γδ intraepithelial lymphocytes are essential mediators of host–microbial homeostasis at the intestinal mucosal surface

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The mammalian gastrointestinal tract harbors thousands of bacterial species that include symbionts as well as potential pathogens. The immune responses that limit access of these bacteria to underlying tissue remain poorly defined. Here we show that γδ intraepithelial lymphocytes (γδ IEL) of the small intestine produce innate antimicrobial factors in response to resident bacterial “pathobionts” that penetrate the intestinal epithelium. γδ IEL activation was dependent on epithelial cell-intrinsic MyD88, suggesting that epithelial cells supply microbe-dependent cues to bacterial stimulation of epithelial cell-intrinsic MyD88 signaling, and were an essential component of the hierarchy of immune defenses that maintain homeostasis with the intestinal microbiota.

antibacterial defense | mucosal immunity | microbiota

All mammals engage in a mutually beneficial symbiosis with a diverse microbial community that can change dynamically through acquisition of organisms from the environment (1, 2). Despite the mutually beneficial nature of the mammalian host–bacterial relationship, resident microbiota can invade intestinal tissues. This occurs during acquisition of new bacterial species from the environment, before the development of species-specific adaptive immune responses (3, 4). Tissue invasion can also occur in the presence of resident “pathobionts,” which have an intrinsic capacity to enter host cells (5). Thus, a key challenge for the mucosal immune system is to immediately detect barrier breach and rapidly limit invasion of bacteria into host tissue.

Large numbers of γδ T-cell receptor-bearing intraepithelial lymphocytes (γδ IEL) inhabit the body’s epithelial barriers. γδ IEL intercalate between epithelial cells, and are thus poised to provide a first line of defense against environmental challenges. In the colon, γδ IEL function predominantly in the response to tissue injury, stimulating repair of damaged epithelia (6) and limiting bacterial penetration of injured tissue (7). However, γδ IEL are abundant even in undamaged intestinal tissues, suggesting that they perform homeostatic functions that extend beyond the response to tissue injury.

Here we show that γδ IEL are a critical component of the mucosal immune response against resident intestinal bacteria. We demonstrate that γδ IEL of the small intestine express innate antibacterial effectors, including the antibacterial lectin RegIIIγ, in response to a resident bacterial pathobiont that enters intestinal epithelial cells. The γδ IEL antibacterial response depends on bacterial stimulation of epithelial cell-intrinsic MyD88 signaling, indicating that epithelial cells supply microbe-dependent cues to γδ IEL. Finally, γδ T cells protect against tissue invasion by resident and pathogenic bacteria specifically during the first hours after bacterial encounter, suggesting that γδ IEL occupy a unique temporal niche among intestinal immune defenses. Together, our findings reveal that γδ IEL are one of the unique adaptations of the intestinal immune system that maintain homeostasis with a diverse resident microbiota.

Results

Intestinal Microbiota Direct an Antimicrobial Response in Small-Intestinal γδ IEL. To characterize small-intestinal γδ IEL responses to the microbiota, we purified γδ IEL from germfree and conventionally raised mice. Similar numbers of small-intestinal γδ IEL were recovered from both groups of mice (Table S1), reflecting the fact that γδ IEL residence in the epithelium is independent of the microbiota (8). We established that our γδ IEL were free of contaminating epithelial cells by showing that the TCRδ+ cells expressed CD103, which marks lymphocytes but not epithelial cells (Fig. S1A).

We next used microarrays to compare the gene expression profiles of these two γδ IEL populations. Of particular interest was the finding that intestinal microbiota elicited expression of a number of known or putative antimicrobial factors in small-intestinal γδ IEL (Fig. S1B). This response encompassed members of the regenerating islet-derived (Reg) protein family of C-type lectins, including RegIIIγ, which kills Gram-positive bacteria (9), and the related lectin RegIIIβ. In contrast to the colonic γδ IEL response to injury, we did not observe elevated expression of the proinflammatory cytokines KC, IL-β, MIP-2α, or CXCL-9 (7). Elevated RegIIIγ and RegIIIβ expression was confirmed by quantitative PCR (Q-PCR) analysis of isolated γδ IEL (Fig. L4 and Fig. S2). To confirm that the observed RegIIIγ expression was in γδ IEL and did not result from epithelial cell contamination, we analyzed the RegIIIγ+/TCRδ+ population by flow cytometry. Virtually all RegIIIγ+/TCRδ+ cells expressed CD103, marking them as lymphocytes and not contaminating epithelial cells (Fig. 1B). These results indicate that γδ IEL respond to the microbiota not only in the context of injury but also under homeostatic conditions.

We next asked whether microbial induction of the γδ IEL antibacterial response was reversible. Antibiotic treatment of conventional mice resulted in γδ IEL RegIIIγ transcript levels


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that were similar to those of germfree mice, demonstrating reversible expression upon microbiota depletion. Conversely, γδ IEL RegIIIγ expression was restored by “conventionalizing” germfree mice with a microbiota harvested from conventionally raised mice (Fig. 1 A and C). Conventionalization of germfree mice results in bacterial penetration of the epithelial barrier during the first 14 d after bacterial exposure, before the development of specific IgA (3). We noted higher RegIIIγ expression in conventionalized mice compared with mice that were conventional from birth (Fig. 1 A and C). Total numbers of small-intestinal bacteria did not differ between the two groups, arguing that this difference did not arise from higher bacterial numbers in the conventionalized mice. This suggests that enhanced γδ IEL antimicrobial responses in conventionalized compared with conventionally raised mice might be linked to this transient bacterial penetration of host tissue. We have addressed this possibility below.

RegIIIγ Expression Is Induced in γδ IEL by a Select Subset of the Microbiota. We next investigated which microbiota components elicit RegIIIγ expression in γδ IEL. Colonization with the Gram-negative symbiont Bacteroides thetaiotaomicron, a prominent member of the human and mouse intestinal microbiota, was not sufficient to trigger γδ IEL expression of RegIIIγ (Fig. 2A). This suggested that γδ IEL respond to a distinct subset of microbiota species rather than simply to the presence of bacteria. TH17 cells also respond to specific microbiota components, requiring segmented filamentous bacteria (SFB) for their differentiation (10). We therefore asked whether SFB could also activate γδ IEL. SFB are present in C57BL/6 mice from Taconic Farms but not in genetically identical mice from Jackson Laboratories (10). We verified increased SFB abundance in Taconic mice (Fig. S3A), but found no significant difference in RegIIIγ transcript abundance between γδ IEL isolated from Taconic and Jackson C57BL/6 mice (Fig. S3B). We therefore conclude that RegIIIγ expression by γδ IEL does not require SFB.

We next searched for a microbiota species resident in our conventional mice with the capacity to activate the γδ IEL antimicrobial response. We cultured total small-intestinal bacteria and used 16S rRNA gene sequencing to identify a predominant facultative aerobe as an Escherichia coli strain (Fig. S4). We further characterized the localization of this E. coli isolate in small-intestinal tissue by fluorescence in situ hybridization (FISH) analysis of monocolonized mice, using a fluorescent probe directed against the bacterial 16S rRNA gene. We detected bacteria in the intestinal lumen, associated with epithelial cells, and in the lamina propria (Fig. 2B), but did not detect bacteria in spleen and blood. Enumeration of tissue-associated bacteria revealed ~6 bacterial cells per crypt-villus unit (Fig. 2C). As some bacteria appeared to be located within epithelial cells, we further analyzed the tissues by confocal microscopy. Z stacks reconstructed from confocal images verified that bacteria were located within epithelial cells (Fig. S5A and Movie S1). The intraepithelial localization correlated with the ability of the E. coli strain to enter the mouse intestinal epithelial cell line MODE-K (Fig. S5B). In contrast, B. thetaiotaomicron was confined to the intestinal lumen and was not detected in tissue (Fig. 2C and Fig. S5 C and D).

To test whether the native E. coli strain was sufficient to trigger γδ IEL expression of RegIIIγ, we monocolonized germfree mice and quantified RegIIIγ mRNA in γδ IEL 48 h later. In contrast to
B. thetaiotaomicron-monoassociated mice, γδ IEL from E. coli-monoassociated mice exhibited a sevenfold increase in RegIIIγ expression relative to germfree mice (Fig. 2A). We therefore conclude that this resident E. coli strain is one member of the indigenous microbiota that can induce γδ IEL to express RegIIIγ, and can in part account for the ability of the steady-state microbiota to activate γδ IEL.

The capacity of the native E. coli strain to cross the mucosal barrier was a distinctive characteristic relative to B. thetaiotaomicron. The correlation of bacterial invasion with γδ IEL activation suggested that mucosal invasion might be required to trigger the γδ IEL antimicrobial response. Because the molecular basis for the ability of this resident E. coli strain to invade epithelial layer is currently unknown, we addressed this question using Salmonella typhimurium, an enteric pathogen whose capacity to penetrate the epithelial barrier has a well-defined genetic basis. S. typhimurium invades intestinal epithelial cells as a first step in barrier translocation and systemic dissemination. This process requires genes contained within the SPI-1 pathogenicity island, and a mutant strain lacking SPI-1 (ASPI-1) thus cannot invade the epithelial barrier (11). Colonization of germfree mice with wild-type S. typhimurium elicited an ∼44-fold increase in the abundance of γδ IEL RegIIIγ transcripts (Fig. 2D). Wild-type and ASPI-1 strains each colonized the small intestine to equivalent levels, but wild-type S. typhimurium induced approximately eightfold higher γδ IEL RegIIIγ mRNA expression relative to the mutant (Fig. 2D). The RegIIIγ expression levels correlated with bacterial entry into the intestinal epithelium (Fig. S6A and B). These data argue that bacterial penetration of the mucosal surface is required to trigger RegIIIγ production by γδ IEL.

Epithelial Cell MyD88 Is Required for RegIIIγ Expression in Small-Intestinal γδ IEL. We next examined the host factors that regulate bacterial induction of the γδ IEL antimicrobial response. We have previously shown that small-intestinal Paneth cells, specialized epithelial cells, detect intestinal bacteria through Toll-like receptors (TLRs) that require the signaling adaptor protein MyD88. Paneth cell TLR engagement elicits production of antimicrobial proteins, including RegIIIγ, that promote homeostasis with the microbiota (12). We therefore tested whether MyD88 governs RegIIIγ expression in small-intestinal γδ IEL. Similar numbers of γδ IEL were recovered from wild-type and MyD88−/− mice (Table S1), in agreement with published data (13) and with the fact that germfree mice harbor normal numbers of γδ IEL. However, γδ IEL from MyD88−/− mice showed decreased expression of RegIIIγ relative to wild-type mice (Fig. 3A), and flow cytometry confirmed fewer RegIIIγ+ γδ IEL from MyD88−/− mice (Fig. 3B). The related antibacterial lectin RegIHIβ showed a similar dependence on MyD88 (Fig. S2). Thus, expression of RegIIIγ and RegIHIβ in small-intestinal γδ IEL requires MyD88.

We next addressed the cellular origin of the MyD88 signaling that triggers γδ IEL production of RegIIIγ. As a first step, we generated bone marrow chimera in which MyD88 was predominantly expressed in lymphocytic lineages such as epithelial cells (Fig. 3C). Although we were also able to generate the reverse chimera, we were unable to recover viable γδ IEL from these mice, suggesting that nonhematopoietic MyD88 might be important for γδ IEL viability after irradiation. As previously reported, chimerism was lower in the intestine than in the circulation (40% donor γδ IEL in intestine compared with ∼90% donor lymphocytes in the circulation) (14). Therefore, the chimeric mice had a mixed population of recipient wild-type γδ IEL and donor MyD88−/− γδ IEL that could be tracked based on the congenic markers Ly5.1 and Ly5.2 (Fig. 3C). Adoptive transfer of MyD88−/− bone marrow into lethally irradiated wild-type mice restored expression of RegIIIγ in donor MyD88−/− γδ IEL to wild-type levels (Fig. 3D). Thus, RegIIIγ expression does not depend on γδ IEL MyD88, establishing that bacterial activation of γδ IEL is cell-nonautonomous.

![Figure 3](image_url)

**Fig. 3.** Epithelial cell MyD88 is required for RegIIIγ expression in small-intestinal γδ IEL. RegIIIγ was quantified by (A) Q-PCR of sorted small-intestinal γδ IEL and (B) flow cytometry of total IEL, performed as in Fig. 1. Gated γδ IEL populations are shown, and percentages of gated populations are given. Four to five mice were pooled per group; results are representative of three independent experiments. (C) Schematic of bone marrow chimera experiment. Ly5.1 wild-type mice were irradiated and reconstituted with bone marrow cells from Ly5.2 MyD88−/− mice. The chimeric intestines retained residual recipient cells while also supporting engraftment of transplanted cells (40% donor/60% recipient γδ IEL), EC, epithelial cells. (D) Eight weeks after reconstitution, Ly5.1 and Ly5.2 γδ IEL were isolated by FACS and analyzed individually for RegIIIγ mRNA by Q-PCR. Each point represents an individual mouse, and the conventional experimental groups were cohoused for 5 d before sacrifice to ensure a shared microbiota. (E) Q-PCR for RegIIIγ expression in sorted small-intestinal γδ IEL. MyD88−/− mice with an epithelial cell-specific MyD88 deletion, MyD88fl/fl, littersmates harboring two floxed MyD88 alleles. Littersmates were cohoused to ensure a shared microbiota. Each point represents an individual mouse. Error bars represent ± SEM. *P < 0.05; **P < 0.01; ns, not significant.
γδ IEL make direct contact with neighboring epithelial cells, suggesting that epithelial cells might provide the MyD88 signals that activate γδ IEL. To test this idea, we generated mice with an epithelial cell-specific deletion of MyD88 (MyD88<sup>fl/flΔ</sup>). We crossed mice carrying the epithelial cell-restricted Villin-Cre transgene (15) with mice harboring a loxP-flanked MyD88 allele (MyD88<sup>fl/flΔ</sup>) (16). This yielded progeny with an epithelial cell-specific MyD88 deletion (Fig. S7). RegIIγ expression in γδ IEL from MyD88<sup>fl/flΔ</sup> mice was decreased relative to MyD88<sup>fl/fl</sup> littermates (Fig. 3E). This indicates that RegIIγ induction in γδ IEL requires epithelial cell MyD88, and suggests that epithelial cells supply γδ IEL with microbe-dependent cues that govern the expression of antimicrobial factors.

γδ T Cells Limit Bacterial Penetration at Early Time Points After Bacterial Exposure. The finding that penetrant bacteria stimulate an antibacterial response in γδ IEL suggested that a key function of γδ IEL might be to limit bacterial invasion of mucosal tissues. To test this idea, we studied TCR<sup>δδ</sup>−/− mice, which lack all γδ T cells including γδ IEL (17). We orally inoculated conventional wild-type and TCR<sup>δδ</sup>−/− mice with 10<sup>7</sup> cfu of S. typhimurium and then quantified splenic bacteria at various times after inoculation. At 3 h there was a 100-fold increase in splenic S. typhimurium in TCR<sup>δδ</sup>−/− compared with wild-type mice (Fig. 4A). This difference was limited to early time points, as numbers of S. typhimurium recovered from spleen at 24 and 48 h after infection were similar in TCR<sup>δδ</sup>−/− and wild-type mice (Fig. 4A). The increased splenic dissemination at 3 h correlated with increased bacterial entry into epithelial cells in TCR<sup>δδ</sup>−/− mice (Fig. S8A). The elevated bacterial loads in spleen were not due to increased luminal loads in TCR<sup>δδ</sup>−/− mice (Fig. S8B) or to impaired bacterial killing in the spleens of TCR<sup>δδ</sup>−/− mice (Fig. S8C). These results support the idea that γδ T cells, and most likely γδ IEL, limit S. typhimurium penetration and dissemination immediately following bacterial encounter in the intestinal mucosa.

We next investigated whether γδ T cells also limit penetration of resident microbiota. We performed FISH analysis on wild-type and TCR<sup>δδ</sup>−/− mice to visualize the locations of intestinal bacteria relative to tissue. We observed small numbers of invading bacteria in the intestines of wild-type mice (~3–4 bacteria per crypt-villus unit), consistent with the stimulation of γδ IEL antibacterial responses by the microbiota in conventional mice (Fig. 4B and C). However, we did not detect increased numbers of tissue-associated bacteria in TCR<sup>δδ</sup>−/− mice (Fig. 4B and C). Given that γδ T cells limit S. typhimurium dissemination at early time points after infection, we reasoned that perhaps these cells also function specifically at early time points following exposure to new resident bacteria. Germfree TCR<sup>δδ</sup>−/− mice are not available, precluding analysis of the protective effects of γδ T cells against mucosal penetration by single resident bacterial species. However, cohousing of mice that have been previously caged separately introduces new resident bacterial species into the gastrointestinal tract and/or alters the abundances of species already present (3, 18, 19). We therefore used cohousing to test whether γδ T cells protect against penetration of resident bacteria during acquisition of new resident species from other mice and from the environment. Wild-type and TCR<sup>δδ</sup>−/− mice that had been separately caged were cohoused for 4 h and their small intestines were examined by FISH. Numbers of intracellular bacteria increased in TCR<sup>δδ</sup>−/− mice (~11 bacteria per crypt-villus unit; Fig. 4B and C). In contrast, numbers of penetrant bacteria in wild-type mice remained similar to the numbers observed before cohousing (Fig. 4B and C). Further, γδ IEL isolated from wild-type mice after cohousing showed increased RegIIγ protein relative to noncohoused mice (Fig. 4D). Measurements of serum fluorescence following FITC-dextran administration revealed that the bacterial penetration in the TCR<sup>δδ</sup>−/− mice did not arise from increased nonspecific barrier permeability (Fig. S9). These results demonstrate that γδ IEL play an essential role in limiting mucosal penetration by intestinal bacteria during shifts in microbiota composition and/or acquisition of new organisms from the environment.

Discussion

γδ IEL represent a large proportion of intestinal T cells, yet their biological functions are poorly understood. Here we show that the biological functions of γδ IEL extend beyond contributions to...
epithelial wound repair and immunoregulation to include a general role in maintaining homeostasis with the intestinal microbiota. γδ IEL provide a rapid first line of mucosal defense that promotes homeostasis with the microbiota by detecting and limiting bacterial penetration of intestinal tissue. The mucosal protection afforded by γδ IEL is of critical importance during the first hours after bacterial exposure, suggesting that γδ IEL occupy a unique temporal niche among mucosal immune responses.

Our studies have yielded key mechanistic insights into how γδ IEL sense intestinal bacteria. First, our data argue that γδ IEL respond specifically to invasive microorganisms, whether they are resident pathobionts or exogenous overt pathogens. Second, we found that bacterial stimulation of γδ IEL was indirect, requiring activation of MyD88 signaling in neighboring epithelial cells. Interestingly, the MyD88 dependence of small-intestinal γδ IEL RegIIIγ expression contrasts with γδ IEL from chemically injured colon, which exhibit MyD88-independent RegIIIγ expression (7). Such differences suggest that γδ IEL function may differ in the two organs, or that γδ IEL may be regulated by distinct mechanisms in damaged and undamaged mucosal tissues. Analysis of skin γδ T cells suggests that γδ IEL–epithelial cell crosstalk could occur via recognition of epithelial cell stress antigens or bacterial antigens by γδ IEL T-cell receptors (20, 21). Other studies indicate that epithelial cell cytokines could stimulate γδ IEL antibacterial responses (22, 23). Further investigation will be required to determine which of these mechanisms induces γδ IEL antimicrobial responses to intestinal bacteria.

Epithelial cells produce antimicrobial proteins that defend the apical surfaces of epithelial cells against bacterial attachment and invasion (12, 24). Our findings suggest that by signaling to γδ IEL, epithelial cells could also direct the expression of antibacterial effectors that defend the intraepithelial and basolateral compartments against microbial invasion. An important unresolved issue is whether antibacterial factors, such as RegIIIγ and RegIIIδ, account for the protective functions of γδ IEL. These effectors would likely be secreted into the intraepithelial and basolateral spaces, thus suggesting a molecular mechanism by which γδ IEL could limit bacterial penetration into mucosal tissue. A second possibility is that γδ IEL cytolytic functions promote the removal of infected epithelial cells, thus preventing further bacterial invasion (25). Testing these models awaits the development of new tools for cellular mosaic genetic manipulations of γδ IEL in mice.

Mucosal immune responses that follow a bacterial challenge are complex, evolving sequentially over many hours and days and involving contributions from multiple cells and effectors. We show here that γδ IEL provide essential antibacterial protection of the mucosal surface specifically during the first hours following bacterial exposure. Like cohoused mice, humans are continually exposed to new intestinal bacteria through ingestion of food and water, contact with other humans, and exposure to organisms in the surrounding environment. Our findings suggest that γδ IEL could be critical mediators of homeostasis between humans and their microbiota, and thus represent attractive therapeutic targets for the management of intestinal inflammatory diseases.

Materials and Methods

Animals. Wild-type C57BL/6 mice, MyD88−/− mice, Villin-Cre mice, MyDBD−/− mice, and TCRα−/− mice were bred at the University of Texas Southwestern Medical Center. For certain experiments, C57BL/6 mice were purchased from Jackson Laboratories and Taconic Farms and used immediately. Germfree C57BL/6 mice were maintained in isolators as described (9). All protocols were approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center.

Bacterial Strains. B. thetaiotaomicron (VPI-5482) and S. enterica serovar typhimurium (SL1344) and its isogenic mutant Δspt-1 were cultured as described (12). An E. coli strain native to the mouse microbiota was isolated by plating total small-intestinal contents on LB agar and growing aerobically at 37 °C. The species was identified by amplifying the 16S RNA gene using primers that anneal to conserved regions (forward: 5′-ACTCCTACGGGGAGGCAGCAGGT-3′; reverse: 5′-ATTACCAGGGCGTCTGGC-3′) (24). The amplicon sequence was compared with all known RNA genes through BLAST, revealing 100% identity with rRNAs from multiple E. coli strains (Fig. S4).

γδ IEL Isolation. Intestines were flushed with PBS, ejected, and washed in cold PBS. Tissues were gently agitated for 30 min at 37 °C in 25 mL extraction buffer (1 mM EDTA, 1% BSA, 1 mM DTT, PBS). IEL were shaken off the intestinal lining by vigorous vortexing for 2 min, and then were filtered through a 100 μm cell strainer, a 40 μm strainer, and a glass wool column. Total IEL were stained with phycoerythrin-labeled anti-TCRδ (GL3; BD Pharmingen), and γδ IEL were purified on a Beckman Coulter MoFlo cell sorter. γδ IEL purity was assayed postsorting and was ≥98% (Fig. S1A).

Quantitative PCR. Total RNA was isolated from purified γδ IEL using the Arcturus PicoPure RNA Kit and was used to generate template cDNA. mRNA levels were normalized to 18S RNA abundance and are expressed relative to conventional raised mice. Sequences of RegIIIγ and 18S RNA primers are published (9).

Flow Cytometry. For surface staining, IEL were suspended in PBS/0.5% BSA and stained for 20 min with PE-conjugated anti-TCRδ (BD Pharmingen). For intracellular staining, cells were fixed using the BD Pharmingen Cytofix/Cytoperm Kit, and stained with preimmune IgG or anti-RegIIIγ (9), followed by anti-rabbit secondary antibody (Jackson Immunoresearch) and anti-CD103 (eBioscience).

Antibiotic Treatment. Conventional C57BL/6 mice were given ampicillin (1 mg/mL), vancomycin (0.50 mg/mL), neomycin sulfate (1 mg/mL), and metronidazole (1 mg/mL) in drinking water for 4 wk. All antibiotics were from Sigma.

Flow Cytometry. For surface staining, IEL were suspended in PBS/0.5% BSA and stained for 20 min with PE-conjugated anti-TCRδ (BD Pharmingen). For intracellular staining, cells were fixed using the BD Pharmingen Cytofix/Cytoperm Kit, and stained with preimmune IgG or anti-RegIIIγ, followed by anti-rabbit secondary antibody (Jackson Immunoresearch) and anti-CD103 (eBioscience).

Statistical Analysis. Statistical significance was determined by unpaired two-tailed Student’s t test.

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Fig. S1. Intestinal microbiota direct a complex transcriptional program in small-intestinal \( \gamma^{\delta} \) intraepithelial lymphocytes \( (\gamma^{\delta} \text{IEL}) \). (A) Purity of isolated \( \gamma^{\delta} \) IEL. TCR\( ^{\delta} \) cells were isolated by flow cytometry as described in Materials and Methods. Presorted and postsorted cells were analyzed for TCR\( ^{\delta} \) expression. Postsorted TCR\( ^{\delta} \) cells were further analyzed for expression of CD103, which marks lymphocytes but not epithelial cells. CD103 expression in small-intestinal epithelial cells is shown for comparison. (B) Microarray analysis of \( \gamma^{\delta} \) IEL. Total RNAs were isolated from purified \( \gamma^{\delta} \) IEL using the PicoPure RNA Isolation Kit (Arcturus). For each experimental condition (cv-L, conventionally raised; gf, germfree), RNA was isolated from \( \gamma^{\delta} \) IEL recovered from two independent groups of 5–8 mice. Five nanograms of total RNA was amplified using an Arcturus RiboAmp HS Kit. Biotinylated cRNAs were generated by substituting the Enzo T7 BioArray Transcript Kit during the last step and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips in the University of Texas Southwestern Microarray Core. To identify \( \gamma^{\delta} \) IEL genes whose expression was altered by bacteria, we performed two-way comparisons between germfree and conventional groups, with germfree samples designated as baseline. Raw data were imported into Affymetrix GeneChip software for analysis, and previously established criteria were used to identify differentially expressed genes (1). Briefly, a ≥2-fold difference was considered significant if three criteria were met: (i) The GeneChip software returned a Difference Call of Increased or Decreased; (ii) the mRNA was called Present by GeneChip software in either germfree or conventional samples; and (iii) the difference was observed in duplicate microarray experiments. GeneChip quality and amplification linearity were assessed using polyadenylated spike-in control transcripts and oligo-B2 hybridization control (Affymetrix). Heat maps to visualize signal intensities were generated using GeneTraffic software (Iobion). Signal intensity data were converted to Z scores \[ z = \frac{x - \mu}{\sigma}, \] where \( x \) = signal intensity, \( \mu \) = mean signal intensity for all samples, and \( \sigma \) = SD across all samples] and subjected to unsupervised hierarchical clustering using GeneTraffic software. Each line represents an Affymetrix probe set and each column represents \( \gamma^{\delta} \) IEL pooled from 5–8 mice. Key functional groups were delineated using Gene Ontology terminology, and are displayed as heat maps in which expression level is defined by Z score. Functional groups representing cytokine/chemokine responses and innate immune/antibacterial responses are shown.

Fig. S2. Microbiota induce RegIIIβ expression in γδ IEL. RegIIIβ mRNA was quantified by quantitative PCR (Q-PCR) of sorted small-intestinal γδ IEL, and mRNA expression was calculated relative to conventionally raised mice. wt, wild-type. n = 5 mice per group. Error bars represent ±SEM. **P < 0.01. ns, not significant.

Fig. S3. RegIIIγ expression by γδ IEL does not require the presence of segmented filamentous bacteria. (A) Quantification of segmented filamentous bacteria (SFB) in the small intestines of Jackson and Taconic C57BL/6 mice. Genomic DNA was isolated from intestinal contents using the QIAamp DNA Stool Mini Kit (Qiagen). To detect SFB, we used specific primers directed against the SFB 16S rRNA gene (forward: 5′-GACGCTGAGGCATGAGAGCAT-3′; reverse: 5′-GACGGCACGGATTGTTATTA-3′) (1). Relative abundances of SFB in both groups of mice were calculated against total bacteria as assessed by kingdom-specific 16S rRNA gene primers (forward: 5′-ACTCCTACGGGAGGCAGCAGT-3′; reverse: 5′-ATTACCGCGGCTGCTGGC-3′) (1). No SFB were detected in the small-intestinal contents of Jackson mice (nd). (B) Q-PCR analysis of small-intestinal γδ IEL isolated from Jackson or Taconic mice. Error bars represent ±SEM; n = 3 mice per group. *P < 0.05.

Fig. S4. (Continued)
Fig. S4. Identification of a resident *Escherichia coli* strain from the mouse intestine that can stimulate γδ IEL expression of RegIIIγ. Intestinal contents were harvested from specified pathogen-free (SPF) C57BL/6 mice and plated on LB. The 16S rRNA gene was amplified from individual colonies by PCR, cloned into plasmids, and sequenced. The majority of cultured isolates produced amplicon sequences that aligned with 100% identity to the 16S rRNA gene of *E. coli* O111: H- strain 11128.
Characterization of a resident E. coli strain that induces RegIIIγ expression in γδ IEL. (A) Confocal microscopy analysis of the E. coli-colonized small-intestinal tissue shown in Fig. 28. Carnoy-fixed, paraffin-embedded ileal sections were prepared for FISH as described in Materials and Methods. The universal 16S rRNA gene probe was used to visualize bacteria in ileal tissues from E. coli-monoassociated mice. Images were acquired with a Leica SPE system fitted with a Leica 63x objective NA 1.4. The datasets were processed with Leica Advanced Fluorescence software. For Z-stack reconstructions, images were captured at 0.2-μm intervals. The Z-stack reconstructions (Movie S1) verified that bacteria were located within the intracellular compartment of intestinal epithelial cells. Fluorescence intensities are displayed through a false-color designation such that tissue autofluorescence is designated in a red channel whereas 16S rRNA gene probe-specific staining is designated in a green channel. (Scale bar, 10 μm.) (B) E. coli entry into cultured mouse intestinal epithelial cells. MODE-K cells (1) were cultured in DMEM supplemented with 10% FBS, 2 mM l-glutamine, and 10 mM Hepes. Cells were incubated at 37 °C in a 5% CO2 incubator and passaged using trypsin-EDTA. E. coli isolated from the small intestines of SPF mice were grown to log phase and used to assess bacterial entry into cells by gentamicin protection assay. Bacteria were added to MODE-K cells at a multiplicity of infection of 100 bacteria per mammalian cell. After the indicated times, the culture medium was replaced with 100 μg/mL gentamicin. Cells were washed and bacteria were quantified by dilution plating. Numbers of intracellular bacteria are expressed as a percentage of starting bacterial infection dose. In the right panel, the numbers of internalized E. coli are compared with numbers of Salmonella typhimurium internalized into MODE-K cells under similar conditions. Note that the S. typhimurium-infected cells were not viable at 6 h postinfection. (C) Bacteroides thetaiotaomicron strain that induces RegIIIγ expression in γδ IEL is likely to be determined by the fact that in vivo, S. typhimurium escapes from the epithelial intracellular niche and disseminates to distal tissue sites (1). Thus, although higher numbers of S. typhimurium enter enterocytes in vivo are lower. This is likely due to the fact that in vivo, S. typhimurium escapes from the epithelial intracellular niche and disseminates to distal tissue sites (1). Thus, although higher numbers of S. typhimurium enter enterocytes, they are probably not retained to the same extent as E. coli. Activation of γδ IEL is likely to be determined by both rate of entry and retention time of a given bacterial strain, which could explain why E. coli and S. typhimurium elicit similar levels of RegIIIγ expression (Fig. 2 A and D). **P < 0.01.


**Fig. 56.** S. typhimurium entry into small-intestinal epithelial cells. Germfree mice monoassociated with wild-type S. typhimurium or the invasion-deficient isogenic mutant ΔSPI-1 were subjected to FISH analysis with a universal bacterial 16S rRNA gene probe. (A) Images of wild-type S. typhimurium-colonized intestines are shown. (Scale bars, 10 μm.) (B) Quantification of intracellular bacteria. Error bars represent ±SEM. Note that although higher numbers of S. typhimurium enter cultured MODE-K cells compared with E. coli (Fig. 55B), the numbers of S. typhimurium detected in enterocytes in vivo are lower. This is likely due to the fact that in vivo, S. typhimurium escapes from the epithelial intracellular niche and disseminates to distal tissue sites (1). Thus, although higher numbers of S. typhimurium enter enterocytes, they are probably not retained to the same extent as E. coli. Activation of γδ IEL is likely to be determined by both rate of entry and retention time of a given bacterial strain, which could explain why E. coli and S. typhimurium elicit similar levels of RegIIIγ expression (Fig. 2 A and D). **P < 0.01.

Intestinal permeability measurements in wild-type and MyD88fl/fl mice. Deletion efficiency of the floxed MyD88 allele was assayed by Q-PCR on DNA from small-intestinal epithelial cells isolated from MyD88fl/fl mice (offspring of a MyD88fl/+ x Villin-Cre cross). Genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit. The presence of the floxed MyD88 allele was quantified by Q-PCR using primers (forward: 5′-GTTGTGTGTGGCCAGCGTG-3′; reverse: 5′-TCTCAATTAGCTGGCAGC-3′) (1). Cycle thresholds (Ct) were normalized to 18S rRNA levels to yield ΔCt values. ΔΔCt is the difference between ΔCt (MyD88fl/+ and MyD88fl/fl). % deletion values were calculated as indicated. The products were also visualized by agarose gel electrophoresis.


Fig. 57. Efficiency of deletion of the floxed MyD88 allele in MyD88fl/fl mice. Deletion efficiency of the floxed MyD88 allele was assayed by Q-PCR on DNA from small-intestinal epithelial cells isolated from MyD88fl/fl mice (offspring of a MyD88fl/+ x Villin-Cre cross). Genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit. The presence of the floxed MyD88 allele was quantified by Q-PCR using primers (forward: 5′-GTTGTGTGTGGCCAGCGTG-3′; reverse: 5′-TCTCAATTAGCTGGCAGC-3′) (1). Cycle thresholds (Ct) were normalized to 18S rRNA levels to yield ΔCt values. ΔΔCt is the difference between ΔCt (MyD88fl/+ and MyD88fl/fl). % deletion values were calculated as indicated. The products were also visualized by agarose gel electrophoresis.

Fig. 58. Colonization of wild-type and TCRδ−/− mice with S. typhimurium. (A) Quantification of intracellular S. typhimurium in the small intestine 3 h after oral challenge of conventionally raised wild-type and TCRδ−/− mice. Mice were challenged with S. typhimurium SL1344 expressing green fluorescent protein (a gift of V. Sperandio, University of Texas Southwestern Medical Center, Dallas) and bacterial entry was quantified by fluorescence microscopy. Error bars represent ±SEM. **P < 0.01. These results show that S. typhimurium entry into epithelial cells is elevated in TCRδ−/− mice, consistent with the increased dissemination to spleen shown in Fig. 4A. The ΔSPI-1 strain, which shows low levels of epithelial cell entry (Fig. S6B) was undetectable in the spleens of TCRδ−/− mice. (B) Small-intestinal colonization levels of S. typhimurium. The results show that the increased numbers of splenic bacteria recovered from TCRδ−/− mice after a 3-h oral infection are not due to differences in luminal colonization levels relative to wild-type mice. (C) Spleen bacteria were quantified after a 3-h i.p. inoculation with 106 cfu of wild-type S. typhimurium. The results show that the increased numbers of splenic bacteria recovered from TCRδ−/− mice after a 3-h oral infection are not due to altered bacterial killing in the spleen. n = 3–8 mice per group.

Fig. 59. Intestinal permeability measurements in wild-type and TCRδ−/− mice. Barrier permeability was assessed by measuring serum concentrations of FITC-dextran after oral gavage based on a previously established method (1). Wild-type C57BL/6 mice and TCRδ−/− mice were administered 200 µl of FITC-dextran (600 mg/kg body weight; 4 kDa; Sigma-Aldrich) by gavage. Total blood was collected 4 h later at sacrifice, and the serum concentration of FITC-dextran was determined using a fluorimeter (Molecular Devices) with an excitation wavelength at 485 nm and an emission wavelength of 530 nm. For comparison, one group of mice was pretreated by gavage with indomethacin (15 mg/kg in 10% DMSO) for 1 h before FITC-dextran administration to induce intestinal epithelial damage, whereas the control group was treated with 10% DMSO alone. n = 6 mice per group from two independent experiments. Error bars represent ±SEM. **P < 0.01.

### Table S1. γδ IEL recovery from mouse small intestine

<table>
<thead>
<tr>
<th>Colonization status</th>
<th>Genotype</th>
<th>Absolute numbers of sorted γδ IEL per small intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventionally raised</td>
<td>Wild-type</td>
<td>$8 \times 10^5 \pm 1 \times 10^5$</td>
</tr>
<tr>
<td>Germfree</td>
<td>Wild-type</td>
<td>$8 \times 10^5 \pm 0.9 \times 10^5$</td>
</tr>
<tr>
<td>Conventionally raised</td>
<td>MyD88−/−</td>
<td>$1 \times 10^6 \pm 4 \times 10^5$</td>
</tr>
<tr>
<td>Conventionalized</td>
<td>Wild-type</td>
<td>$9 \times 10^5$</td>
</tr>
</tbody>
</table>

γδ IEL were isolated from mouse small intestines by flow cytometry as described in Materials and Methods. Absolute numbers of sorted small-intestinal γδ IEL per mouse small intestine are reported. Numbers for conventionally raised wild-type, germfree wild-type, and conventionally raised MyD88−/− mice are the mean ± SEM ($n = 8$–20 mice). Numbers for conventionalized mice are from cells pooled from eight mice. We note that our reported total numbers of sorted γδ IEL are lower than those reported in prior studies (1). This is due to the fact that we based our numbers on sorted cell populations where we have been extremely stringent about excluding cellular aggregates that might compromise purity, which resulted in a reduced overall yield.


**Movie S1.** *E. coli* from SPF mouse intestine is internalized within epithelial cells. Germfree mice were orally inoculated with $10^8$ cfu of an indigenous *E. coli* strain isolated from the microbiota of SPF mice. Tissues were stained by FISH as shown in Fig. 2B, and were further analyzed by confocal microscopy to assess whether bacteria were internalized into epithelial cells. Z-stack reconstructions demonstrate that bacteria are located within the intracellular compartment of intestinal epithelial cells. Images were acquired with a Leica SPE system fitted with a Leica 63× objective NA 1.4. The datasets were processed with Leica Advanced Fluorescence software. Fluorescence intensities were displayed through a false-color designation such that tissue autofluorescence was designated in a red channel whereas 16S rDNA probe-specific staining was designated in a green channel. Red and green fluorescence images were acquired independently at each z plane.

**Movie S1**