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Gut bacteria induce granzyme B expression in human colonic ILC3s *in vitro* in an IL-15-dependent manner

Moriah J. Castleman[*], **Stephanie M. Dillon**[*], **Tezha A. Thompson**[*], **Mario L. Santiago**[*], **Martin D. McCarter**[†], **Edward Barker**[‡], **Cara C. Wilson**[*]

[*]Department of Medicine, Division of Infectious Disease, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

[†]Department of Surgery, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

[‡]Department of Microbial Pathogens and Immunity, Rush University Medical Center, Chicago, Illinois, USA

Abstract

Group 3 Innate Lymphoid cells (ILC3s) in the gut mucosa have long been thought to be non-cytotoxic lymphocytes, critical for homeostasis of intestinal epithelial cells through secretion of IL-22. Recent work using human tonsillar cells demonstrated that ILC3s exposed to exogenous inflammatory cytokines for a long period of time acquired expression of granzyme B, suggesting that under pathological conditions ILC3s may become cytotoxic. We hypothesized that inflammation associated with bacterial exposure might trigger granzyme B expression in gut ILC3s. To test this, we exposed human colon lamina propria mononuclear cells to a panel of enteric bacteria. We found that the Gram-negative commensal and pathogenic bacteria induced granzyme B expression in a subset of ILC3s that were distinct from IL-22-producing ILC3s. A fraction of granzyme B^+ ILC3s co-expressed the cytolytic protein perforin. Granzyme B expression was mediated in part by IL-15, produced upon exposure to bacteria. ILC3s co-expressing all three IL-15R subunits (IL15Rα/β/γ) increased following bacterial stimulation, potentially allowing for cis-presentation of IL-15 during bacterial exposure. Additionally, a large frequency of colonic myeloid dendritic cells (mDCs) expressed IL-15Rα, implicating mDCs in trans-presentation of IL-15 to ILC3s. Tonsillar ILC3s minimally expressed granzyme B when exposed to the same bacteria or to recombinant IL-15. Overall, these data establish the novel finding that human colonic ILC3s can express granzyme B in response to a subset of enteric bacteria through a process mediated by IL-15. These observations raise new questions about the multi-functional role of human gut ILC3s.

Correspondence: Cara Wilson, cara.wilson@cuanschutz.edu.

AUTHOR CONTRIBUTION

MC, SD, and CW designed the study, interpreted the work and wrote the manuscript. MC and TT performed the experiments. MM and MS provided tissue specimens. EB provided expertise. All authors contributed to manuscript revision, and read and approved the submitted version.

DISCLOSURE STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Keywords

ILC3s; granzyme B; IL-15; human; bacteria; gut

INTRODUCTION

The Innate Lymphoid Cell (ILC) family descend from a common lymphoid progenitor. Effector ILCs reside in tissues and are comprised of a heterogeneous collection of cells categorized into three distinct groups based on expression of a select group of cytokines(1, 2). Group 1 ILCs include NK cells and ILC1s which traditionally produce IFNγ, Group 2 ILCs (ILC2s) produce IL-4, IL-13, and IL-5, and Group 3 ILCs (ILC3s) produce the cytokine IL-22(1). Multiple studies have demonstrated that ILC3s play an important role in gut mucosal immunology via regulation of gut homeostasis(3–6). ILC3s facilitate epithelial barrier maintenance through secretion of IL-22, which acts directly on epithelial cells to induce survival, proliferation, mucus, fucosylation, and antimicrobial peptide production(7–11). Our group demonstrated that *in vitro* exposure of primary human colonic lamina propria mononuclear cells (LPMC) to enteric bacteria induced IL-22 expression in ILC3s via a process mediated by myeloid dendritic cell (mDC) production of IL-23 and IL-1β(12). Other studies also examining anti-bacterial defense demonstrated that during murine bacterial enteric infections, gut ILC3s produced IL-17A in response to *Helicobacter hepaticus,*(13) IFNγ in response to *Salmonella typhimurium*(14) or IL-22 in response to *Citrobacter rodentium.*(15–17) To control over-growth of gut microflora and regulate host responses to commensal bacteria, ILC3s also expressed IL-22,(7) lymphotoxin ($LT\alpha_1B_2$),(18) or GM-CSF(19). Additionally, ILC3 production of $LT\alpha_1B_2$ protected against enteric *C. rodentium* infection in mice.(20) Therefore ILC3s secrete many different cytokines to deal with the plethora of microorganisms that insult the gut mucosa, and it is likely that additional anti-microbial functions of these cells remain to be discovered.

Granzyme B is a serine protease that cleaves substrates after the amino acid glutamic acid or aspartic acid and is expressed by a variety of immune cells including CD8 T cells, Natural Killer (NK) cells, CD4 T cells, B cells, mast cells and basophils.(21) This multi-functional protein can induce apoptosis in target cells, facilitate the alteration of cytokine precursors into active cytokines thereby contributing to inflammation, cleave components of the extracellular matrix for tissue remodeling, and directly induce bacterial cell death as a mechanism of anti-bacterial defense(21–26). We previously demonstrated that *in vitro* exposure of LPMCs to antibiotic-killed Gram-negative bacteria (commensal and pathogenic) induced granzyme B expression in Group 1 Innate Lymphoid Cells (ILCs)(27). Two recent studies using human tonsillar cells suggested that granzyme B expression by ILC3s may be possible under certain circumstances. Long-term exposure (3 weeks) of purified human tonsillar ILC3s to the combination of IL-12 and IL-15 induced ILC3 plasticity, altering the cells into NK-like cells which expressed *gzmb* mRNA, a variety of NK cell surface markers (CD94, NKG2A, NKG2C) and had cytotoxicity against the classical NK cell target cell line K562 (28). Another study demonstrated that purified human tonsillar ILC3s exposed to the combination of IL-12 and IL-1β (for 4–6 days) resulted in the downregulation of the transcription factor Helios, upregulation of Aiolos and expression of *gzmb* mRNA,

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thought to be indicative of ILC3s turning into Group 1 ILCs. (29) Overall, these reports indicated that in certain inflammatory environments ILC3s can express gene transcripts for granzyme B, and this is likely mediated through robust *in vitro* cytokine stimulation. However, it remains to be determined if more biologically relevant stimuli would be capable of inducing granzyme B in ILC3s in mucosal tissue such as the intestine. We hypothesized that the inflammatory insult associated with epithelial barrier breakdown and microbial translocation, a process common to a number of inflammatory intestinal conditions, might induce granzyme B expression in gut ILC3s. This prompted us to utilize the primary human LPMC model to examine whether exposure of LPMCs to gut bacteria induced granzyme B expression in ILC3s *in vitro*, a process which could contribute to anti-bacterial defense during disease states where microbial translocation is a key feature (e.g., Inflammatory Bowel Disease-IBD, HIV infection) or in the setting of pathogenic bacterial infections.

In the current study, we report the novel finding that human colonic ILC3s expressed granzyme B following LPMC exposure to specific Gram-negative enteric bacteria. We further demonstrate that IL-15 signaling in response to bacteria exposure was a key driver of granzyme B induction in ILC3s, potentially mediated through both cis- and trans-presentation of IL-15. Overall, our data raise new questions about the heterogeneity of human gut ILC3s, their role in anti-microbial defense and contribution to the homeostatic environment of the human gut.

MATERIALS AND METHODS

Human tissue specimens

The use of human colonic tissue in this study was approved by the Colorado Multiple Institutional Review Board (COMIRB) at the University of Colorado Anschutz Medical Campus. Colonic tissue specimens were received from patients undergoing elective abdominal surgery at the University of Colorado Hospital and represent the surgical margins that would have otherwise been discarded. All patients signed a release to allow unrestricted use of their discarded tissue. Patient information was protected and de-identified to all laboratory investigators. Colonic samples used within this study were macroscopically normal. Exclusion criteria for colonic tissue samples are as follows: patients who underwent chemotherapy or radiation within six weeks of tissue procurement, patients with a history of inflammatory bowel disease, patients infected with HIV, and patients who received immunosuppressive drugs such as steroids within four weeks of tissue procurement. LPMCs were isolated as previously described(30, 31) and stored in liquid nitrogen until use.

The use of human tonsillar tissue in this study was approved by the Colorado Multiple Institutional Review Board (COMIRB) at the University of Colorado Anschutz Medical Campus. Tonsillar tissue specimens were acquired from pediatric patients undergoing a tonsillectomy at Colorado Children's Hospital. Patient information was protected and de-identified to all laboratory investigators. Tonsillar mononuclear cells (TMCs) were isolated as previously described(32) and stored in liquid nitrogen until use.

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Bacterial stock generation

Ruminococcus bromii (ATCC# 27255) was grown in liquid chopped meat broth (Hardy Diagnostics) for 1–2 days under anaerobic conditions at 37°C using the BD GasPak EZ Anaerobe Pouch System according to manufacturer's instructions (BD Diagnostics, Franklin Lakes, NJ). *Acinetobacter junii* (ATCC 17908) was grown using Nutrient Agar plates (Edge Biologicals, Memphis, TN) for 1–2 days under aerobic conditions at 26°C. *Salmonella typhimurium* (ATCC 35986) was grown on LB agar plates (Sigma-Aldrich) for 1–2 days under aerobic conditions at 37°C. Single-use working bacterial stocks were generated using 1X DPBS and long-term bacterial stocks were generated using 10% glycerol. All stocks were stored at −80°C until use. Bacterial concentrations were determined using the BD Cell Viability Kit (BD Bioscience) according to manufacturer's instructions.

In vitro stimulation of ILC3s

Human colonic LPMCs or TMCs were thawed as previously described(30–32) and resuspended in culture media. Culture media was RPMI supplemented with 10% human AB serum (Gemini Bioproducts, West Sacramento, CA), 1% Penicillin/Streptomycin/Glutamine (Life Technologies, Grand Island, NY), and 500μg/ml Zosyn- Piperacillin and Tazobactam (Wyeth, Madison, NY).

For the stimulation with whole bacteria, cells were plated at a concentration of 1.0×10^6 cells per mL in 48 well plates. Broad-spectrum antibiotics were present for the entire culture time period in order to prevent bacterial overgrowth and to standardize exposure of LPMCs to differing bacteria.(12, 27) Whole bacteria were added to cell cultures at a ratio of 1 LPMC/TMC to 2.5 bacteria and incubated for 16 hours at 37°C + 5% CO_2, followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 4 hours. Cells were then collected for flow cytometry as described below.

For the stimulation with recombinant cytokines, cells were plated at a concentration of 1.0×10^6 cells per mL in 48 well plates. 50ng/mL of recombinant IL-15 (R&D Systems (R & D Systems, catalog no. 247-ILB-05), the combination of 50ng/mL IL-15 and 1000 U/mL IL-2 (Tonbo) or the combination of 50ng/mL IL-15 and 50ng/mL IL-12p70 (Biolegend) were added to cell cultures and incubated for 16 hours at 37°C + 5% CO_2, followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 4 hours. Cells were then collected for flow cytometry as described below.

For blocking experiments, cells were plated at a concentration of 1.0×10^6 cells per mL in 48 well plates and exposed to 5μg/mL blocking antibody targeting IL-15 (R & D Systems, catalog no. MAB247, clone #34593) or the matched isotype control antibody (Mouse IgG1; R & D Systems, catalog no. MAB002, clone #11711) for 30 minutes followed by the addition of whole bacteria as described above. Cells were incubated for 16 hours at 37°C + 5% CO_2, followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 4 hours. Cells were collected for flow cytometry as described below. The appropriate dose off blocking antibody was determined by exposing LPMCs to 50ng/mL of recombinant IL-15 in the presence of increasing amounts of blocking antibody (data not shown) and evaluating the reduction in the percentage of granzyme B-expressing ILC3s.

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For the measurement of secreted cytokines, cells were plated as described above. Supernatant was collected and stored at −20°C until use. IL-15 was measured in the supernatant using the U-PLEX Assay according to manufacturer's instructions and quantified on the QuickPlex SQ 120 Instrument (Mesoscale Discovery, Rockville, MD).

Staining of cells for flow cytometry

For examination of ILC3s in human colonic LPMCs, ILC3s were identified as CD45 (clone HI30)+ viable lineage (CD3 (clone UCHT1), CD20 (Clone 2H7), CD13 (clone WM15), CD123 (clone 6H6), CD303 (clone 201A), CD34 (clone 561), FCεR1α (clone AER-37), CD11c (clone 3.9), CRTH2 (clone BM16))− CD127 (clone A019D5)+CD117 (clone 104D2)+ cells, a method of identification based on the universal nomenclature proposed by Spits and colleagues (1). In some studies, NKp44 (Clone P44–8) was also used. We previously reported(12) the majority of ILC3s identified using this gating strategy directly *ex vivo* expressed numerous molecules associated with ILC3 phenotype including NKp44, CCR6 and the transcription factor RORγt whereas few ILC3s expressed EOMES (<5% of ILC3s) or T-bet (<0.5%),(12) transcription factors associated with NK and ILC1 cell differentiation and function.(33) Cells were surfaced stained to identify ILC3s followed by intracellular staining for granzyme B (clone GB11), perforin (clone B-D48), IL-22 (clone 22URTI), IFNγ (4S.B3) using Fix and Perm Cell fixation and permeabilization buffer set according to manufacturer's instructions (Thermo Fisher Scientific, Frederick, MD). For analysis of surface expression of IL-15Rα (clone JM7A4), IL-15Rβ (TU27), or IL-15Rγ (clone TUGh4), CD45+ viable single cells were identified then B cells were defined as CD3-CD19+ (clone SJ25C1), mDCs were defined as CD3−CD19− HLA-DR (clone L243) + CD11c+, and Macrophages were defined as CD3−CD19− HLA-DR+ CD11c−(12, 34, 35). All flow cytometry data were acquired on an LSRII flow cytometer (BD Biosciences). Routine quality control using the Cytometer Setup and Tracking feature within the BD FACSDiva software version 6.1.2 (BD Biosciences) was performed daily.

Data analysis

FlowJo v10.0 was used for analysis of flow cytometry data. Only data sets with a minimum of 25 ILC3 events were included in analysis. GraphPad Prism v6.00 for Windows was used for statistical analysis and graphing. Statistical differences between conditions were determined by paired t test, unpaired t test or one-way ANOVA as indicated in figure legend. For this study, each tissue specimen provided by a human donor was considered a single sample for data analysis. A total of 39 independent gut tissue donors and 5 independent tonsil tissue donors were examined for this study. Each figure legend details how many donors were examined for each independent assay.

RESULTS

Gut bacteria induce granzyme B expression in human colonic ILC3s

To determine if human colon ILC3 constitutively expressed granzyme B, percentages of granzyme B+ ILC3s (identified as CD45+ viable lineage- CD127+ CD117+,(1, 12) were measured using multi-color flow cytometry prior to *in vitro* culture, and compared to percentages of granzyme B-expressing NK cells (identified as CD45+ viable lineage-

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CD127− CD56+)(27). As expected, a fraction of colon NK cells expressed granzyme B at baseline; however, constitutive granzyme B expression was completely lacking in colon ILC3s (Supplementary Figure 1).

To investigate if gut bacteria induced granzyme B expression in human colonic ILC3s, LPMCs were exposed to a panel of enteric bacteria reported to be of clinical interest in GI disease. Gram-positive commensal *Ruminococcus bromii* is decreased in relative abundance in colonic mucosa of people with HIV (PWH) and Gram-negative commensal *Acinetobacter junii* is increased in relative abundance in PWH. (36, 37) Gram-negative pathogen *Salmonella typhimurium* may contribute to the onset of IBD symptoms (38) and PWH are at an increased risk of acquiring *S. typhimurium*–bacteremia.(39, 40) Percentages of granzyme B-expressing ILC3s were determined using flow cytometry as detailed above (Figure 1A). Exposure of LPMCs to Gram-negative *A. junii* or *S. typhimurium* at matched ratios of 2.5 bacteria: 1 LPMC, significantly increased the average percentage of granzyme B-expressing ILC3s to 13.7%±3.0 (of total ILC3s) and 25.4%±5.5 respectively above no stimulation (2.2%±1.1) (Figure 1B,C). In contrast, exposure of LPMCs to Gram-positive *R. bromii* (2.5 bacteria: 1 LPMC) led to a smaller increase (7.9%±3.3) that did not reach statistical significance (Figure 1B,C). To determine whether a higher dose of Gram-positive bacteria could induce a more robust granzyme B response from ILC3s, LPMCs were exposed to increasing ratios of *R. bromii*. Frequencies of granzyme B-expressing ILC3s were not significantly increased upon LPMC exposure to 2x, 5x, or 10x the amount of *R. bromii* used above (Supplemental Figure 2).

A recent study identified a population of CD117+ precursor ILCs with the potential to migrate to various tissue sites including the gut, and differentiate into multiple ILC populations including not only ILC3s, but also NK cells.(41) To confirm bacteria-induced granzyme B expression was primarily measured in bone fide ILC3s, we began by examining expression of the canonical transcription factor for ILC3s, RORγt.(42) However, we observed a down-regulation of RORγt expression in ILC3s following 16hrs of culture versus direct *ex vivo* expression (Supplement Figure 1C, Supplemental Table 1). Therefore, we elected to use NKp44 as a defining ILC3 marker. NKp44 was originally used to identify ILC3s(43), and more recently has been shown to be expressed on mature ILC3s(41). As expected, the majority of ILC3s in the lamina propria expressed NKp44 *ex vivo* (74%±5.0 of ILC3s) whereas few ILC1s (5.2%±1.6) or NK cells (8.6%±3.4) expressed this marker, showing specificity for lamina propria ILC3s (Figure 2A). NKp44 expression remained high on ILC3s following *in vitro* stimulation of LPMC with *S. typhimurium* (71%±3.3; N=3). Critically, the majority (60%) of granzyme B+ Lin-CD127+CD117+ ILC3s induced in response to *S. typhimurium*, co-expressed NKp44 (Figure 2B,C) confirming that these cells were indeed ILC3s.

Given that some functions of granzyme B require the pore-forming protein perforin in order to access substrates in target cells,(21) we asked whether human gut ILC3s expressed perforin. Perforin expression was present in a subset of ILC3s after LPMCs were cultured for twenty hours *in vitro* in the absence of stimulation and exposure of LPMCs to bacteria did not significantly alter the percentage of perforin-expressing ILC3s (Figure 3A,B). ILC3s co-expressing granzyme B and perforin were next enumerated. Stimulation of LPMCs with

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R. bromii, A. junii or *S. typhimurium* increased the percentage of ILC3s that co-expressed granzyme B and perforin (Figure 3C). *A. junii* and *S. typhimurium* increased the percentage of ILC3s that only expressed granzyme B and not perforin, whereas *R. bromii* did not increase this subset of ILC3s (Figure 3D). These data indicate that certain gut bacteria may induce perforin-dependent and perforin-independent subsets of granzyme B within ILC3s.

Granzyme B-expressing ILC3s are a distinct subset of Group 3 ILCs

Given that we and others have shown that bacteria indirectly induce IL-22 expression in ILC3s,(12, 15–17) it was next determined if granzyme B-expressing ILC3s were a distinct subset of ILC3s from those that express IL-22 in response to *in vitro* exposure of LPMC to gut bacteria. Exposure of LPMCs to *R. bromii, A.junii*, or *S. typhimurium* significantly increased the percentage of IL-22 expressing ILC3s as expected (Figure 4A,B).(12) Few (<6.0%) of the granzyme B-expressing ILC3s generated in response to gut bacteria expressed IL-22 (Figure 4C,D). In fact, the majority of granzyme B-expressing ILC3s did not co-express IL-22 (Figure 4E). Furthermore, examination of co-expression of perforin and IL-22 revealed that majority of perforin-expressing ILC3s did not co-express IL-22 (Figure 4F–H).

We previously demonstrated that Group 1 ILCs expressed IFNγ and TNFα in response to exposure to enteric gut bacteria *in vitro*, and those that expressed IFNγ also co-expressed granzyme B.(27) In this current study, we investigated co-expression of these cytokines by granzyme B^{+} ILC3s. Similar to an additional study on Group 3 ILCs,(12) we observed no significant induction of either IFNγ or TNFα in ILC3s after LPMC exposure to enteric bacteria (Supplemental Table 1). Therefore, as expected, examination of bacteria-exposed ILC3s revealed minimal co-expression of those cytokines with granzyme B (Supplemental Table 1).

Gut bacteria induction of IL-15 drives granzyme B expression in ILC3s

Granzyme B expression is enhanced in human peripheral blood NK cells(44, 45) or induced in NK cells derived from umbilical cords(46, 47) by *in vitro* exposure to IL-2 and/or IL-15 and purified human tonsillar ILC3s expressed granzyme B after long-term exposure to IL-12 and IL-15.(28) To determine if these cytokines induced granzyme B in human colonic ILC3s, LPMCs were exposed to recombinant IL-15 (rIL-15) alone or in combination with rIL-2 or rIL-12 and granzyme B expression was evaluated. Exposure to rIL-15 increased the percentage of granzyme B-expressing ILC3s (Figure 5A,B). Similarly, exposure to the combinations of rIL-15 and rIL-2 or rIL-15 and rIL-12 increased the percentage of granzyme B-expressing ILC3s (Figure 5A,B); however the percentage was not significantly different to what was induced by rIL-15 alone. In comparing the capacity of bacteria to induce ILC3s to express granzyme B, *S. typhimurium* lead to a higher percentage of granzyme B-expressing ILC3s versus recombinant IL-15 (Figure 5A,B). Exposure to recombinant cytokines did not alter the percentage of perforin-expressing ILC3s (Supplemental Figure 3A). Exposure of LPMCs to rIL-15 alone or in combination with rIL-2 or rIL-12 did not induce IL-22, IFNγ or TNFα-expressing ILC3s and therefore, preferentially induced granzyme B+ ILC3s that did not co-express these cytokines, similar to the observations made in response to stimulation with bacteria (Supplemental Table 1).

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To determine whether IL-15 production is implicated in bacteria-induced granzyme B expression by ILC3s, levels of secreted IL-15 were measured in LPMC cultures after exposure to bacteria. IL-15 levels were significantly increased in cultures stimulated with *A junii* and *S. typhimurium,* but not those stimulated with *R. bromii* (Figure 5C). Antibody-mediated blockade of IL-15 in bacteria-stimulated LPMC cultures resulted in significant reductions in the frequency of granzyme B-expressing ILC3s generated in response to *A. junii* (Figure 5D) or *S. typhimurium* exposure (Figure 5E). By comparison, antibody-mediated blockade of IL-15 did not alter frequencies of perforin-expressing ILC3s during exposure to either of these bacteria (Supplement Figure 3B,C).

Signaling of IL-15 during bacteria stimulation

The IL-15 receptor is composed of three subunits including IL-15Rγ (also called the common gamma chain), IL-15Rβ (also called IL-2Rβ), and IL-15Rα.(48) IL-15 signals through its receptor following cis- or trans-presentation.(48) Cis-presentation is the mechanism by which a cell expresses all three subunits of the IL-15R and thus responds directly to IL-15 without additional cellular help. Trans-presentation is the mechanisms by which a cell only expresses IL-15Rγ and IL-15Rβ and thus requires a different cell to provide the third subunit, IL-15Rα, bound to IL-15, in order to trigger a response to IL-15. (48) Trans-presentation of IL-15 is a critical signaling mechanism for induction of granzyme B in NK cells in both mice and humans and demonstrates the requirement for an additional cell type (*e.g.,* mDCs, macrophages) in stimulating IL-15 signaling pathways through the IL-15 receptor. (49–53) To probe whether trans- or cis-presentation of IL-15 plays a role in induction of granzyme B in human gut ILC3s during bacteria stimulation we first quantified expression of IL-15R subunits on ILC3s. The majority of ILC3s expressed the IL-15Rγ subunit (79.9%±8.6) and the expression was not altered following LPMC exposure to *A. junii* or *S. typhimurium* (Figure 6A). A smaller portion of ILC3s expressed the IL-15Rβ subunit (20.8%±2.7), and similarly, the percentage of ILC3s expressing this subunit was not altered by LPMC exposure to either bacteria (Figure 6B). There was minimal expression of the third subunit, IL-15Rα (2.8%±0.8) on ILC3s in the absence of stimulation (Figure 6C). However, exposure of LPMC to *A. junii* or *S. typhimurium* significantly increased the percentage of IL-15Rα-expressing ILC3s, with the greatest response observed with *S. typhimurium* (Figure 6C). Co-expression of all three subunits on ILC3s for a functional IL-15R increased with bacterial stimulation (Figure 6D), suggesting that a subset of ILC3s are able to directly respond to IL-15. Based on the percentage of ILC3 expressing granzyme B in response to *S. typhimurium* (25.4%±5.5) and the percentage of ILC3s expressing all three subunits in response to *S. typhimurium* (10.0%±2.9), we postulate that cis-presentation accounts for nearly 40% of the granzyme B response to bacteria in ILC3s.

We next investigated the potential for trans-presentation of IL-15 and examined expression of IL-15Rα on other cells within the LPMC population. In the absence of stimuli, IL-15Rα was expressed at low percentages (< 5%) on CD8+ T cells, CD4+ T cells, NK cells, and B cells, with a slightly higher frequency of macrophages (7.9%±1.9) expressing this receptor component (Figure 6E). By comparison, nearly half of mDCs (49.4%± 10.7) expressed the IL-15Rα receptor subunit (Figure 6E), and this expression was not significantly altered

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following LPMC exposure to bacteria (Figure 6F). Thus, human gut mDCs have the potential to trans-present IL-15 to gut ILC3s through expression of IL-15Rα.

Granzyme B-expressing ILC3s are unique to the human gut

A recent report demonstrated that purified human tonsillar ILC3s expressed *gzmb* mRNA after long-term exposure to IL-12 and IL-15.(28) To determine if human tonsillar ILC3s expressed granzyme B after exposure to either bacteria or following short-term exposure to cytokines, tonsillar mononuclear cells (TMCs) were cultured with the same bacteria that induced robust granzyme B in gut ILC3s (*A. junii* and *S. typhimurium*), to rIL-15 or to rIL-12 and rIL-15 and then ILC3s were evaluated for granzyme B. Minimal expression of granzyme B was detected in tonsillar ILC3s (3.0%±0.7) in the absence of stimulation (Figure 7A). The percentage of tonsillar ILC3s expressing granzyme B did not increase after exposure of TMCs to either the bacteria *A. junii* or *S. typhimurium* (Figure 7A) or to recombinant cytokines (Figure 7B), in stark contrast to similar stimulation in colonic LPMCs (Figure 1C, 5B).

To determine if the failure to induce granzyme B in tonsillar ILC3s in response to bacteria was due to a lack of IL-15 signaling, expression of IL-15 subunits were assessed. The percentages of ILC3s expressing IL-15Rγ and IL-15Rβ were not altered after TMC exposure to *A. junii* or *S. typhimurium* (Figure 7C,D) and were similar to frequencies observed on colonic ILC3s (Figure 6A,B). Minimal expression of IL-15Rα was detected on tonsillar ILC3s in the absence of stimulation (similar to the colon) (Figure 7E); however, unlike the colon, there was no statistical increase in the frequency of IL-15Rα-expressing tonsillar ILC3s after exposure to *A. junii* or *S. typhimurium* (Figure 7E). Since mDCs likely play a role in trans-presentation of IL-15 to ILC3s in the gut, expression of IL-15Rα on tonsillar mDCs was also examined. Similar to the colon, about half of mDCs in the tonsil expressed IL-15Rα and expression was not altered after exposure to *A. junii* or *S. typhimurium* (Figure 7F). However, TMC contained fewer mDCs (0.18%±0.02 of viable, CD45+ cells) versus frequencies of mDCs in colon LPMCs (1.35%±0.1 of viable CD45+ cells). Thus, despite similar levels of IL-15Rα between colonic and tonsillar mDCs, the lack of granzyme B induction in tonsillar cells may be due to the presence of fewer mDCs and therefore limited trans-presentation of IL-15 to tonsil ILC3s.

DISCUSSION

To our knowledge, this study is the first to report that exposure of human colonic LPMC to enteric bacteria resulted in expression of granzyme B in ILC3s, through an IL-15-dependent process. Granzyme B has important cytolytic and non-cytolytic functions in mucosal immunity, and thus bacteria-induced granzyme B production by ILC3s could be an immune mediator involved in protection against enteric bacterial infections. However, a possible unintended consequence of this innate immune function of ILC3s is that granzyme B may be a mediator of inflammation and/or tissue destruction of the gut mucosa observed in disease settings characterized by epithelial barrier breakdown and microbial translocation, such as IBD or HIV-1 infection. Our study raises questions about how ILC3s, often described as

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being a primarily regulatory cell type, balance roles in maintaining gut homeostasis versus pathogen protection.

In this study we identify three distinct populations of ILC3s generated in response to enteric bacteria; one subset expressing the homeostatic cytokine IL-22, one subset expressing both granzyme B and perforin, and one subset expressing only granzyme B. Granzyme B-producing ILC3s did not co-produce IFNγ or TNFα in response to these same bacteria in contrast to human gut NK cells, which we previously demonstrated to be capable of poly-functional cytokine and cytolytic protein production in response to enteric Gram-negative bacteria.(27) Although it is tempting to describe these Granzyme B-producing ILC3 subsets as "NK-like," we hesitate to do so given that they do not express the other cytokine traditionally attributed to NK cell function (e.g. IFNγ). Recent studies making use of advanced transcriptomics, epigenetics, or mass-spectrometry have also described the heterogeneous nature of ILC3s within mice and humans.(54–58)

Here the Gram-negative bacteria tested (*A. junii* and *S. typhimurium*) induced robust fractions of distinct granzyme B-expressing and IL-22-expressing subsets within the ILC3 population, whereas the Gram-positive bacteria tested (*R. bromii*) only induced IL-22-expressing ILC3s. This differential granzyme B ILC3 response to specific bacteria is likely related to differences in IL-15 production by LPMCs when triggered by distinct bacterial species. Intriguingly, our data show that *S. typhimurium* induced higher fractions of granzyme B-expressing ILC3s when compared to recombinant IL-15 exposure (alone and in combination with IL-2 or IL-12p70), despite a substantially greater amount of IL-15 being added to the cultures than that detected in culture supernatants following bacterial stimulation. Measurement of low levels of secreted IL-15 in the setting of a robust granzyme B response may be attributed to the possibility that a large portion of IL-15 produced following bacterial exposure remains bound to IL-15Rα expressed on the surface of other immune cells and thereby capable of signaling to ILC3s. Indeed, others have reported that dendritic cells pre-assemble IL-15 bound to its receptor subunit and present this complex to NK cells rather than secrete IL-15.(49) It is also possible that the low amount of secreted IL-15 measured is ample to induce a certain level of granzyme B, and other factors independent of IL-15 are required for maximal induction. Indeed, neutralizing IL-15 in the presence of bacteria did not fully abrogate granzyme B expression, supporting the concept that other factors not yet identified and in addition to IL-15 may contribute to the bacteria-induced response in an ILC3. In other studies, activation of NK cells with IFNα induced granzyme B expression through suppression of microRNAs,(59) and exposure of colonic LPMCs to IL-21 increased expression of granzyme B in B cells and enhanced their cytotoxicity against the HCT-116 cell line.(60) Investigations into the contribution of these cytokines to bacteria-induced expression of granzyme B by human colonic ILC3s in addition to IL-15 will need to be undertaken.

The high level of constitutive expression of IL-15Rα on colonic mDCs suggests that trans-presentation of IL-15 to ILC3s may be a homeostatic system in place for colonic ILC3 responses to IL-15. However, cis-presentation of IL-15 to colonic ILC3s appears to be a regulatable unit which can be turned on or modified as needed. Bacteria exposure induced self-expression of IL-15Rα on gut ILC3s that, in conjunction with the high constitutive

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expression of IL-15Rβ and IL-15Rγ, resulted in a population of ILC3s that co-expressed all three subunits of the IL-15R. Bacteria-induced IL-15Rα expression on ILC3s was specific to the gut and not observed in tonsillar ILC3s, suggesting that cis-presentation of IL-15 could be a key driver of granzyme B production in gut ILC3s. This provides a potential mechanism by which the gut immune system can modulate ILC3 responses when needed during certain inflammatory states such as bacteria exposure.

Future studies are needed to determine the exact function of granzyme B produced by ILC3s in the gut mucosa since granzyme B is a multi-functional enzyme with various roles in mucosal immunology. Granzyme B induces apoptosis in target cells, (21, 61) can directly cleave pro-IL-18 and pro-IL-1α in order to induce inflammatory cytokine secretion,(62–64) and can cleave extracellular matrix (ECM) components and positively impacts wound healing at low levels or further disrupts the connective fibers holding the structure of the gut together when present at high levels(23, 24). It has also been reported to disrupt processes such as metabolism and biosynthesis within various bacteria to induce apoptosis-like cell death of the bacteria.(25, 26) Here, the identification of an ILC3 subset expressing both granzyme B and perforin suggests the possibility that ILC3s have cytolytic roles against target cells and/or inflammatory roles via activation of IL-18 or IL-1α. Additional studies are needed to directly determine the cytolytic potential of these cells, including evaluation of other cytotoxic effector pathways (e.g. FasL, TRAIL), the functional activity of cytolytic ILC3s (e.g. ability to degranulate and release cytolytic molecules) and the specificity of the target cells. Detection of an additional ILC3 subset expressing only granzyme B but not perforin raises the possibility that low levels of granzyme B produced by ILC3s could assist in gut tissue remodeling of the ECM. Additionally, it is tempting to speculate that granzyme B from an ILC3 could directly target bacteria for death, which would be a clear mechanism by which ILC3s engage in anti-bacterial activity.

Granzyme B expression has been linked to a number of gastrointestinal (GI) diseases and may be a contributor to disease pathology. For example, HIV infection is considered a disease of the GI tract(65) and granzyme B may be a biomarker of HIV infection with elevated levels reported in the plasma of untreated HIV-infected subjects as compared to uninfected controls.(66) Plasmacytoid DCs (pDCs) in duodenal biopsies from HIV-infected subjects were shown to have upregulated granzyme B expression, interpreted as pDCs contributing to gut pathology through granzyme B production.(67) During acute SIV infection (non-human primate model of HIV infection), intestinal NKp44+ ILCs (which may be equivalent to human ILC3s) had increased expression of CD107a, a cellular marker of degranulation, suggesting that during viral infection functional changes in the ILC population may exacerbate GI disease.(68, 69) In other GI diseases, microarray analysis of intestinal tissue from patients with active Crohn's disease (a type of IBD) showed a significant increase in Granzyme B gene expression (*gzmb*) and these patients displayed increased serum granzyme B levels.(70) In the gut of patients with IBD, there was an accumulation of granzyme B-expressing plasma B cells in areas with active disease, and these cells displayed cytotoxicity against an epithelial cell line(60). More work will be needed to determine whether granzyme B from an ILC3 is protective or harmful for gut function and homeostasis.

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A population of CD117+ precursor ILCs in human peripheral blood have the potential to migrate to various tissue sites including the gut, and differentiate into multiple ILC populations including not only ILC3s, but also EOMES+ NK cells.(41) Therefore, the utilization of CD117 as the defining marker of ILC3s may have resulted in the inclusion of other ILC/NK subsets in the ILC3 population. However, our current observations that 1) gut ILC3s did not express granzyme B without bacterial or cytokine exposure, 2) did not co-express cytokines (e.g. IFNγ) previously shown to be co-expressed with granzyme B+ colon NK and ILC1s(27), and 3) the majority of granzyme B+ ILC3s co-expressed NKp44, a marker of mature ILC3s,(41) together with our previous report that similarly identified ILC3s only minimally expressed transcription factors or cytokines traditionally associated with NK cells and Group 1 ILCs(12), provide strong evidence that these granzyme B-expressing cells are likely bone fide ILC3s. Given that not all granzyme B+ ILC3s co-expressed NKp44, it will be important to conduct additional phenotypic and functional studies to shed further light on the origin of enteric bacteria-expanded granzyme B-producing ILC3s.

Together, our work brings into light potential new functions of human gut ILC3s and identifies additional mechanisms by which translocating microbes might disrupt gut homeostasis. We demonstrate that bacterial induction of the cytokine IL-15 by LPMCs is important in eliciting granzyme B production in colonic ILC3s, in a process potentially mediated through both cis- and trans-presentation of IL-15. Importantly, we demonstrate that this function is specific to gut ILC3s, as minimal expression of granzyme B was detected in similarly stimulated tonsillar ILC3s. In different GI diseases, our data highlight that gut ILC3s are uniquely poised to respond to enteric bacteria in a multitude of diverse ways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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KEY POINTS

- Gut bacteria induce a unique subset of ILC3s expressing Granzyme B.
- Gut bacteria induce IL-15 which stimulates Granzyme B expression in ILC3s.
- ILC3s respond to IL-15 via cis- and trans-presentation during bacteria exposure.

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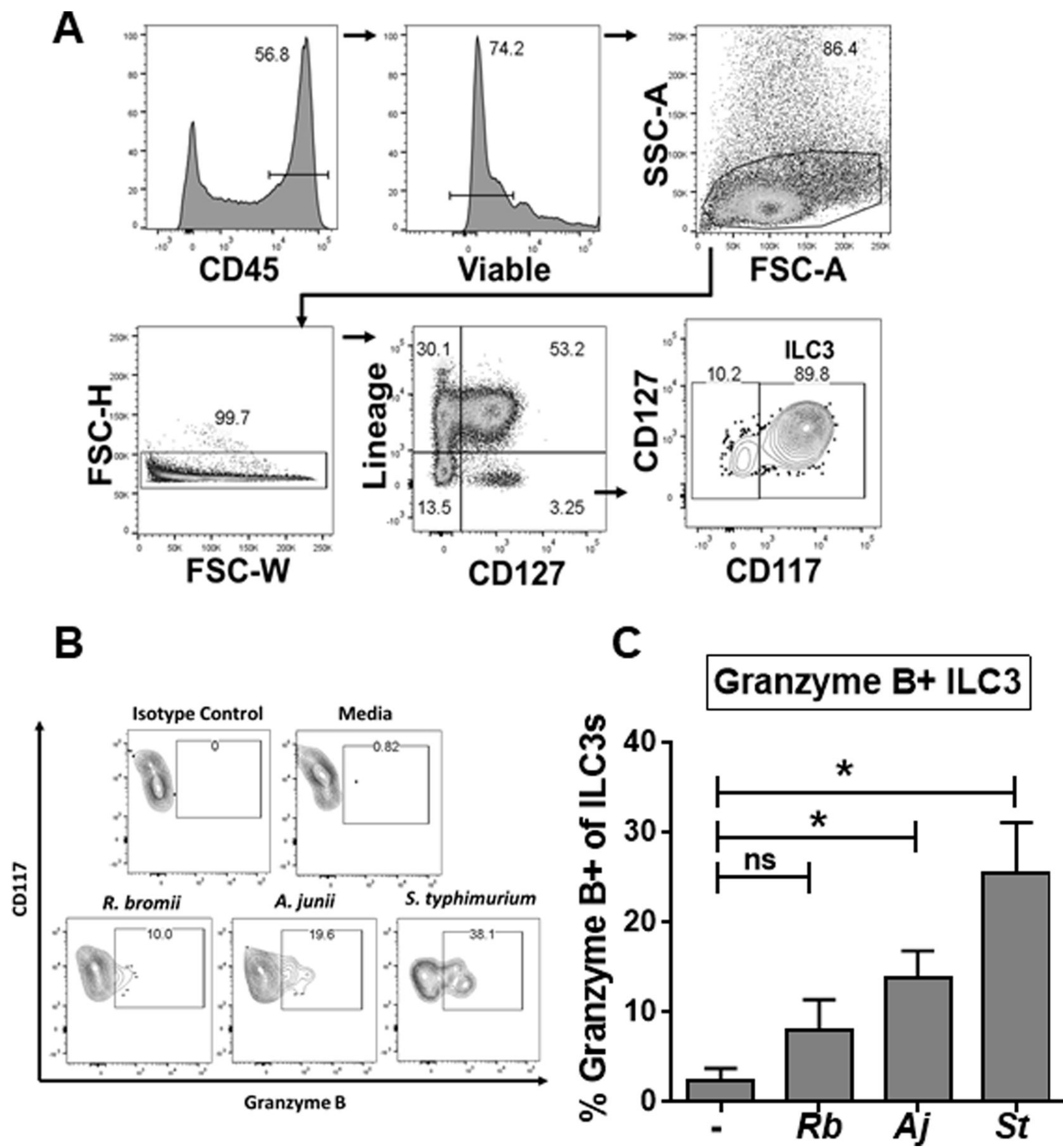

Figure 1. Gut bacteria induce granzyme B-expressing ILC3s.

(A) Gating strategy for identification of ILC3s via flow cytometry. ILC3s were defined as CD45+Viable Lymphocyte Single Cells that were lineage- CD127+ and CD117+. (B) Flow plots from a representative sample illustrating granzyme B expression in ILC3s after LPMCs were exposed to *R.bromii (Rb), A.junii (Aj),* or *S. typhimurium (St)*. Isotype controls were used to establish positive granzyme B staining. (C) Percentages of ILC3s expressing granzyme B after LPMC exposure to bacteria. N=5. Statistical analysis was one-way ANOVA. *p<0.05, n.s. not significant.

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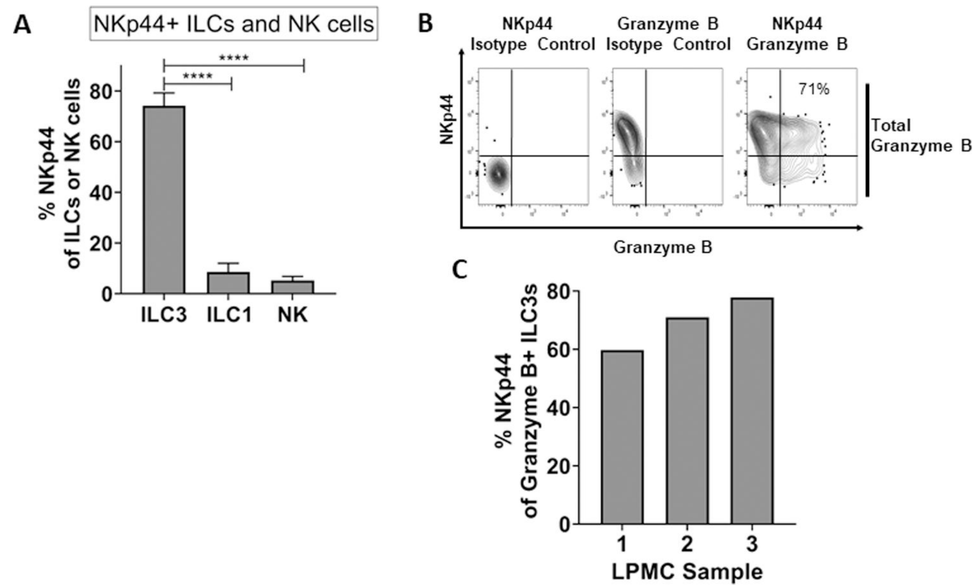

Figure 2. The majority of granzyme B-expressing ILC3s co-express NKp44.

(A) Percentages of ILC3s (identified as Lin-CD127+CD117+), ILC1s, (Lin-CD127+CD117−) and NK cells (Lin-CD127−CD56+) expressing NKp44 *ex vivo* (N=9). Isotype controls were used to established NKp44 staining. Statistical analysis was one-way ANOVA. ****p<0.0001. (B) Flow plots from a representative sample illustrating granzyme B and NKp44 expression in ILC3s after LPMCs were exposed to *S. typhimurium (St)*. Isotype controls were used to establish positive granzyme B and NKp44 staining as shown. (C) Percentages of ILC3s expressing granzyme B after LPMC exposure to *S. typhimurium*. Values are shown for 3 LPMC samples.

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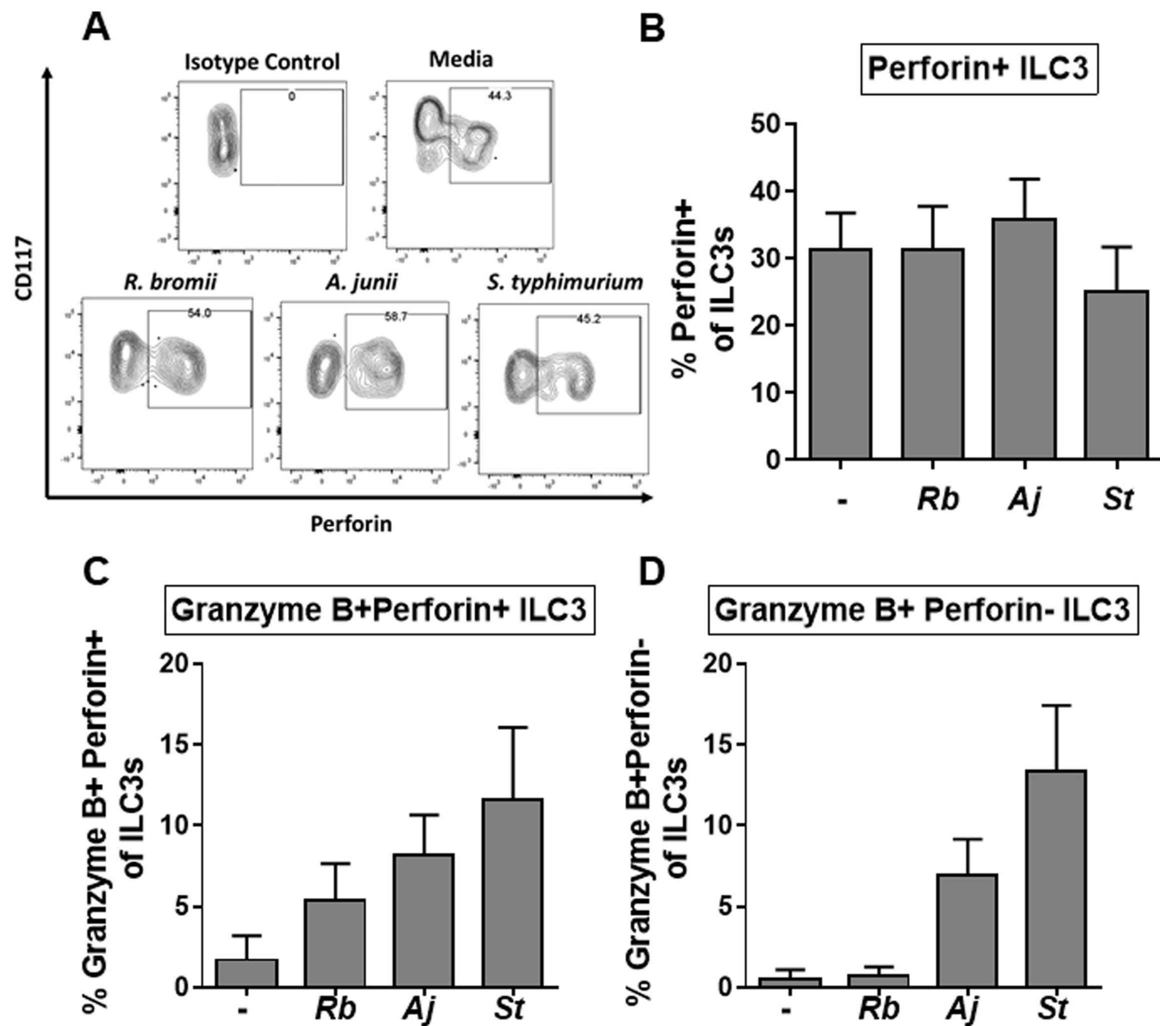

Figure 3. A subset of ILC3s express both granzyme B and perforin.

(A) Flow plots from a representative sample illustrating perforin expression in ILC3s after LPMCs were exposed to *R.bromii (Rb), A.junii (Aj),* or *S. typhimurium (St).* Isotype controls were used to establish positive perforin staining. (B) Percentages of ILC3s expressing perforin, (C) granzyme B and perforin, or (D) granzyme B and not perforin after LPMC exposure to bacteria. N=5.

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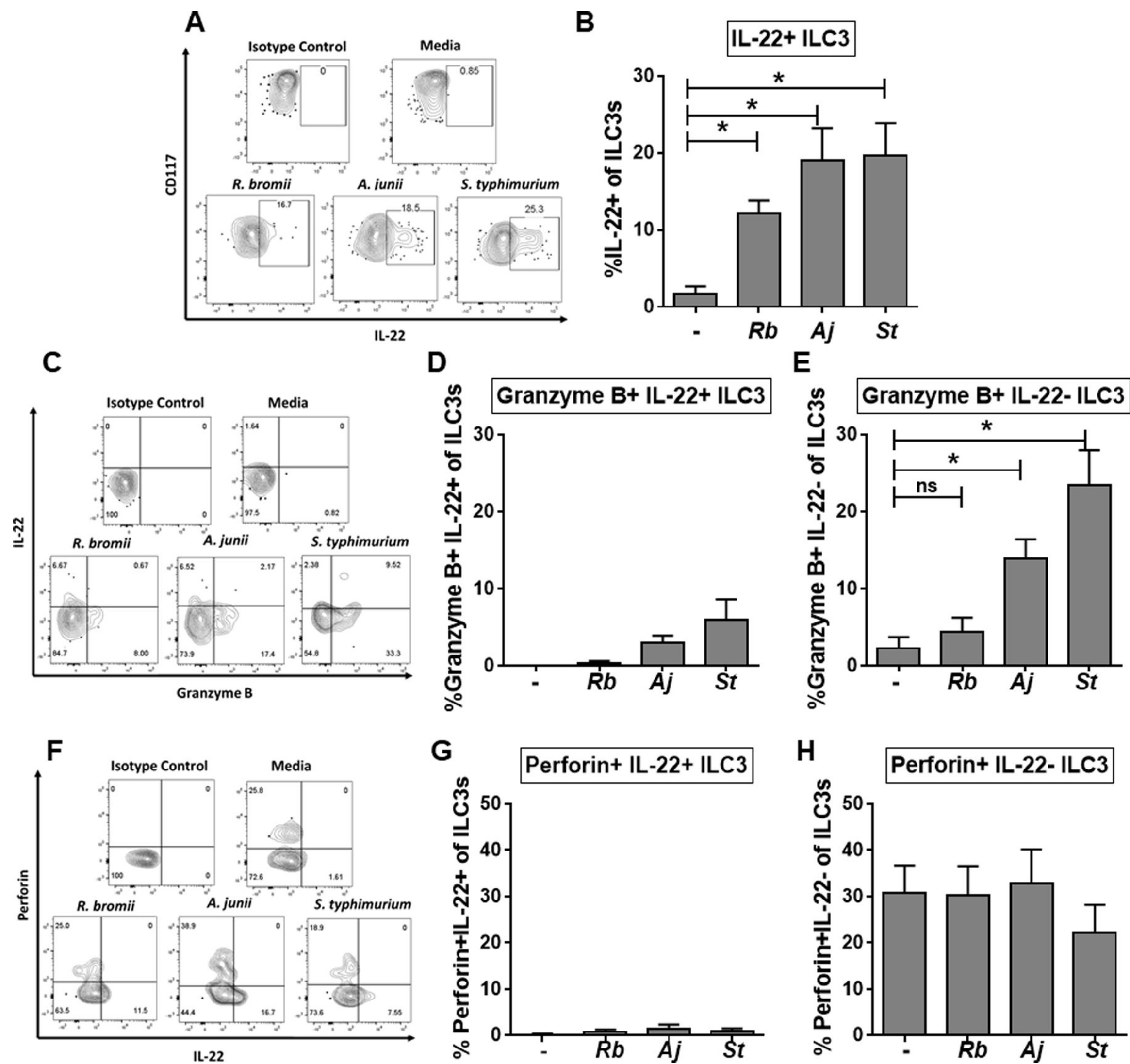

Figure 4. Granzyme B-expressing ILC3s are a unique subset of Group 3 ILCs.

(A, C, F) Flow plots from a representative sample illustrating (A) IL-22, (C) IL-22 and granzyme B or (F) IL-22 and perforin expression in ILC3s after LPMCs were exposed to *R.bromii (Rb), A.junii (Aj),* or *S. typhimurium (St).* Isotype controls were used to establish positive IL-22, granzyme B and perforin staining. (B) Percentages of ILC3s expressing IL-22, (D) co-expressing granzyme B and IL-22, (E) expressing granzyme B only and not IL-22, (G) co-expressing perforin and IL-22 or (H) expressing perforin only and not IL-22, after LPMC exposure to bacteria *R.bromii (Rb), A.junii (Aj),* or *S. typhimurium (St).* N=5. Statistical analysis was one-way ANOVA. *p<0.05, n.s. not significant.

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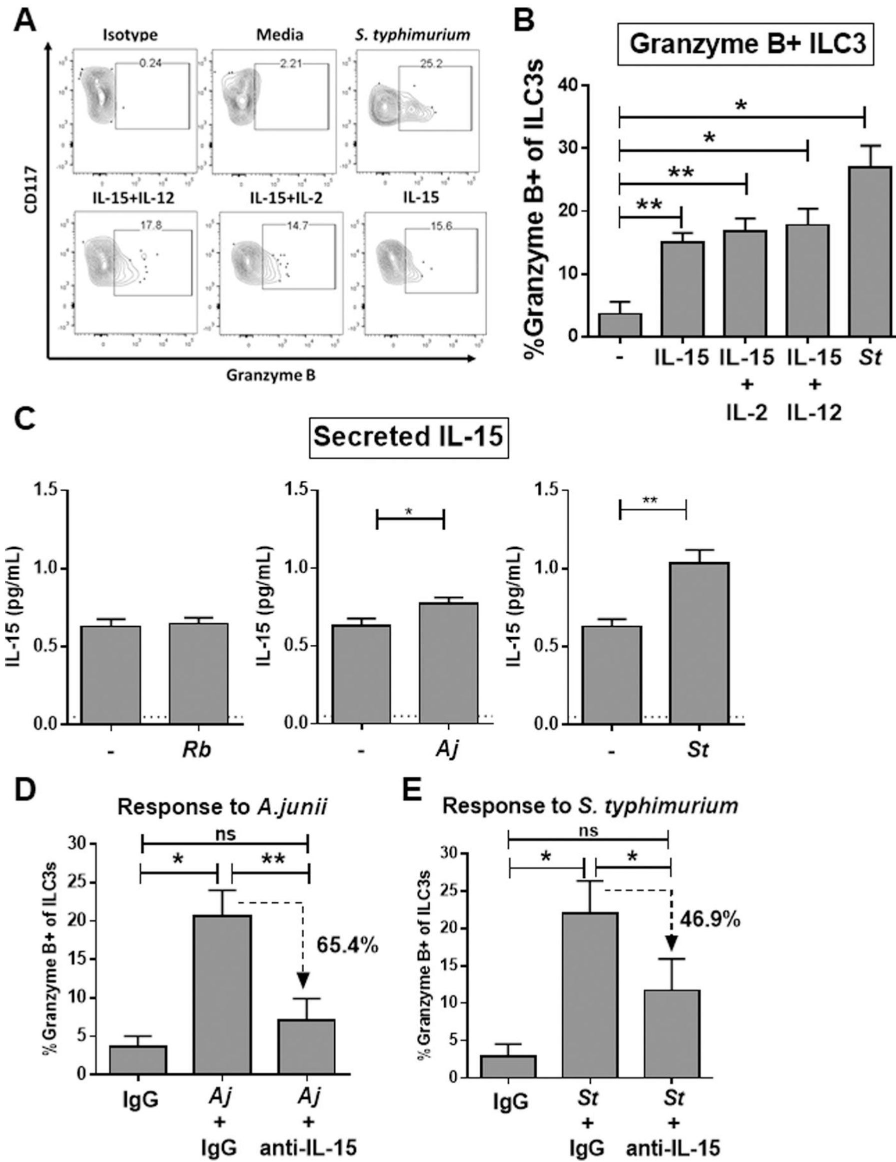

Figure 5. Bacteria induction of IL-15 drives granzyme B expression in ILC3s.

(A) Flow plots from a representative sample illustrating granzyme B expression in ILC3s after LPMCs were exposed to *S. typhimurium* or recombinant IL-15 alone (IL-15) or in combination with (IL-15+IL-12) or IL-2 (IL-15+IL-2). Isotype controls were used to establish positive granzyme B staining. (B) Percentages of granzyme B expression in ILC3s after LPMCs were exposed to IL-15, IL-15+IL-2, IL-15+IL-12p70, or *S. typhimurium.* N=4. (C) Measurement of IL-15 (pg/mL) in cell supernatant after LPMC exposure to bacteria *R.bromii (Rb), A.junii (Aj),* or *S. typhimurium (St).* N=5. (D) Percentages of ILC3s expressing granzyme B after LPMC exposure to *A. junii* in the presence of antibody targeting IL-15 or control IgG antibody. N=5. (E) Percentages of ILC3s expressing granzyme B after LPMC exposure to *S. typhimurium* in the presence of antibody targeting IL-15 or control IgG antibody. N=4. Statistical analysis was one-way ANOVA or paired t test. $^{**}p<0.01$, $^{*}p<0.05$, n.s. not significant.

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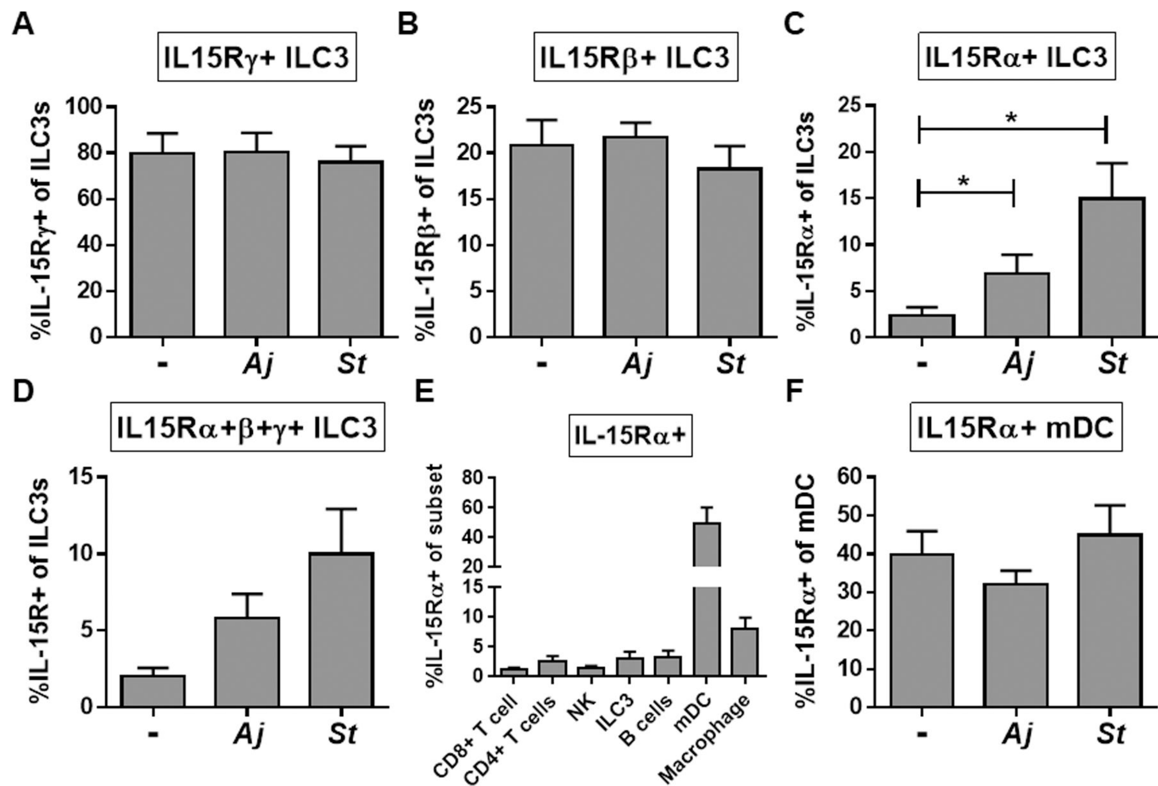

Figure 6. Bacteria induce cis- and trans-presentation of IL-15.

(A) Percentages of ILC3s expressing IL-15Rγ, (B) IL-15Rβ, (C) IL-15Rα or (D) all three subunits (IL-15Rα/ IL-15Rβ/ IL-15Rγ) after LPMC exposure to *A.junii (Aj)* or *S. typhimurium (St)*. N=6. (E) Percentages of other immune cells expressing IL-15Rα after LPMC incubation in the absence of stimuli. N=4. (F) Percentages of mDCs expressing IL-15Rα after LPMC exposure to *A.junii* or *S. typhimurium*. N=4. Statistical analysis was one-way ANOVA. *p<0.05.

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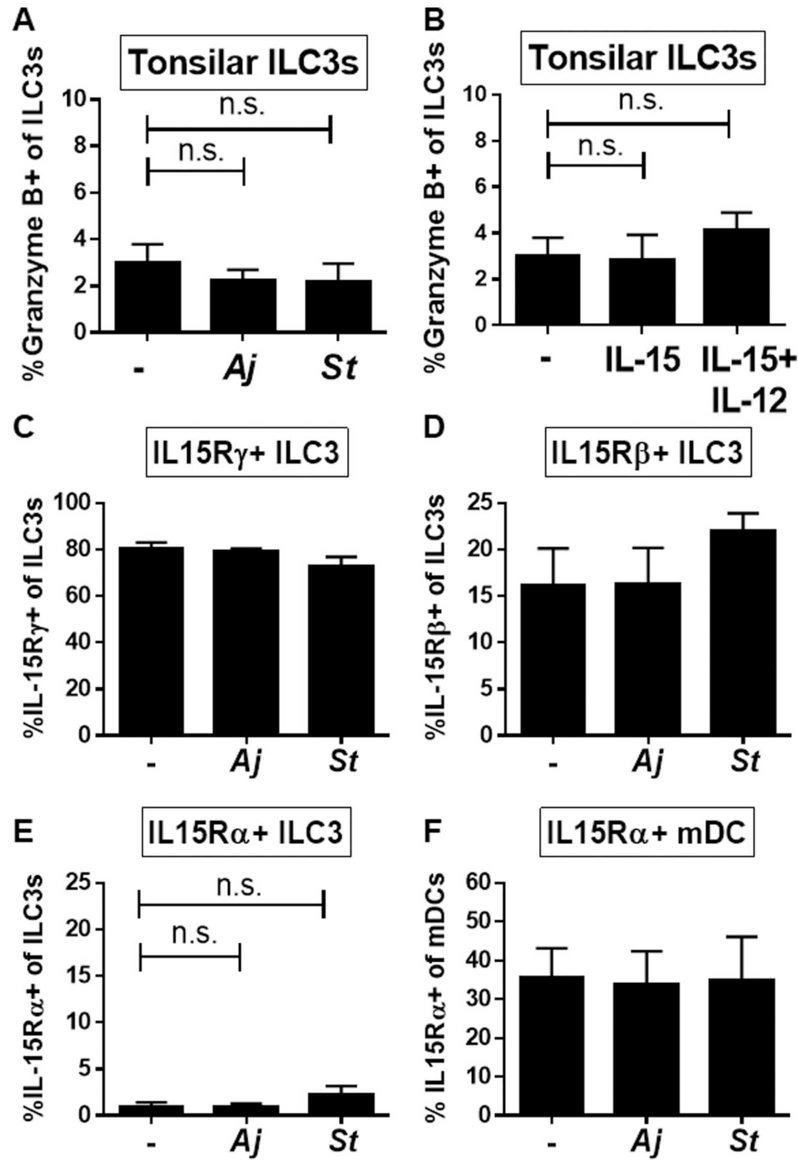

Figure 7. Bacteria do not induce granzyme B in tonsillar ILC3s.

(A) Percentages of ILC3s expressing granzyme B after TMC exposure to *A.junii (Aj),S. typhimurium (St)* or (B) IL-15, IL-15+IL-12p70. N=5. (C) Percentages of IL-15Rγ-expressing ILC3s, (D) IL-15Rβ-expressing ILC3s, (E) IL-15Rα-expressing ILC3s, or (F) IL-15Rα-mDCs after TMC exposure to *A.junii* or *S. typhimurium*. N=5. Statistical analysis was one-way ANOVA. n.s. not significant.