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Peyer's patch innate lymphoid cells regulate commensal bacteria expansion

Masaaki Hashiguchi*, Yuji Kashiwakura, Hidefumi Kojima, Ayano Kobayashi, Yumiko Kanno, Tetsuji Kobata

Department of Immunology, Dokkyo Medical University School of Medicine, Mibu, Tochigi 321-0293, Japan

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ABSTRACT

Anatomical containment of commensal bacteria in the intestinal mucosa is promoted by innate lymphoid cells (ILCs). However, the mechanism by which ILCs regulate bacterial localization to specific regions remains unknown. Here we show that Peyer's patch (PP) ILCs robustly produce IL-22 and IFN-γ in the absence of exogenous stimuli. Antibiotic treatment of mice decreased both IL-22$^+$ and IFN-γ$^+$ cells in PPs. Blockade of both IL-2 and IL-23 signaling *in vitro* lowered IL-22 and IFN-γ production. PP ILCs induced mRNA expression of the antibacterial proteins RegIIIβ and RegIIIγ in intestinal epithelial cells. Furthermore, *in vivo* depletion of ILCs rather than T cells altered bacterial composition and allowed bacterial proliferation in PPs. Collectively, our results show that ILCs regulate the expansion of commensal bacteria in PPs.

1. Introduction

The mammalian intestinal tract is colonized by trillions of bacteria comprising thousands of different species [1–3]. Indigenous bacteria maintain appropriate homeostatic conditions for physiological processes such as metabolism of dietary materials. The bacteria reside in defined anatomical locations and exist in a symbiotic relationship with their host, limiting pathogenic microbes [4–9]. Most commensal bacteria reside in the intestinal lumen, separated from the underlying connective tissues of the body by a single layer of intestinal epithelial cells. Disorder or imbalance of commensal bacteria is related to many diseases such as inflammatory bowel disease [3]. Therefore, understanding the mechanisms that regulate anatomical containment of commensal bacteria could help in the development of novel therapeutics to prevent diseases.

Innate lymphoid cells (ILCs) are a heterogeneous population of innate immune cells. ILCs are classified into three groups based on the cytokines they produce [10]. Group 1 ILCs (ILC1s) are characterized by T-bet expression, and they produce IFN-γ. Group 2 ILCs (ILC2s) require RORα and GATA-3 and express IL-5 and/or IL-13. Group 3 ILCs (ILC3s) depend on RORγt and GATA-3 for their development and produce IL-17A and IL-22 [11–14]. ILC3s are further categorized into at least two types: lymphoid tissue inducer (LTi) cells and IL-22-producing ILCs (ILC22s) [10]. IL-22 production, which is induced by cytokines such as IL-1 and IL-23, requires the aryl hydrocarbon receptor (AHR) [12,15,16]. IL-22 produced by Th17 cells and ILC3s induces inflammation and the production of antibacterial proteins such as RegIIIβ and RegIIIγ [15,17]. Therefore, IL-22 is believed to be important for anatomical containment of commensal bacterial [18]. Microflora maintains ILC22s and enhances the production of IL-22 by NKp46$^+$ ILC22s of the intestinal lamina propria [11,19]. For example, indole-3-aldehyde, a tryptophan catabolite produced by lactobacilli, increases the number of ILC22s and upregulates IL-22 production [20]. On the other hand, ILC22s play important roles in intestinal inflammation. For example, ILC22s can induce colitis in a murine model [21] and sustains murine colon cancer [22].

Peyer's patches (PPs) are well-characterized gut-associated lymphoid tissue [23]. PPs contain immunocompetent cells and are thought to be inductive sites for immune responses [23,24]. PPs acquire foreign antigens directly from the intestinal lumen *via* specialized epithelial microfold cells (M cells) [25]. They also incorporate a variety of pathogens such as listeria and rotavirus [26]. These pathogens do not diffuse throughout the whole body, and only certain microbes such as *Alcaligenes* spp., *Ochrobactrum* spp.,

Abbreviations: AHR, aryl hydrocarbon receptor; GAT, gonadal adipose tissue; IEC, intestinal epithelial cell; ILC, innate lymphoid cell; LI, large intestine; LP, lamina propri; LTi, lymphoid tissue inducer; MLN, mesenteric lymph node; PP, Peyer's patch; SI, small intestine.

* Corresponding author at: Department of Immunology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Tochigi 321-0293, Japan.
Tel.: +81 282 87 2401; fax: +81 282 86 2705.
E-mail address: mhashi@dokkyomed.ac.jp (M. Hashiguchi).

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Serratia spp., *Burkholderia* spp. inhabit PPs, whereas Segmented filamentous bacteria reside on the luminal surface of PPs [27]. After depletion of CD90/Thy1$^+$ cells, which include ILCs, in *Rag1*-deficient mice, Alcaligenaceae, normally detected only in the intestinal tract, can be systemically detected [18]; this suggests that ILC3s of the intestinal lamina propria regulate the spread of bacteria. However, *Rag1/2*-deficient mice do not have mature PPs [28], the entry site for microorganisms under physiological conditions. Furthermore, analyses of intestinal ILCs were mainly executed using ILCs from the lamina propria. Therefore, the involvement of PP ILCs in the anatomical containment of bacteria remains unclear.

In this study, we compared the phenotypes and the cytokine production of ILCs in various tissues and found that PP ILCs specifically produced IFN-γ and IL-22 *ex vivo*. This cytokine production was dependent on IL-2 and IL-23. We demonstrated that PP ILCs activate epithelial cells to produce antibacterial proteins and regulate bacterial expansion in PPs.

2. Materials and methods

2.1. Mice

C57BL/6J (B6) mice were purchased from CLEA Japan (Tokyo, Japan) and were maintained in specific pathogen-free conditions in the Laboratory Animal Research Center of Dokkyo Medical University. Age- and sex-matched mice, 8–16 weeks old, were used in all experiments. The animal protocol was approved by and carried out in accordance with the guidelines of the committee for the Care and Use of Laboratory Animals, Dokkyo Medical University (protocol # 0663).

2.2. Cell preparation and cell culture

Spleens, lungs, mesenteric lymph nodes (MLNs), or PPs were minced with scissors, stirred in 1 mg/ml collagenase IV at 37 °C, and passed through a 70-μm filter to obtain a single-cell suspension. Splenocytes and lung cells were depleted of erythrocytes using a 0.15 M ammonium chloride solution. Small intestine or large intestine lamina propria cells were prepared as previously described [29]. Intestinal epithelial cells were prepared as previously described [30] and cultured with 10 μg/ml insulin (Sigma-Aldrich) for 1 week in 24-well plates coated with collagen type IV (Nitta Gelatin, Osaka, Japan). In some experiments, ILCs were sorted using a FACSAria cell sorter based on the expression of lineage markers and CD90. In some experiments, prepared cells were cultured in RPMI with 10% FBS and 10 ng/ml recombinant cytokine (rmIL-2, rmIL-23, or rmIL-12), 10 μg/ml mAb (anti-p40 [C17.8], anti-IL-2 [S4B6], or anti-p19 [MMp19, BioLegend]), or 25 ng/ml phorbol 13-myristate 12-acetate (Sigma-Aldrich) plus 0.5 μg/ml ionomycin (Calbiochem) for 18–24 h to measure cytokine production. Cytokines used in culture were purchased from BioLegend unless otherwise indicated. mAbs used in culture were purified from the ascitic fluid of *Rag2*-deficient mice unless otherwise described. Isotype control mAbs (rat IgG2a and mouse IgG2b) were purchased from BioLegend.

2.3. Flow cytometry

The culture supernatant from the 2.4G2 (anti-CD16/CD32) hybridoma was used to block nonspecific binding. Fluorochrome-conjugated mAbs and streptavidin conjugates were purchased from BioLegend or eBioscience. The following mAbs were used for flow cytometry: FITC- or biotin-anti-Lin (anti-CD3ε, 145-2C11; anti-B220, RA3-6B2; anti-MHC II, M5/114.15.2), PerCP-Cy5.5-anti-CD45.2 (104), APC-anti-CD90.2 (53-2.1), PE-anti-Sca-1 (E13-161.7), PE-anti-CD25 (PC61.5), APC-anti-CD127 (A7R34), PE-anti-IL-33Rα (DIH9), APC-anti-KLRG1 (2F1), APC-anti-ICOS (C398.4A), PE-anti-NK1.1 (PK136), PE-NKp46 (29A1.4), PE-anti-RORγ (B2D), PE-anti-T-bet (4G10), PE- or APC-anti-IL-5 (TRFK5), PE-anti-IL-13 (eBio13A), APC-anti-IL-4 (11B11), PE-anti-IL-17A (TC11-18H10.1), APC-anti-IL-22 (IL22JOP), and PE-anti-IFN-γ (XMG1.2). Isotype and fluorochrome-matched mAbs were used for control staining.

For intracellular cytokine staining, 2 mM monensin was added to cultures 4 h prior to harvest. After staining the surface molecules, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with cytokine-specific mAbs.

Flow cytometry experiments were performed using a FACSCalibur flow cytometer, and data analyses were performed using CellQuest Pro (BD Biosciences) and FlowJo (TreeStar, Ashland, OR) software.

2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen). cDNA was synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen), and qPCR was performed using Thunderbird SYBR qPCR mix (Toyobo, Japan, Tokyo) and an ABI 5700 (Invitrogen) instrument. Data were normalized to *Actb* (tissues) or *Hprt* (cells) gene expression levels. The specific primers are listed as follows: *Actb*, 5′-cct gtg ctg ctc acc gag gc-3′ and 5′-gca cag tgt ggg tga ccc cg-3′; *Hprt*, 5′-cgt gat tag cga tga tga acc ag-3′ and 5′-atc cag cag gtc agc aaa gaa c-3′; *Il23r*, 5′-aca gct cgg att tgg tat aaa ggc-3′ and 5′-tga cac atg tca gat tgc tgg g-3′; *Il17rb*, 5′-gac tga gga gag tga agg tgc g-3′ and 5′-cct tcc ttg cct cca agt tag g-3′; *Il22*, 5′-ggt gcc ttt cct gac caa act c-3′ and 5′-gcc ttg atc tct cca ctc tct c-3′; *Ifng*, 5′-ttc agc aac agc aag gcg aaa a-3′ and 5′-gag gct gga ttc cgg caa ca-3′; *Ahr*, 5′-aga gct gtg cac aag agg atc g-3′ and 5′-aat caa gcg tgc att gga ctg-3′; *Reg3b*, 5′-tgt gct caa tag cgc tga gg-3′ and 5′-cag aaa gca cgg tct aag gc-3′; and *Reg3g*, 5′-acc atc atg tcc tgg atg ctg-3′ and 5′-aat agg agc cat agg cac gg-3′.

2.5. In vivo *treatment*

To kill intestinal bacteria, a cocktail of antibiotics (1 mg/ml ampicillin, 1 mg/ml neomycin, 0.5 mg/ml vancomycin, and 1 mg/ml metronidazole) was continuously administered *via* drinking water for 2 weeks.

To deplete CD90$^+$ cells in mice, 0.5 mg of anti-CD90.2 mAb (30-H12, ATCC; prepared from ascites using caprylic acid) was administered ip 4 times (once every 3 days). Control rat IgG was purchased from MP Biomedicals (Santa Ana, CA).

2.6. 16S rRNA analysis

PPs were isolated, washed vigorously with PBS, and dispersed using collagenase type IV. The supernatant of the collagenase-treated fluid was centrifuged at 1000 × *g* for 10 min to remove host mononuclear cells and then centrifuged at 18,000 × *g* for 10 min. The pellets were lysed with Proteinase K, and nucleic acid was prepared by phenol/chloroform extraction followed by ethanol precipitation. The 16S rRNA gene was amplified by PCR with two universal primers (27F: 5′-aga gtt tga tcc tgg ctc ag-3′; 1492R: 5′-ggt tac ctt gtt act act g-3′) and ligated into TA cloning vector pTAC-2 (BioDynamics, Tokyo, Japan). Plasmid DNA of randomly selected transformants was prepared and sequenced using the M13F primer. All sequences were examined by BLAST search to identify the closest relatives.

For 16s qPCR analyses, DNA was prepared form a homogenate of PP cells. qPCR analyses were performed as described in Section 2.4. The phylum- or class-specific *16s* and control primers are listed as follows: Bacteroidetes, 5′-ccg gaw tya ttg ggt tta aag gg-3′ and 5′-ggt aag gtt cct cgc gta-3′; Firmicutes, 5′-ctg atg gag caa cgc cgc