Supporting Information

Ismail et al. 10.1073/pnas.1019574108

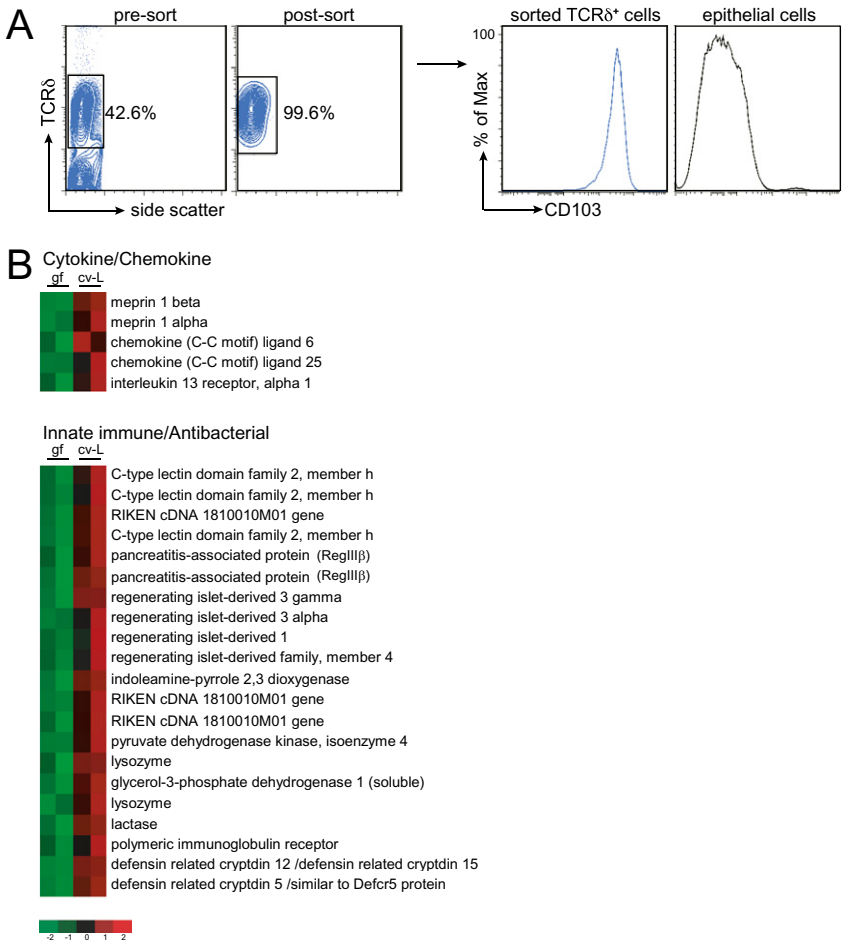

Fig. S1. Intestinal microbiota direct a complex transcriptional program in small-intestinal γδ intraepithelial lymphocytes (γδ IEL). (*A*) Purity of isolated γδ IEL. TCRδ$^+$ cells were isolated by flow cytometry as described in *Materials and Methods*. Presorted and postsorted cells were analyzed for TCRδ expression. Postsorted TCRδ$^+$ 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 γδ IEL. Total RNAs were isolated from purified γδ IEL using the PicoPure RNA Isolation Kit (Arcturus). For each experimental condition (cv-L, conventionally raised; gf, germfree), RNA was isolated from γδ 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 γδ 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 [$z = (x - \mu)/\sigma$, where x = signal intensity, μ = mean signal intensity for all samples, and σ = SD across all samples] and subjected to unsupervised hierarchical clustering using GeneTraffic software. Each line represents an Affymetrix probe set and each column represents γδ 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.

1. Cash HL, Whitham CV, Behrendt CL, Hooper LV (2006) Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313:1126–1130.

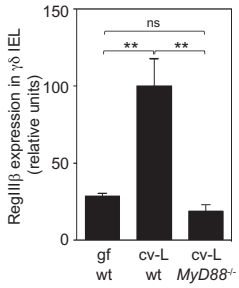

Fig. S2. Microbiota induce RegIIIβ expression in γδ IEL. *RegIIIβ* mRNA was quantified by quantitative PCR (Q-PCR) of sorted small-intestinal γδ IEL, and mRNA expression was calculated relative to conventionally raised mice. wt, wild-type. $n = 5$ mice per group. Error bars represent ±SEM. $^{**}P <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

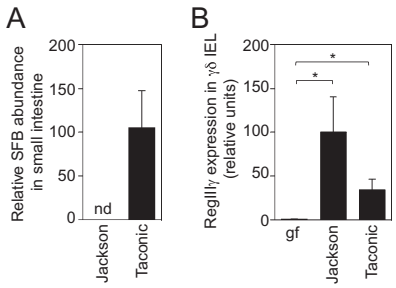

Fig. S3. RegIIIγ expression by γδ IEL does not require the presence of segmented filamentous bacteria. (*A*) Quantification of segmented filamentous bacteria (SFB) in the small intestines of Jackson and Taconic C57BL/6 mice. Genomic DNA was isolated from intestinal contents using the QIAamp DNA Stool Mini Kit (Qiagen). To detect SFB, we used specific primers directed against the SFB 16S rRNA gene (forward: 5′-GACGCTGAGGCATGAGAGCAT-3′; reverse: 5′-GACGGCACGGATTGTTATTCA-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. $^{*}P < 0.05$.

1. Salzman NH, et al. (2010) Enteric defensins are essential regulators of intestinal microbial ecology. *Nat Immunol* 11:76–83.

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E.coli              AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAA 60
sm_int_isolate      ------------------TGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAA 41
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E.coli              GTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAA 120
sm_int_isolate      GTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAA 101
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E.coli              TGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCAT 180
sm_int_isolate      TGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCAT 161
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E.coli              AACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATG 240
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E.coli              GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCA 600
sm_int_isolate      GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCA 581
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E.coli              GATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTC 660
sm_int_isolate      GATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTC 641
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E.coli              GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC 720
sm_int_isolate      GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC 701
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E.coli              GGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCA 780
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Fig. S4. (Continued)

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sm_int_isolate      AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT 941
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sm_int_isolate      TCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGA 1001
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E.coli              TTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA 1080
sm_int_isolate      TTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA 1061
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sm_int_isolate      AATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGC 1121
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E.coli              CGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTC 1200
sm_int_isolate      CGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTC 1181
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sm_int_isolate      ATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCG 1241
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E.coli              ACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAAC 1320
sm_int_isolate      ACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAAC 1301
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E.coli              TCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGT 1380
sm_int_isolate      TCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGT 1361
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E.coli              TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGT 1440
sm_int_isolate      TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGT 1421
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sm_int_isolate      AGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAA 1481
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E.coli              CAAGGTAACCGTAGGGGAACCTGCGGTTGGATCACCTCCTT 1541
sm_int_isolate      CAAGGTA---------------------------------- 1488
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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.

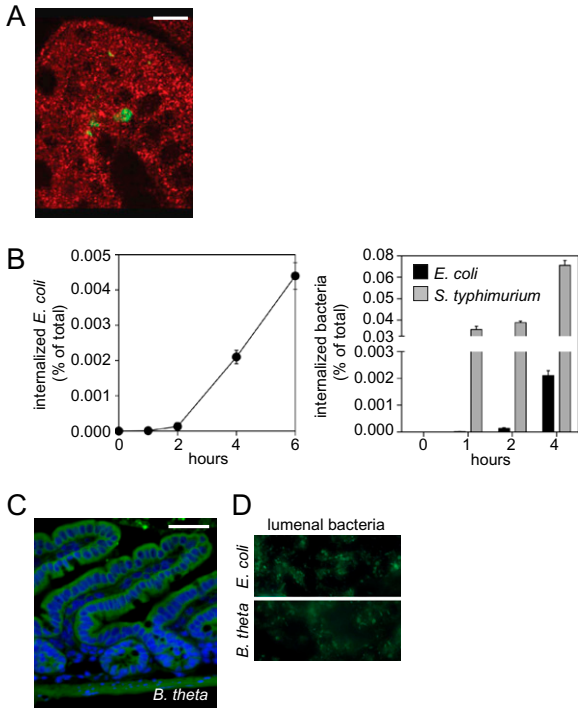


Fig. S5. Characterization of a resident *E. coli* strain that induces RegIIIγ expression in γδ IEL. (*A*) Confocal microscopy analysis of the *E. coli*-colonized small-intestinal tissue shown in Fig. 2*B*. Carnoy-fixed, paraffin-embedded ileal sections were prepared for FISH as described in *Materials 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 63× 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% CO_2 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* (*B. theta*) invasion into intestinal tissue is rare. Germfree mice were orally inoculated with 10^8 cfu of *B. thetaiotaomicron*, and FISH analysis was performed in parallel with that shown in Fig. 2*B* for a native intestinal *E. coli* strain isolated from the microbiota of SPF C57BL/6 mice. (Scale bar, 50 μm.) (*D*) FISH analysis of intestinal luminal contents, demonstrating that the FISH probe hybridizes to both mouse *E. coli* and *B. thetaiotaomicron*.

1. Vidal K, Grosjean I, Revillard JP, Gespach C, Kaiserlian D (1993) Immortalization of mouse intestinal epithelial cells by the SV40-large T gene. Phenotypic and immune characterization of the MODE-K cell line. *J Immunol Methods* 166:63–73.

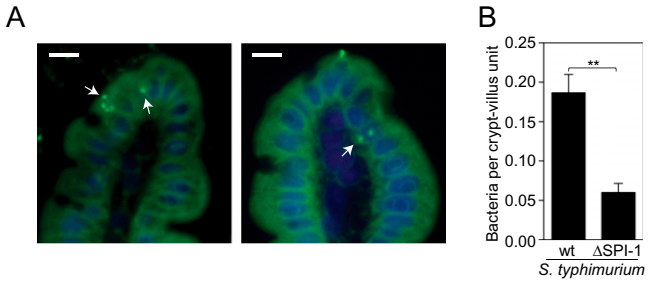


Fig. S6. *S. typhimurium* entry into small-intestinal epithelial cells. Germfree mice monocolonized with wild-type *S. typhimurium* or the invasion-deficient isogenic mutant ΔSPI-1 were subjected to FISH analysis with a universal bacterial 16S rRNA gene probe. (*A*) Images of wild-type *S. typhimurium*-colonized 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. S5*B*), 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 epithelial cells, 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*). $^{**}P < 0.01$.

1. Knodler LA, et al. (2010) Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proc Natl Acad Sci USA* 107:17733–17738.

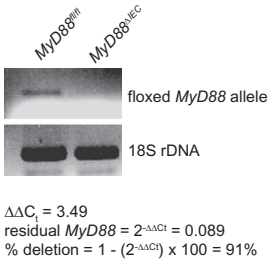


Fig. S7. Efficiency of deletion of the floxed MyD88 allele in *MyD88*$^{\Delta IEC}$ mice. Deletion efficiency of the floxed *MyD88* allele was assayed by Q-PCR on DNA from small-intestinal epithelial cells isolated from *MyD88*$^{\Delta IEC}$ mice (offspring of a *MyD88*$^{fl/fl}$ x Villin-Cre cross). Genomic DNA from epithelial cells isolated from *Myd88*$^{fl/fl}$ mice was used for the no-deletion control. 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′-GTTGTGTGTGTCCGACCGTG-3′; reverse: 5′-TCTCAATTAGCTCGCTGGCA-3′) (1). Cycle thresholds (C_ts) were normalized to 18S rRNA levels to yield ΔC_t values. $\Delta\Delta C_t$ is the difference between ΔC_t (*MyD88*$^{fl/fl}$) and ΔC_t (*MyD88*$^{\Delta IEC}$). % deletion values were calculated as indicated. The products were also visualized by agarose gel electrophoresis.

1. Hou B, Reizis B, DeFranco AL (2008) Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. *Immunity* 29:272–282.

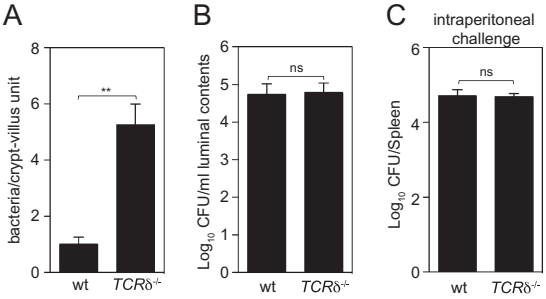


Fig. S8. Colonization of wild-type and *TCRδ*$^{-/-}$ mice with *S. typhimurium*. (*A*) Quantification of intracellular *S. typhimurium* in the small intestine 3 h after oral challenge of conventionally raised wild-type and *TCRδ*$^{-/-}$ mice. Mice were challenged with *S. typhimurium* SL1344 expressing 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. 4*A*. The ΔSPI-1 strain, which shows low levels of epithelial cell entry (Fig. S6*B*) 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 10^6 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.

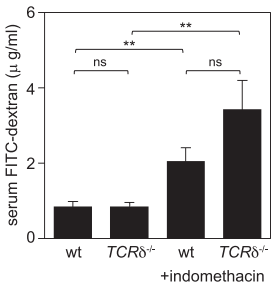


Fig. S9. Intestinal permeability measurements in wild-type and *TCRδ*$^{-/-}$ mice. Barrier permeability was assessed by measuring serum concentrations of FITC-dextran after oral gavage based on a previously established method (1). Wild-type C57BL/6 mice and *TCRδ*$^{-/-}$ mice were administered 200 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. $^{**}P < 0.01$.

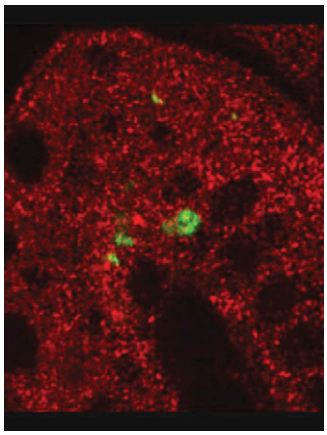
1. Furuta GT, et al. (2001) Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. *J Exp Med* 193:1027–1034.

Table S1. γδ IEL recovery from mouse small intestine

Colonization status	Genotype	Absolute numbers of sorted γδ IEL per small intestine
Conventionally raised	Wild-type	$8 \times 10^5 \pm 1 \times 10^5$
Germfree	Wild-type	$8 \times 10^5 \pm 0.9 \times 10^5$
Conventionally raised	*MyD88*$^{-/-}$	$1 \times 10^6 \pm 4 \times 10^5$
Conventionalized	Wild-type	9×10^5

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.

1. Iiyama R, et al. (2003) Normal development of the gut-associated lymphoid tissue except Peyer's patch in MyD88-deficient mice. *Scand J Immunol* 58:620–627.



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. 2*B*, 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