Figure S1 (Related to Figure 1)

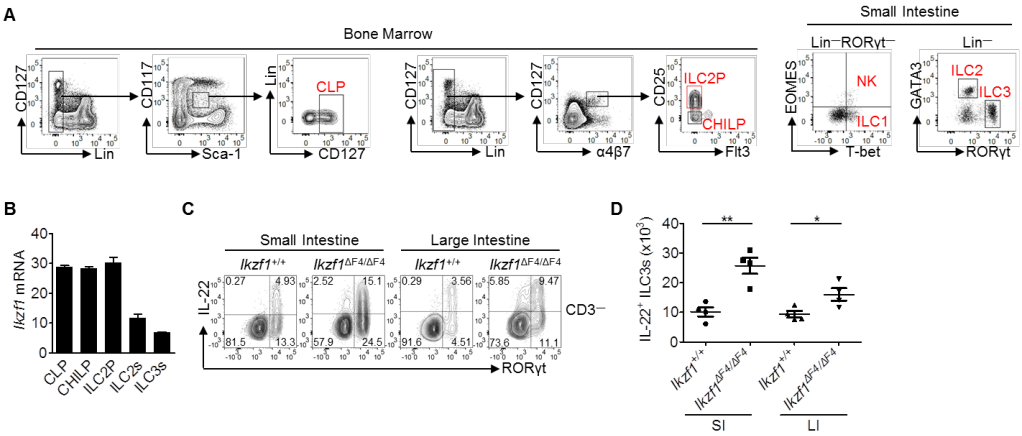

Figure S1 (Related to Figure 1). Ikaros mRNA in different cell populations and increased IL-22$^+$ ILC3s in *Ikzf1*$^{\Delta F4/\Delta F4}$ mice. (A) Gating strategy of CLP (Lin$^-$ CD117^{low}Sca-1^{low}CD127$^+$), CHILP (Lin$^-$CD127$^+$α4β7$^+$CD25$^-$Flt3$^-$), and ILC2P (Lin$^-$ CD127$^+$α4β7$^+$CD25$^+$Flt3$^-$) from bone marrow. Gating strategy of ILC1s (Lin$^-$ T-bet$^+$EOMES$^-$RORγt$^-$), NK cells (Lin$^-$T-bet$^+$EOMES$^+$ RORγt$^-$), ILC2s (Lin$^-$ GATA3$^+$RORγt$^-$), and ILC3s (Lin$^-$RORγt$^+$GATA3$^-$) from the small intestine. (B) CLP (Lin$^-$CD117^{low}Sca-1^{low}CD127$^+$), CHILP (Lin$^-$CD127$^+$α4β7$^+$CD25$^-$Flt3$^-$), ILC2P (Lin$^-$ CD127$^+$α4β7$^+$CD25$^+$Flt3$^-$), ILC2s (Lin$^-$KLRG1$^+$CD90^{hi}), and ILC3s (Lin$^-$ CD45^{low}CD90^{hi}) were sorted from wildtype C57BL/6 mice bone marrow or the small intestine. Expression of Ikaros (*Ikzf1*) mRNA was analyzed by realtime RT-PCR. Data are representative of two independent experiments with one mouse in each experiment and shown as mean ± SEM. The error bars were generated from triplicates in realtime RT-PCR reaction. (C) Expression of RORγt and IL-22 by intestinal LPLs isolated from indicated littermate mice after gating on CD3$^-$ cells. Data are representative of three independent experiments. (D) Numbers of IL-22 producing ILC3s (CD3$^-$RORγt$^+$) in the small (SI) and large (LI) intestinal LPLs are shown. Data are shown as mean ± SEM (n=4 mice per group). *p<0.05, **p<0.01.

Figure S2 (Related to Figure 2 and Figure 3)

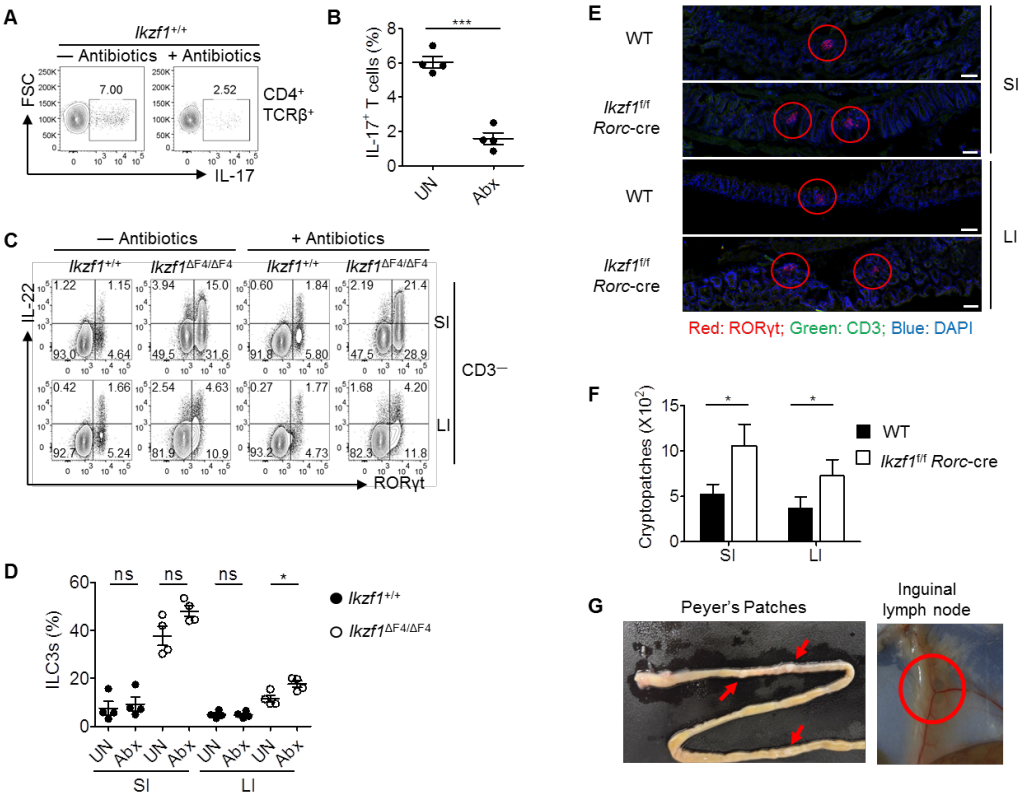

Figure S2 (Related to Figure 2 and Figure 3). Microbiota-independent upregulation of ILC3s in *Ikzf1*$^{\Delta F4/\Delta F4}$ mice, increased intestinal cryptopatches and normal development of secondary lymphoid tissues in *Ikzf1*$^{f/f}$ *Rorc*-cre mice. (A and B) Small intestinal LPLs were isolated from *Ikzf1*$^{+/+}$ mice untreated (UN) or treated with antibiotics (Abx) for 7 days. Expression of IL-17 after gating on CD4^{+}TCRβ^{+} cells in the small intestine was measured by FACS (A). Data are representative of two independent experiments. Percentages of IL-17^{+} cells gating on CD4^{+}TCRβ^{+} small intestinal LPLs are shown (B). Data are shown as mean ± SEM (n=4 mice per group). ***p<0.001. (C) Small (SI) or large (LI) intestinal LPLs were isolated from littermate mice of indicated genotypes untreated or treated with antibiotics for 7 days. Expression of RORγt and IL-22 after gating on CD3^{-} cells was determined by FACS. Data are representative of two independent experiments. (D)

Percentages of ILC3s ($CD3^{-}ROR\gamma t^{+}$) are shown. Data are shown as mean ± SEM (n=4 mice per group). *p<0.05. (E) Cryptopatches in representative sections of small (SI) or large intestines (LI) of *Ikzf1*$^{f/f}$ *Rorc*-cre or wildtype (WT: *Ikzf1*$^{f/f}$ or *Ikzf1*$^{f/+}$) littermate mice were stained with RORγt (red), CD3 (green), and DAPI (blue) and analyzed by fluorescence microscopy. Scale bar, 50 μm. Data are representative of two independent experiments. (F) Numbers of cryptopatches in the small (SI) and large intestines (LI) of indicated mice. Data are shown as mean ± SEM (n=3 mice per group). *p<0.05. (G) Pictures of Peyer's patches and inguinal lymph node of *Ikzf1*$^{f/f}$ *Rorc*-cre mice.

Figure S3 (Related to Figure 4)

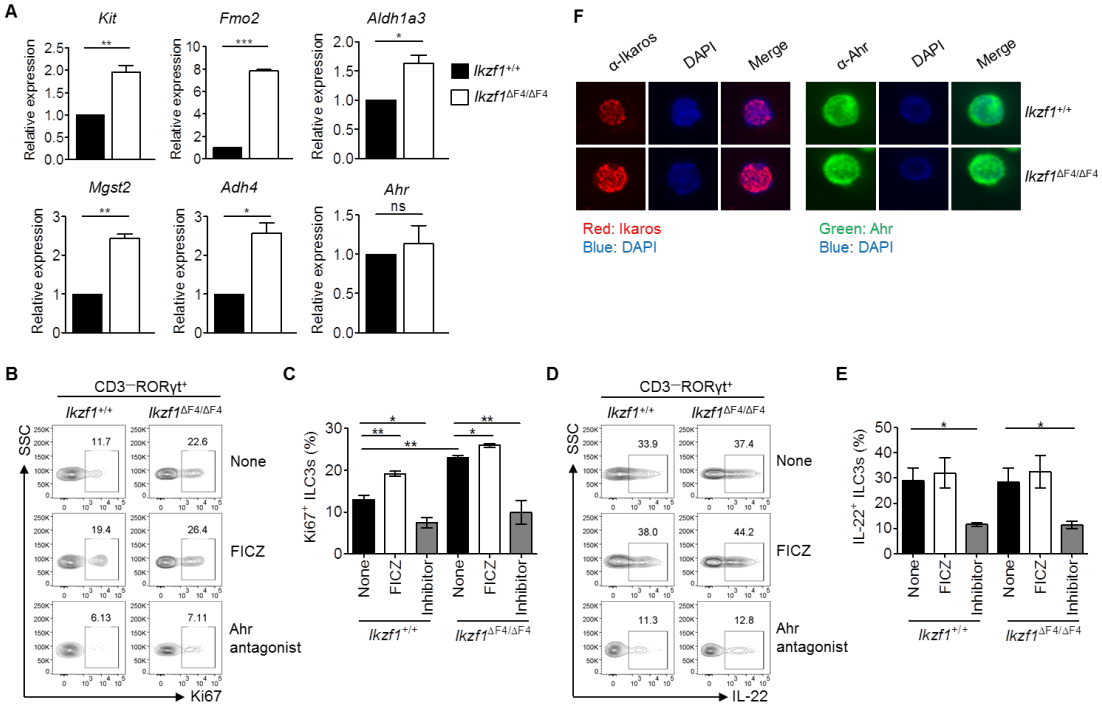

Figure S3 (Related to Figure 4). Upregulation of ILC3s in *Ikzf1*$^{\Delta F4/\Delta F4}$ mice is dependent on Ahr pathway. (A) Small intestinal ILC3s (CD3^{-}CD45^{low}CD90^{hi}) were sorted from *Ikzf1*$^{\Delta F4/\Delta F4}$ or *Ikzf1*$^{+/+}$ littermate mice. Expression of indicated genes was analyzed by realtime RT-PCR, and normalized to the expression in wildtype mice. Data are compiled from three (*Fmo2*, *Aldh1a3*, *Mgst2,* and *Adh4*) or four independent experiments (*Kit* and *Ahr*). Each experiment consisted of one *Ikzf1*$^{\Delta F4/\Delta F4}$ mouse and one *Ikzf1*$^{+/+}$ littermate mouse, and shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 (One-sample t-test). (B to E) Large intestinal LPLs from *Ikzf1*$^{\Delta F4/\Delta F4}$ and *Ikzf1*$^{+/+}$ littermate mice were cultured in the presence of the Ahr ligand FICZ or the Ahr antagonist (CAS 301326-22-7 Millipore) for 24 hours. Expression of Ki67 (B) and IL-22 (D) was measured by flow cytometry after gating on CD3^{-}RORγt^{+} ILC3s. Data are representative of two independent experiments. Percentages of Ki67^{+} ILC3s (C) and IL-22^{+} ILC3s (E) are shown as mean ± SEM (n=3 mice per group), *p<0.05,

**p<0.01. (F) Small intestinal ILC3s ($CD3^{-}CD45^{low}CD90^{hi}$) were sorted from $Ikzf1^{\Delta F4/\Delta F4}$ or $Ikzf1^{+/+}$ littermate mice. Full-length Ikaros or Ikaros that lacks zinc finger 4 (Red), and Ahr (Green) with nucleus (Blue) were stained with indicated antibodies and DAPI, and analyzed by fluorescence microscopy. Data are representative of two independent experiments.

Figure S4 (Related to Figure 4)

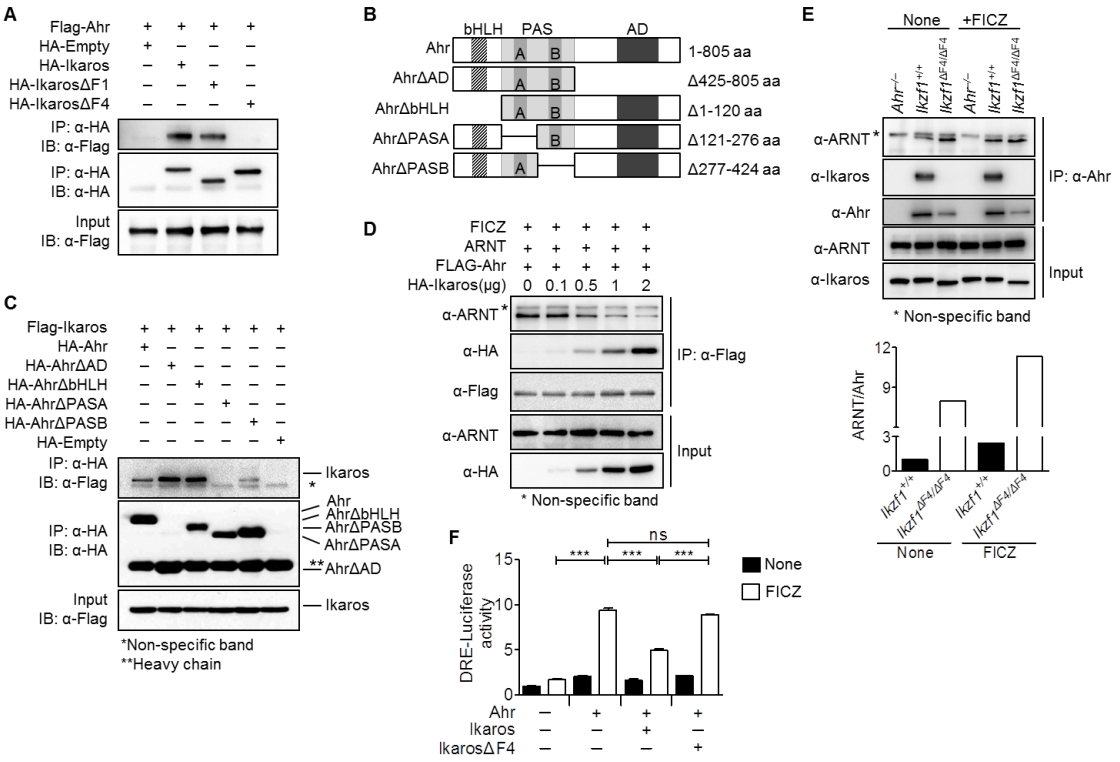

Figure S4 (Related to Figure 4). Ikaros interacts with Ahr and suppresses Ahr-ARNT dimerization and the transcription of DRE-containing genes. (A) HEK293T cells were transfected with Flag-tagged Ahr and HA-tagged full-length Ikaros or zinc finger 1-deleted or zinc finger 4-deleted Ikaros for 24 hours. Total cell lysates were immunoprecipitated (IP) with anti-HA beads and blotted (IB) with indicated antibodies. Data are representative of three independent experiments. (B) Schematic depiction of full-length Ahr and its mutants. (C) HA-tagged Ahr or its mutants were co-expressed with Flag-tagged Ikaros in HEK293T cells for 24 hours. Total cell lysates were immunoprecipitated with anti-HA beads and blotted (IB) with indicated antibodies. Data are representative of three independent experiments. (D) ARNT and Flag-tagged Ahr were expressed with increasing amounts of HA-tagged Ikaros in HEK293T cells for 24 hours. The cells were treated with FICZ for 3 hours.

Total cell lysates were immunoprecipitated (IP) with anti-Flag beads and blotted with indicated antibodies. Data are representative of two independent experiments. (E) Splenic $CD4^+$ T cells were purified from indicated mice and activated by anti-CD3 and anti-CD28 in the presence of TGF-β and IL-6 with or without FICZ for 96 hours. Total cell lysates were immunoprecipitated (IP) with anti-Ahr and blotted with indicated antibodies. The interaction of ARNT and Ahr was represented as the ratio of ARNT and Ahr band intensity. Data are representative of two independent experiments. (F) DRE-firefly luciferase and pRL-TK-Renilla luciferase were transfected into HEK293T cells, together with Ahr and full-length Ikaros or Ikaros that lacks zinc finger 4. Cells were stimulated with or without FICZ for 18 hours and activation of DRE was presented as the firefly luciferase activity normalized to Renilla luciferase activity. Data were compiled from three independent experiments, and are shown as mean ± SEM (n=3). ***$p<0.001$.

Figure S5 (Related to Figure 5)

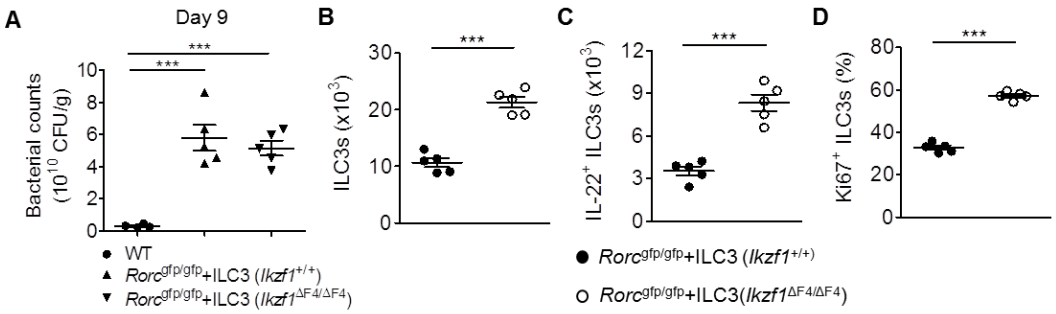

Figure S5 (Related to Figure 5). Ikaros regulates ILC3s cell-intrinsically during *C. rodentium* infection. (A to D) *Rorc*$^{gfp/gfp}$ mice were adoptively transferred with ILC3s (CD3$^-$CD45^{low}CD90^{hi}) sorted from the small intestine of *Ikzf1*$^{+/+}$ (*Rorc*$^{gfp/gfp}$ + ILC3 (*Ikzf1*$^{+/+}$)) or *Ikzf1*$^{\Delta F4/\Delta F4}$ littermate mice (*Rorc*$^{gfp/gfp}$ + ILC3 (*Ikzf1*$^{\Delta F4/\Delta F4}$)). Wildtype C57BL/6 (n=4 mice per group), and *Rorc*$^{gfp/gfp}$ with transfer (n=5 mice per group) were infected with *C. rodentium* 24 hours after adoptive transfer. Day 9 after infection, bacterial counts (CFUs) normalized to per gram of feces (A), numbers of ILC3s (CD3$^-$RORγt$^+$) (B) and IL-22$^+$ ILC3s (C), and percentages of Ki67$^+$ ILC3s (D) are shown. Data are shown as mean ± SEM. ***p<0.001.

Figure S6 (Related to Figure 5)

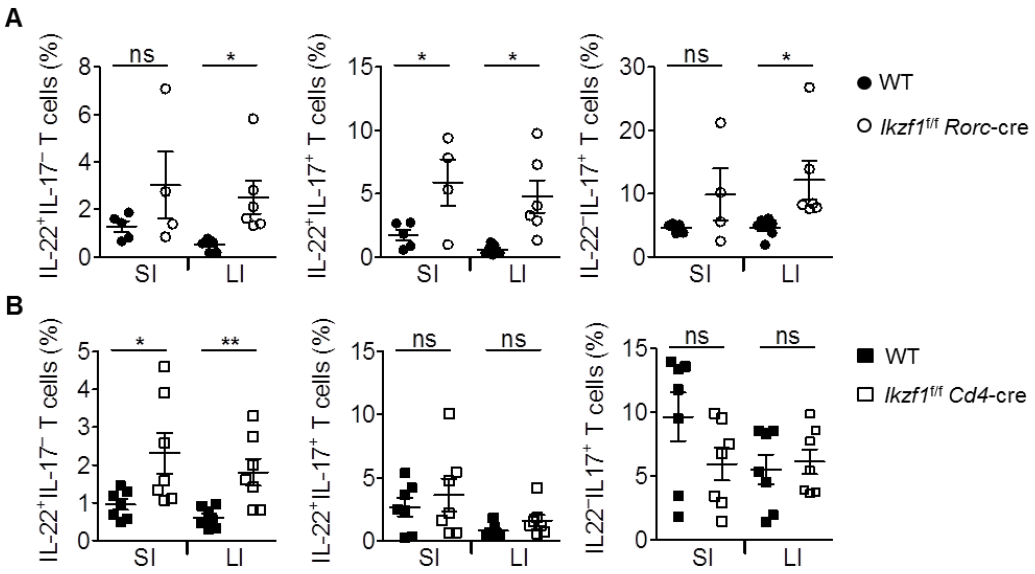

Figure S6 (Related to Figure 5). Ikaros deficiency leads to increased intestinal Th22 and/or Th17 cells at the steady state. IL-17 and IL-22 production by T cells from the small (SI) or large intestine (LI) of *Ikzf1*$^{f/f}$ *Rorc*-cre or wildtype (WT: *Ikzf1*$^{f/f}$ or *Ikzf1*$^{f/+}$) littermate mice (n=4 to 6 mice per group) (A), and *Ikzf1*$^{f/f}$ *Cd4*-cre or wildtype (WT: *Ikzf1*$^{f/f}$ or *Ikzf1*$^{f/+}$) littermate mice (n=7 mice per group) (B) under steady state without infection. Data were compiled from four independent experiments and are shown as mean ± SEM. *p<0.05, **p<0.01.

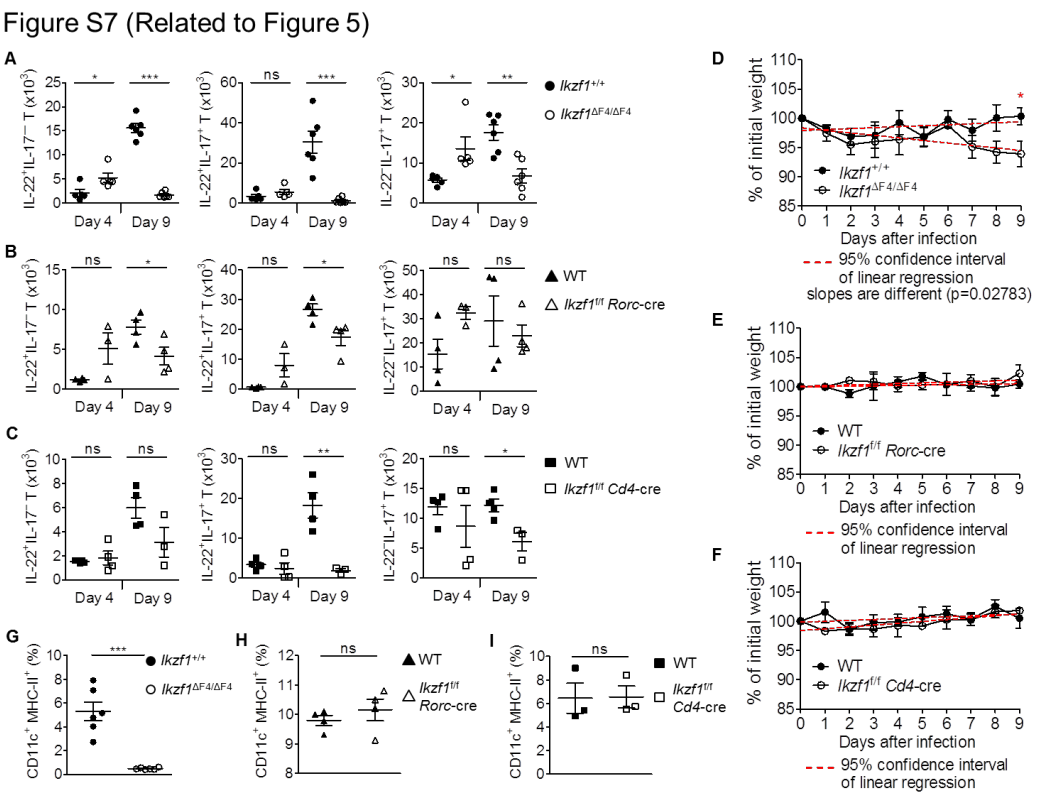

Figure S7 (Related to Figure 5). T cell responses, weight changes, and CD11c$^+$ DC compartment in Ikaros-deficient mice during *C. rodentium* infection. *Ikzf1*$^{\Delta F4/\Delta F4}$ or *Ikzf1*$^{+/+}$ littermate mice (n=5 to 6 mice per group), *Ikzf1*$^{f/f}$ *Rorc*-cre or wildtype (WT: *Ikzf1*$^{f/f}$ or *Ikzf1*$^{f/+}$) littermate mice (n=3 to 4 mice per group), and *Ikzf1*$^{f/f}$ *Cd4*-cre or wildtype (WT: *Ikzf1*$^{f/f}$ or *Ikzf1*$^{f/+}$) littermate mice (n=3 to 4 mice per group) were infected with *C. rodentium*. (A to C) Numbers of large intestinal IL-22$^+$ and/or IL-17$^+$ T cells of indicated mice at day 4 or day 9 after infection are shown. (D to F) Body weight changes were monitored at the indicated time points (n=3 to 6 mice per group). (G to I) MHC-II and CD11c expression by large intestinal LPLs were measured at day 9 after infection (n=3 to 6 mice per group). Data were compiled from two independent experiments and are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Table S1 (Related to Figure 4 and Figure S3). Primers for realtime RT-PCR and ChIP assays

Actin FW	CTTCTTTGCAGCTCCTTCGTT
Actin RV	AGGAGTCCTTCTGACCCATTC
Ikzf1 FW	GCTGGCTCTCAAGGAGGAG
Ikzf1 RV	CGCACTTGTACACCTTCAGC
Ahr FW	GGCTTTCAGCAGTCTGATGTC
Ahr RV	CATGAAAGAAGCGTTCTCTGG
Kit FW	GTGGCTAAAGATGAACCCTCAG
Kit RV	CAACTCTTGCCGAGCTGATA
Fmo2 FW	CCCAACTTCCTGCACAACTC
Fmo2 RV	TCCTTTCCGTTGCTCTGAGT
Aldh1a3 FW	TAGAAAGGGACCGAGCGATC
Aldh1a3 RV	ACGTTGTCATCTGTGGGGAT
Adh4 FW	CCTGGGGTGTGTAGGTCTTT
Adh4 RV	AGGTTTGTCTAGCTCCCTGG
Mgst2 FW	GTAATGCTGTGGATGGCTGG
Mgst2 RV	GACAGGCAGCAAGGTCAAAA
Kit-ChIP FW	CCGGTGGTTGTCCTTTATTGTCTA
Kit-ChIP RV	CATCGGTGGTCCTTCAGTCC
Fmo2-ChIP FW	CTGCCTCTGATTCTGAAAGGTG
Fmo2-ChIP RV	AAAAGCGTTCTGTATTTGCTAC

Il12b-ChIP FW	TAGTATCTCTGCCTCCTTCC
Il12b-ChIP RV	GAACTTTCTGATGGAAACCC

Supplemental Experimental Procedures

Isolation of Intestinal Lamina Propria Lymphocytes (LPLs) and Flow Cytometry

Isolation of intestinal lamina propria cells and flow cytometry were done as previously described (Qiu et al., 2012). CD16/32 antibody (eBioscience) was used to block the non-specific binding to Fc receptors before surface stainings. LPLs were stained with antibodies against following makers: CD3 (FITC, eBioscience), RORγt (PE, eBioscience), IL-17 (PE, eBioscience), GM-CSF (FITC, eBioscience), IL-22 (APC, eBioscience), NKp46 (PerCP-Cy5.5, eBioscience), CD4 (APC-Cy7, eBioscience), CD127 (APC, eBioscience), CD45.2 (PerCP-Cy5.5, eBioscience), CD90.2 (APC-Cy7, eBioscience), CCR6 (Alexa 647, BD Pharmingen), CD127 (PE, eBioscience), CD25 (PE-Cy7, eBioscience), α4β7 (APC, eBioscience), Flt3 (PerCP-Cy5.5, eBioscience), T-bet (PE-Cy7, eBioscience), GATA3 (APC, eBioscience), CD117 (APC, eBioscience), Sca-1 (PE-Cy7, eBioscience), and Ki67 (Alexa 647, BD Pharmingen). To sort CLP, CHILP, ILC2P, ILC2s and ILC3s, lineage marker mix contained FITC-CD3, CD19, B220, Ly6G, FcεR1, CD11c, CD11b, Ter119, NK1.1, and CD16/CD32 (Tonbo). For Ikaros intracellular staining of ILC1s and NK cells, lineage marker mix contained APC-Cy7-CD3, CD5, CD19 and B220 (eBioscience). For Ikaros intracellular staining of CLP, CHILP, ILC2s, and ILC3s, lineage marker mix contained APC-Cy7-CD3, CD19, B220, CD11b, CD11c, and Ter119 (eBioscience). Ikaros primary antibody (raised in rabbit) (Hahm et al., 1998) and Alexa Fluor 488-goat anti-rabbit IgG (H+L) secondary

antibody (Invitrogen) were used in intracellular staining. For nuclear transcription factor staining, cells were fixed and permeabilized with Foxp3 staining buffer Kit (eBioscience). For cytokine staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 hours and Brefeldin A (2 μg/ml) was added 2 hours before cells were harvested. The live and dead cells were discriminated by Live and Dead violet viability kit (Invitrogen).

Antibiotic Treatment

Mice were gavaged daily with 200 μl autoclaved water supplemented with antibiotics (ampicillin 1g/L, gentamicin 1g/L, metronidazole 1g/L, neomycin 1g/L, and vancomycin 0.5g/L) for 7 days.

Quantitative Real-Time RT-PCR

RNA of ILC3s sorted from the small intestines was isolated with Trizol reagent (Invitrogen). cDNA was synthesized using GoScript™ Reverse Transcription kit (Promega). Real-time RT-PCR was performed using SYBR Green (Biorad) and different primer sets (Table S1). Reactions were run using the MyiQ™2 Two-Color Real-Time PCR Detection System (Biorad). The gene expression relative to to β-actin was analyzed by the comparative C_T method, and normalized to the expression in wildtype strain.

Plasmids

cDNA of mouse Ikaros, IkarosΔF1 and IkarosΔF4 were cloned into pMIGR with HA

or Flag tag at N terminus. cDNA of mouse ARNT was cloned into pMIGR. cDNA of mouse Ahr (1-805aa) was cloned into pMIGR with HA tag at N terminus or into pMIR-DFTC (double Flag-tagged construct). The AhrΔAD (425-805aa were deleted), AhrΔbHLH (1-120aa were deleted), AhrΔPAS-A (121-276aa were deleted) and AhrΔPAS-B (277-424aa were deleted) were subcloned into pMIGR with HA tag.

Luciferase Reporter Assay

HEK293T cells were transfected with Ahr and Ikaros and its mutants, together with pGudLuc6.1 luciferase (He et al., 2011) and pRL-TK Renilla constructs for 24 hours. Then the cells were treated with or without FICZ (200 nM) for 4 hours. Luciferase activity was measured in a luminometer (Turner BioSystems) with a Dual-Luciferase assay kit (Promega). Normalized luciferase activity was presented as the ratio of firefly luciferase activity to Renilla luciferase activity.

Immunofluorescence

Sorted ILC3s ($CD3^{-}CD45^{low}CD90^{hi}$) were centrifuged onto the slides using Cytospin (Thermo Scientific), fixed in 4% paraformaldehyde for 10 minutes, and then permeabilized in 1% TritonX-100 for 10 minutes. The cells were stained using Ikaros (rabbit) or Ahr antibody (goat; Santa Cruz) at 4˚C overnight, and the Goat anti-rabbit IgG-Cy3 (Jackson ImmunoResearch) or Bovine anti-goat IgG-Alexa 488 (Jackson ImmunoResearch) were used to detect Ikaros or Ahr, respectively. The immunofluorescence was detected with fluorescent microscope (Leica).

In Vitro Th17 Cell Differentiation

Splenocytes were made into single cell suspension and $CD4^+$ T cells were purified with $CD4^+$ T cell isolation kit (Stem Cell). 24-well-plate was coated with goat anti-hamster antibody (MP Biomedical). $CD4^+$ T cells were cultured for 96 hours in IMDM medium (Sigma Aldrich) supplied with soluble hamster-anti-mouse CD3 (0.25 μg/ml; BioXCell), hamster-anti-mouse CD28 (1 μg/ml; BioXCell), anti-mouse IL-4 (2 μg/ml; BioXCell), anti-mouse IFN-γ (2 μg/ml; BioXCell), TGF-β (5 ng/ml; PeproTech), and IL-6 (20 ng/ml; eBioscience). In some experiments, FICZ was added at a concentration of 200 nM.

In vitro Culture of LPLs

Freshly isolated large intestinal LPLs were cultured in the presence of FICZ (200 nM) or Ahr antagonist (10 μM, EDM Millipore, CAS 301326-22-7) for 24 hours. Cells were subjected to proliferation and cytokine production examination.

Chromatin Immunoprecipitation (ChIP) Assay

ILC3s were fixed in 1% formaldehyde in IMDM medium (Sigma Aldrich) for 10 minutes at room temperature. The reaction was stopped by adding glycine solution (final concentration, 0.125 M). Fixed cells were then washed 3 times with ice cold PBS, and resuspended in Farnham Lysis Buffer (5 mM PIPES (pH 8.0), 85 mM KCl and 0.5% NP-40) with protease inhibitors (Roche). Nuclei were collected and lysed with RIPA Buffer (1XPBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS)

with protease inhibitors. Chromatin was sheared by sonication on ice and the insoluble fraction was removed by centrifugation. The lysates were incubated with Ahr antibody (Novus) or control IgG overnight and then incubated with Dynabeads Protein G (Life Technologies) at 4˚C for 4 hours. Immunocomplexes were collected and washed with LiCl wash buffer (100 mM Tris (pH 7.5), 500 mM LiCl, 1% NP-40 and 1% sodium deoxycholate) and TE buffer (10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA). Precipitated chromatin fragments were eluted with IP Elution Buffer (1% SDS and 0.1 M $NaHCO_3$) at 65 ˚C for one hour. ChIP DNA was obtained by collecting supernatant after centrifuging at 14,000 rpm for 3 minutes and then incubated at 65 ˚C water bath overnight. After proteinase K digestion, DNA was extracted using Phenol-Chloroform (Invitrogen), and precipitated for real-time PCR analyses using specific primers (Table S1).

Calculation of Cryptopatch Number

The number of cryptopatches were calculated under the assumption that each section would be representative of the distribution of cryptopatches throughout the small and large intestines. The intestine, when cut open, has an average width of 5 mm, and thus for each sample, a total of 1000 sections can be cut at 5 μm. The absolute numbers of clusters counted were multiplied by 1000. Cryptopatches have an average size that spans 86 μm (Kanamori et al., 1996). The same cryptopatch would be counted 17 times, and thus the calculated number was divided by 17. The resulting calculation was: (numbers of cryptopatches) × 1000/17.

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