the jejunum. **a**, **b** Percentage (**a**) and cell numbers (**b**) of lymphoid cells in the jejunum lamina propria of WT and NKR-P1B-deficient mice. **c** Intestinal length was normalized to the tibia length in WT and NKR-P1B-deficient mice (WT: $n = 12$, $Nkrp1b^{-/-}$: $n = 14$). **d** Number of Peyer's patches in the small intestine from WT and NKR-P1B-deficient mice. **e** Body weights of WT and NKR-P1B-deficient mice. Each data point represents one mouse, and the horizontal lines indicate the mean values (**c**–**e**). Statistical analysis was performed using a two-tailed unpaired *t*-test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Also, see Suppl. Figure 2

(in *cis*). We have previously reported high NKR-P1B expression in Clr-b-deficient splenic and liver NK cells.[32]

The selective expression of NKR-P1B over Ly49 receptors in the intestine could be due to genetic differences between the two receptor families. While Ly49 receptors and their MHC-I ligands are highly polymorphic and encoded by genes on separate chromosomes, the genes encoding the NKR-P1 receptors and Clr ligands are intermingled in the same genomic region, allowing for the cosegregation and linked conservation of receptor–ligand interactions.[11] The high polymorphism of the Ly49 receptor and its MHC-I ligands may be suboptimal for tolerance induction in an environment as heterogeneous as the intestine and instead may promote positive regulation by unique MHC-restricted gut-associated T cell subsets. In contrast, the more conserved inhibitory NKR-P1:Clr recognition systems (NKR-P1B:Clr-b; NKR-P1G:Clr-f;Clr-a) might promote a more tolerogenic intestinal environment.[24–26]

The genetic deficiency of NKR-P1B resulted in an increased frequency and number of ILC and γδT cells in the intestine, which was also associated with fewer Peyer's patches but an increased intestinal length in the NKR-P1B-deficient mice compared to the WT mice, despite their smaller body weight. This finding could reflect differences in the LTi numbers and function. LTi cells in the postnatal gut are required for the development of intestinal cryptopatches and lymphoid follicles.[5] Our results indicate that the cell number and percentage of $NKp46^{-}$ ILC3, which include LTi cells, in NKR-P1B-deficient mice are significantly higher than those in WT mice. Therefore, it is reasonable to speculate that the NKR-P1B:Clr-b recognition axis plays a role in the modulation of LTi function and development of intestinal lymphoid structures.

Interestingly, consistent with previous data demonstrating the modulatory effects of the microbiome on gene expression in NK, ILC1, ILC2, and ILC3 cells, changes in the expression of NKR-P1B were observed specifically in colonic $NKp46^{+}$ and $NKp46^{-}$ILC3 cells.[31,42] These observations are very intriguing and demonstrate the microbiota-controlled, tissue-specific regulation of NKR-P1B expression in colonic ILC3 cells. Microbiota-derived signals activating the Myd88 pathway in macrophages and DCs drive ILC3-specific cytokine release and support intestinal immune homeostasis in both the small and large intestine.[29] In turn, the inhibitory actions of microbial fermentation products appear to counterbalance these actions specifically in gut-associated

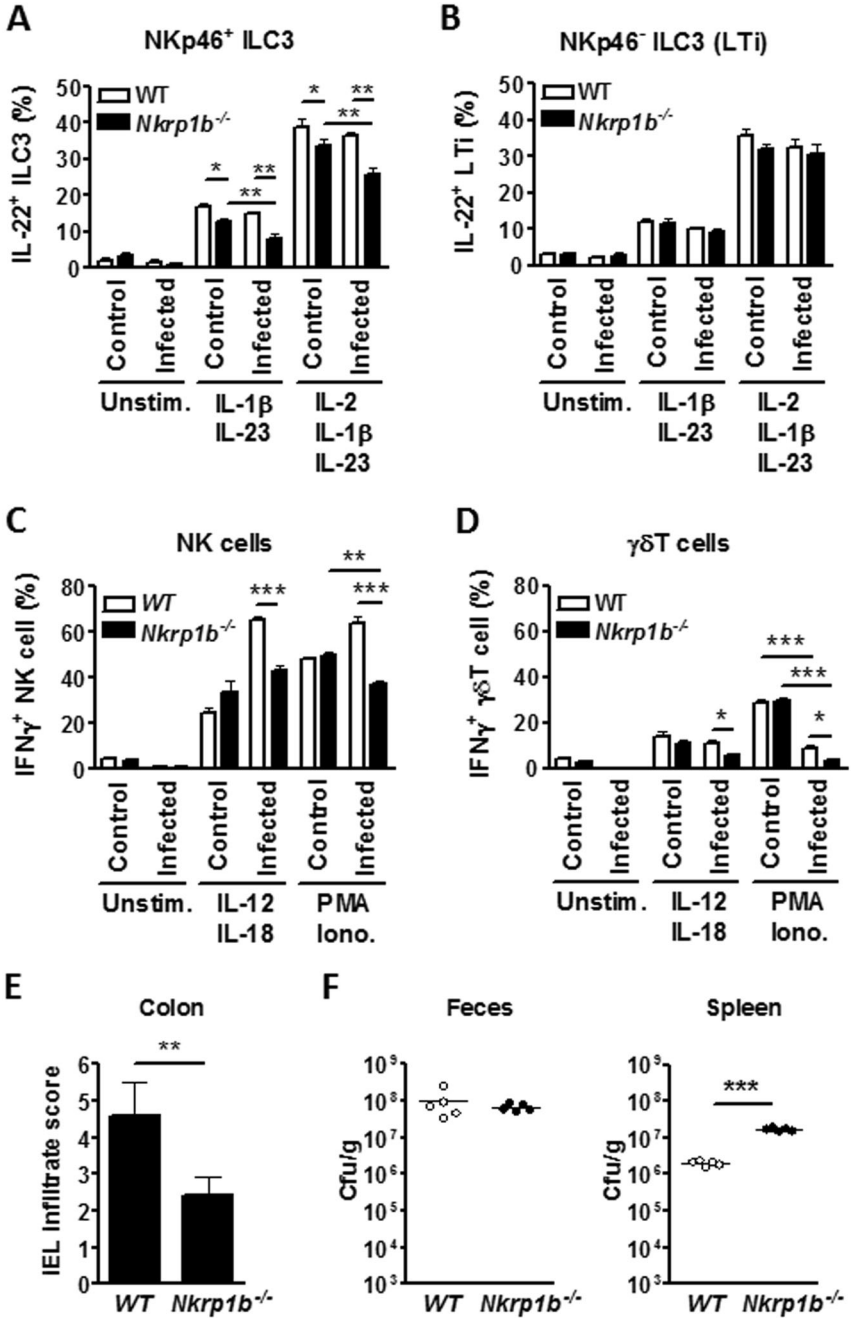

Fig. 6 ILC3, NK/ILC1, and γδT cell responses to bacterial infections. Mice were orally infected with *C. rodentium* (**a**, **b**) or *S. typhimurium* (**c**–**f**). After 5 days, lymphoid cells from the colon lamina propria of infected and uninfected control mice were stimulated in vitro. IL22 and IFNγ production was measured by intracellular staining and flow cytometry in ILC3, NK/ILC1, and γδT cells. **a, b** Bar graphs represent the percent (mean + SD) of IL-22$^+$ NKp46$^+$ (**a**) and IL-22$^+$ NKp46$^-$ (**b**) ILC3 cells in *C. rodentium*-infected mice. **c, d** Bar graphs represent the percent (mean + SD) of IFNγ$^+$ NK (**c**) and IFNγ$^+$ γδT (**d**) cells in *S. typhimurium*-infected mice. The data are representative of at least three independent experiments (performed in triplicate). **e** Histological assessment of intraepithelial layer (iEL) infiltration on day 5 after *S. typhimurium* infection (iEL count per 100 surface enterocytes at ×40 magnification; $n = 5$). **f** CFU counts in feces and spleen of *S. typhimurium*-infected mice on day 5 postinfection. The data ($n = 5$) are representative of at least three independent experiments. Statistical analysis was performed using a two-tailed unpaired *t*-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

lymphoid organs in the caecum, suggesting that microbiota-derived signals have a location-specific, negative regulatory effect on ILC3 function.[30] Future research should address these differential regulatory mechanisms of ILC3 across multiple anatomic locations.

Gut-resident NK/ILC1, γδT, and ILC3 cells play an important role in the immune response against pathogenic bacteria.[8,35,36] Antibody-mediated depletion and adoptive transfer experiments have demonstrated that Thy1$^+$ NK/ILC1 cells are mainly responsible for intestinal immunity against *Salmonella* through the early production of IFNγ.[38] ILC3 cells have also been shown to be involved in immunity against gastrointestinal bacterial infections.[35,43] NKp46$^+$ ILC3 cells are important producers of IL-22, which is required for the health and proliferation of epithelial cells, ensuring the fortification of the intestinal barrier,[44,45] while NKp46$^-$ ILC3 cells have been found to release high amounts of IL-17 and

IFNγ, which are necessary for the recruitment and activation of immune cells and the clearance of invading microbes.[43] The absence of NKp46$^+$ ILC3 cells has been associated with increased susceptibility to infection by *C. rodentium*, which is a murine pathogen that models human infection by the entero-pathogenic *Escherichia coli*,[35] and the absence of NKp46$^-$ ILC3 (LTi) cells in B and T cell-deficient mice ameliorates colitis due to *Helicobacter hepaticus* infection.[43] Although the implications of these innate cell types in gut immunity are becoming clear, the mechanisms regulating their functions are poorly understood.

NKR-P1B might play an important role in the regulation of the functions of not only intestinal NK/ILC1 cells but also ILC3/LTi and γδT cells. Indeed, an increased proportion of γδT and αβT cells expressing NKR-P1A/CD161, which is the human homolog of NKR-P1B, have been detected in peripheral blood from *Salmonella*-infected individuals.[46] Our data demonstrate that compared to their WT counterparts, the absence of the NKR-P1B receptor renders NK/ILC1, γδT, and ILC3 cells hyporesponsive to stimuli. Fewer IFNγ$^+$ NK/ILC1 and γδT cells and fewer IL-22$^+$ ILC3 cells were detected among gut-associated lymphocytes from NKR-P1B-deficient mice infected with *S. typhimurium* and *C. rodentium*, respectively. IFNγ production by mucosal NK/ILC1 cells limits systemic bacterial dissemination and sustains the recruitment of inflammatory leukocytes to the site of infection.[38,39] Compared to the WT mice, we detected fewer IELs in the intestine and significantly higher bacterial counts in the spleens of the NKR-P1B-deficient mice on day 5 postinfection, despite similar bacterial counts in their feces.

Inhibitory MHC-I-specific Ly49 receptors are essential for licensing NK cells to respond to activation signals, which is a process known as NK cell education. Generally, the more inhibitory self-MHC-I-specific Ly49 receptors an NK cell expresses, the more educated and responsive the cell is to activating stimuli; in contrast, NK cells from MHC-I-deficient mice are functionally hyporesponsive.[47] Importantly, this may also be true for MHC-independent inhibitory receptors, including NKR-P1B. Therefore, the lack of NKR-P1B-mediated NK cell education in Clr-b ligand-deficient mice[32] and/or NKR-P1B receptor-deficient mice[13] may render NK cells hyporesponsive, which is specifically important for intestinal NK/ILC1 cells since these cells do not display high or prevalent expression of Ly49 receptors to support MHC-I-dependent education. The mechanisms by which inhibitory NK lineage receptors regulate ILC3/LTi and γδT cell function is unknown. However, surprisingly, NKR-P1B appears to perform a positive regulatory function in ILC3 and γδT cells. Furthermore, we have previously shown that the inhibitory Ly49Q receptor positively regulates type-I IFN production by plasmacytoid DC.[48] Alternatively, this finding could reflect an indirect effect of the regulated production of cytokines and chemokines, which have been shown to modulate immune cell functions. CXCL16 and IL-23 produced by intestinal DC have been shown to influence IL-22 production by ILC3.[49] CXCR6 chemokine receptor-deficient ILC3 cells exhibit impaired IL-22 production, resulting in impaired mucosal immunity to *C. rodentium*.[49] CD11c$^+$ CD11b$^+$ intestinal macrophages have also been shown to activate IL-22-producing ILC3 in an IL-1β-dependent manner.[50] Further studies are needed to determine whether NKR-P1B deficiency results in the functional impairment of intestinal DC and macrophages, in turn resulting in reduced ILC3 responses in NKR-P1B-deficient mice.

The NKR-P1B:Clr-b recognition system plays an important role in NK cell-mediated anti-viral immune responses. We have shown that the murine cytomegalovirus (MCMV)-encoded m12 protein can directly interact with the NKR-P1B receptor to inhibit NK cell activation.[51] Consequently, NK cells lacking the expression of the NKR-P1B receptor confer better protection against MCMV infection than those expressing NKR-P1B.[52] MCMV infection has been detected in the intestine, resulting in the exacerbation of dextran sodium sulphate (DSS)-induced colitis in mice.[53] Whether m12-mediated MCMV immunoevasion can also affect NKR-P1B-expressing gut-associated innate immune cell functions and play a role in IBD pathogenesis remains unknown.

Taken together, our results indicate that the NKR-P1B:Clr-b receptor–ligand pair plays a role in the control of gut innate immunity, particularly in the context of NK cells and other ILC subsets that utilize NK-associated receptors. This system appears to supplant the conventional Ly49:MHC-I axis known for regulating classical NK cell functions, which raises intriguing questions about the possible duality of tissue-restricted "gut" and systemic "nongut" immune responses. Despite advances in our understanding of the etiology of IBDs, knowledge regarding gut immunology and how it may differ from our knowledge of classical immune responses remain limited. We hope that these findings will enable advances in our understanding of GI health and immunity and lead to more effective clinical interventions for diseases, such as Crohn's and colitis. These data may suggest that the human NKR-P1A:LLT1 interaction plays a role in human mucosal infection and disease.

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AUTHOR CONTRIBUTIONS

E.A.-S., Z.H., J.F., O.A.A., M.S., A.B.M., M.M.T., S.P., A.M., and M.M.A.R. performed the experiments and analyzed the data. E.A.-S., J.R.C., A.M., M.M.A.R., and A.P.M. designed the experiments, analyzed the data, and wrote the manuscript. A.P.M. supervised the study.

ADDITIONAL INFORMATION

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