SUPPLEMENTAL FIGURES

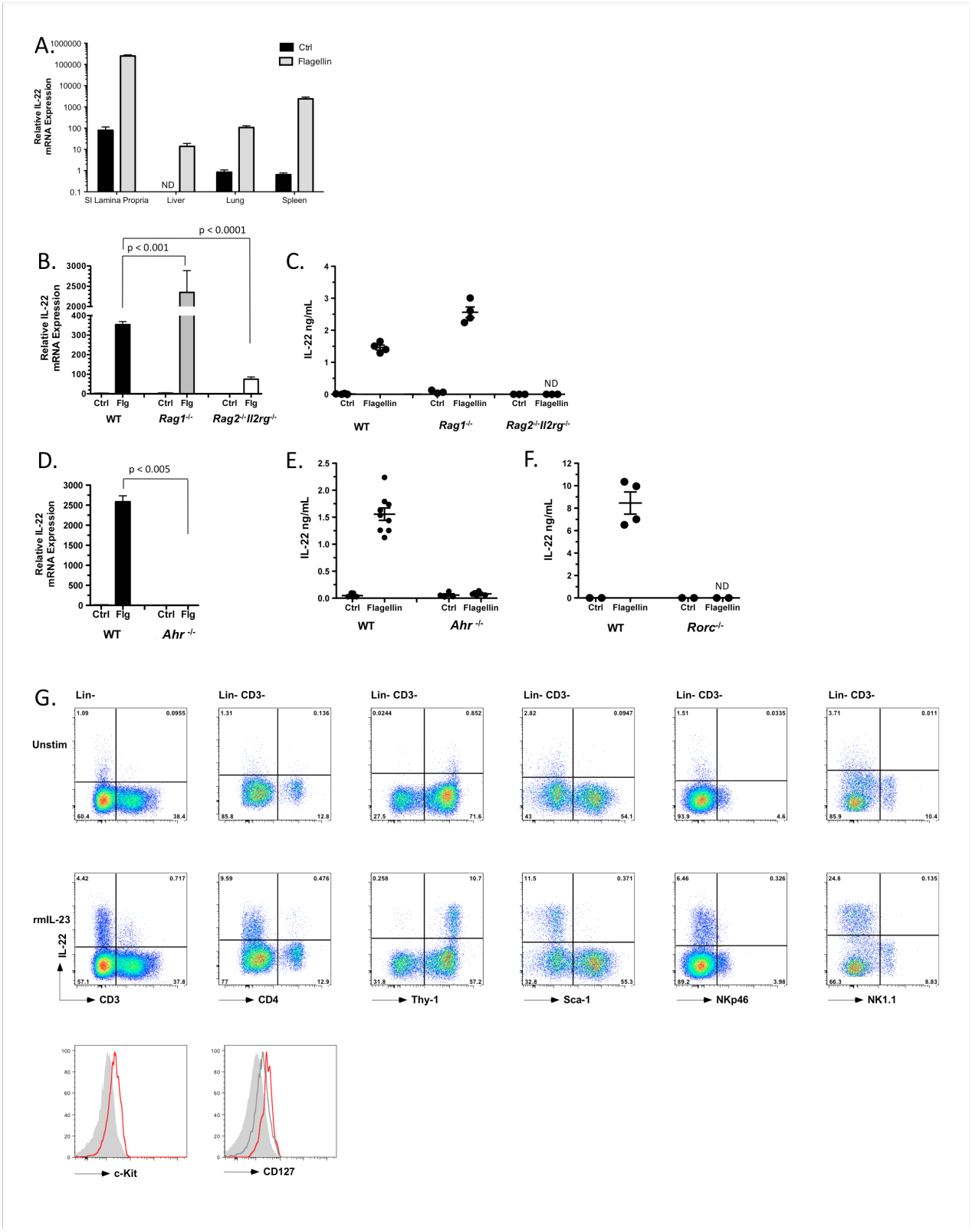

Figure S1. Phenotype of IL-23-responsive innate lymphoid cells.

(A) Flagellin (1 μg) was administered to wild-type (WT) mice via intravenous injection. Lamina propria tissue, liver, spleen, and lung were collected 2 hours post-injection. Tissue was analyzed for IL-22 mRNA expression using quantitative PCR. (n = 3 mice per group).

(B – C) WT, *Rag1*$^{-/-}$, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice received 1 μg of flagellin (Flg) or PBS (Ctrl) by intravenous injection. *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice are deficient in both RAG2 and the common γ receptor chain. Three hours post-injection, the small intestine lamina propria was harvested and analyzed for IL-22 mRNA expression (B) using quantitative PCR. IL-22 protein levels were measured in the blood serum (C) using ELISA. Data are representative of 3 independent experiments. Error bars denote ± s.e.m.

(D – E) WT and *Ahr*$^{-/-}$ mice received 1 μg of flagellin or PBS by intravenous injection and sacrificed three hours later. Lamina propria IL-22 mRNA expression in *Ahr*$^{-/-}$ mice was compared to WT mice using qPCR (C). Serum concentrations of IL-22 were assessed by ELISA (D) (n = 2 – 4 mice per group). Data are representative of 2 independent experiments.

(F) Flagellin-mediated IL-22 expression was evaluated in mice deficient in *Rorc*$^{-/-}$ mice (gene name for RORγ) and compared to wild-type controls three hours following injection of 1 μg of flagellin. Serum concentrations of IL-22 were assessed by ELISA (n = 2 – 4 mice per group). Error bars denote ± s.e.m.

(G) Lamina propria cells were isolated from the small intestine and stimulated with rmIL-23 for 2.5 hours with Brefeldin A. IL-23 was demonstrated to be the soluble factor

that stimulates IL-22 secretion following flagellin administration in Figure 3 and Figure 4. Intracellular cytokine was preformed to analyze the phenotype of IL-22-producing cells. The cells displayed were gated on viable $CD45^+$ cells. The solid gray histogram shows c-kit and CD127 staining for all viable $CD45^+$ cells. The lined gray histogram shows c-kit and CD127 staining for IL-22^- Lin^- $CD3\varepsilon^-$ $Thy1^+$ cells while the red line shows IL-22^+ Lin^- $CD3\varepsilon^-$ $Thy1^+$ cells. Data are representative dot plots of at least 2 independent experiments (n = 2 mice per group).

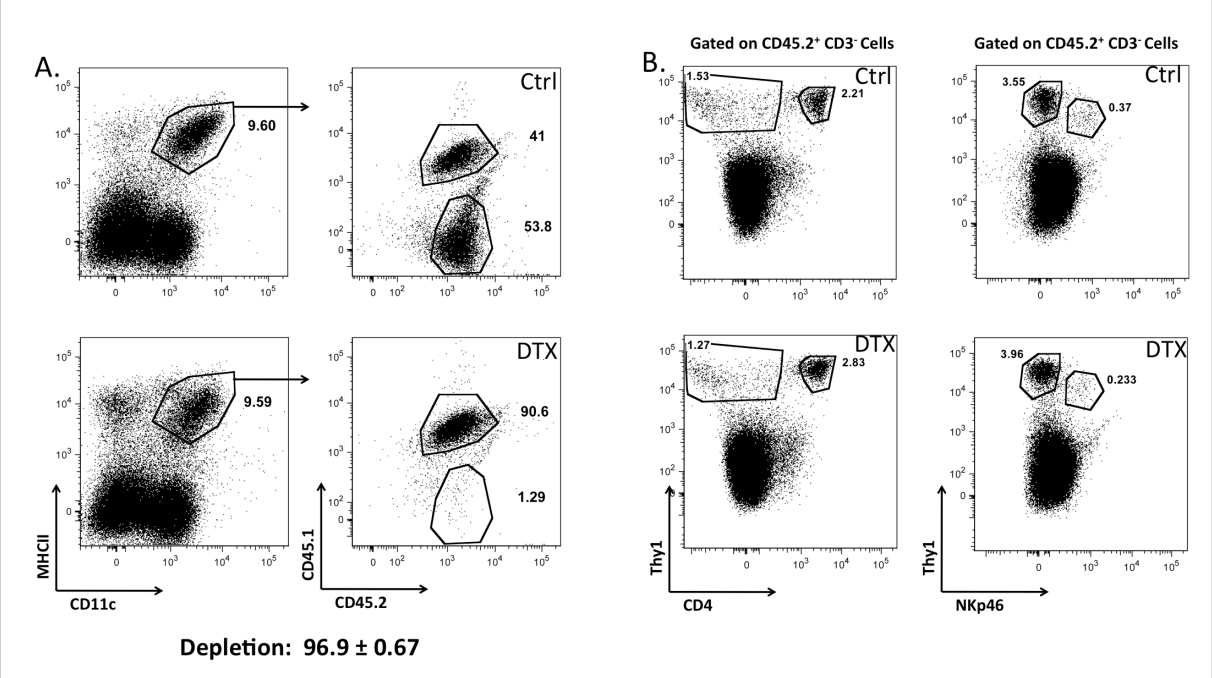

Figure S2. Diphtheria toxin treatment of TLR5 KO:CD11c.DTR mixed chimeric mice specifically depletes TLR5-sufficient dendritic cells and not innate lymphocytes.

A 1:1 mixture of TLR5-deficient bone marrow and CD11c.DTR bone marrow was transferred into lethally irradiated wild-type mice. After 7 weeks of reconstitution, mice received 3 doses of diphtheria toxin (DTX, 10 ng/g) by intraperitoneal injection administered every other day over the course of 6 days. Mice were sacrificed 1 day after the last dose of flagellin. Lamina propria cells were isolated from the small intestine to assess depletion by flow cytometry.

(A) The composition of $CD11c^+MHCII^+$ lamina propria dendritic cells was assessed by CD45.1/CD45.2 (TLR5 KO) and CD45.2 (CD11c.DTR) congenic markers.

(B) Frequency of innate immune cells, gated by $CD45^+CD3\varepsilon^-$, after DTX treatment. Numbers on dot plots are the percentage of gated cells out of $CD45^+$ cells. Data are representative of a least 2 independent experiments (n = 2 – 4 mice per group).

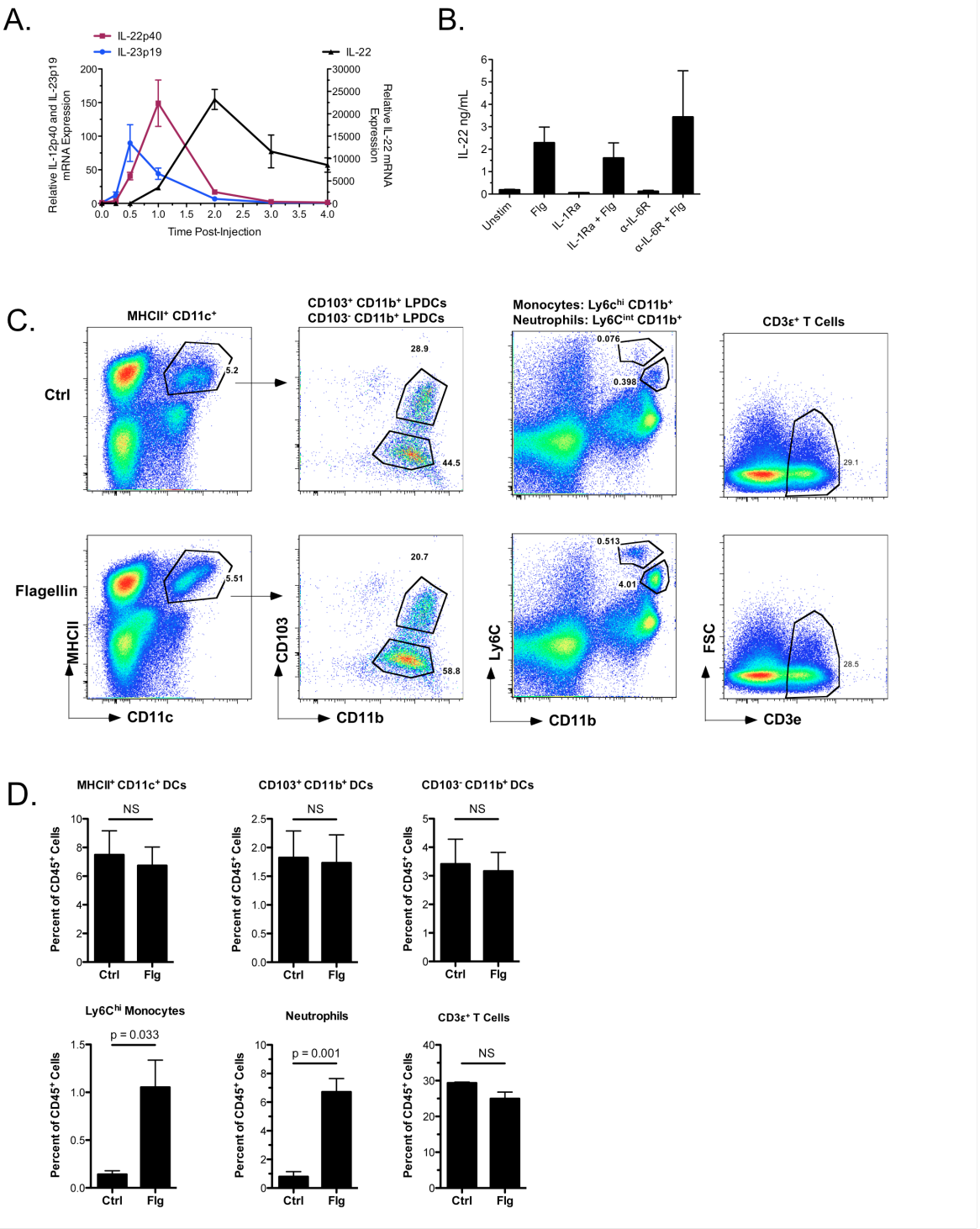
A.
IL-22p40
IL-23p19
IL-22
Relative IL-12p40 and IL-23p19 mRNA Expression
Relative IL-22 mRNA Expression
Time Post-Injection
B.
IL-22 ng/mL
Unstim
Flg
IL-1Ra
IL-1Ra + Flg
α-IL-6R
α-IL-6R + Flg
C.
MHCII+ CD11c+
CD103+ CD11b+ LPDCs
CD103- CD11b+ LPDCs
Monocytes: Ly6chi CD11b+
Neutrophils: Ly6Cint CD11b+
CD3ε+ T Cells
Ctrl
Flagellin
MHCII
CD11c
CD103
CD11b
Ly6C
CD11b
FSC
CD3e
5.2
28.9
44.5
0.076
0.398
29.1
5.51
20.7
58.8
0.513
4.01
28.5
D.
MHCII+ CD11c+ DCs
CD103+ CD11b+ DCs
CD103- CD11b+ DCs
Ly6Chi Monocytes
Neutrophils
CD3ε+ T Cells
Percent of CD45+ Cells
NS
p = 0.033
p = 0.001
Ctrl
Flg

Figure S3. Characterization of lamina propria IL-23 expression and cell populations following stimulation with flagellin.

(A) Flagellin (1 μg) was administered to WT mice via tail vein injection. Mice were sacrificed at 15, 30, 60, 120, 180, and 240 minutes after flagellin injection. The lamina propria of the small intestine was harvested and processed to extract mRNA. Quantitative PCR was used to measure the relative upregulation of IL-12/23p40, IL-23p19, and IL-22. Expression was normalized to Hprt1. Data are expressed as relative induction compared to levels at 0 hours post-injection. Data are representative of 2 independent experiments (n = 3 mice per group).

(B) Lamina propria cells were isolated from WT mice and stimulated overnight in the absence or presence of flagellin with or without the indicated cytokine-blocking agent. IL-22 protein levels were measured in the supernatant using ELISA. Data are representative of three independent experiments. Error bars denote ± s.e.m.

(C) To determine if TLR5 stimulation induces migration of immune cells within 2 hours following injection of flagellin, wild-type mice received 1 μg of flagellin by intravenous injection. Mice were sacrificed 2.5 hours post-injection. Lamina propria cells were harvested from the small intestine and analyzed by flow cytometry. Dot plots show a representative sample from each group. Cells were pre-gated on $CD45^+$ cells. Data shown in (D) is pooled from two independent experiments (n = 2 – 3 mice per group). Error bars denote ± s.e.m.

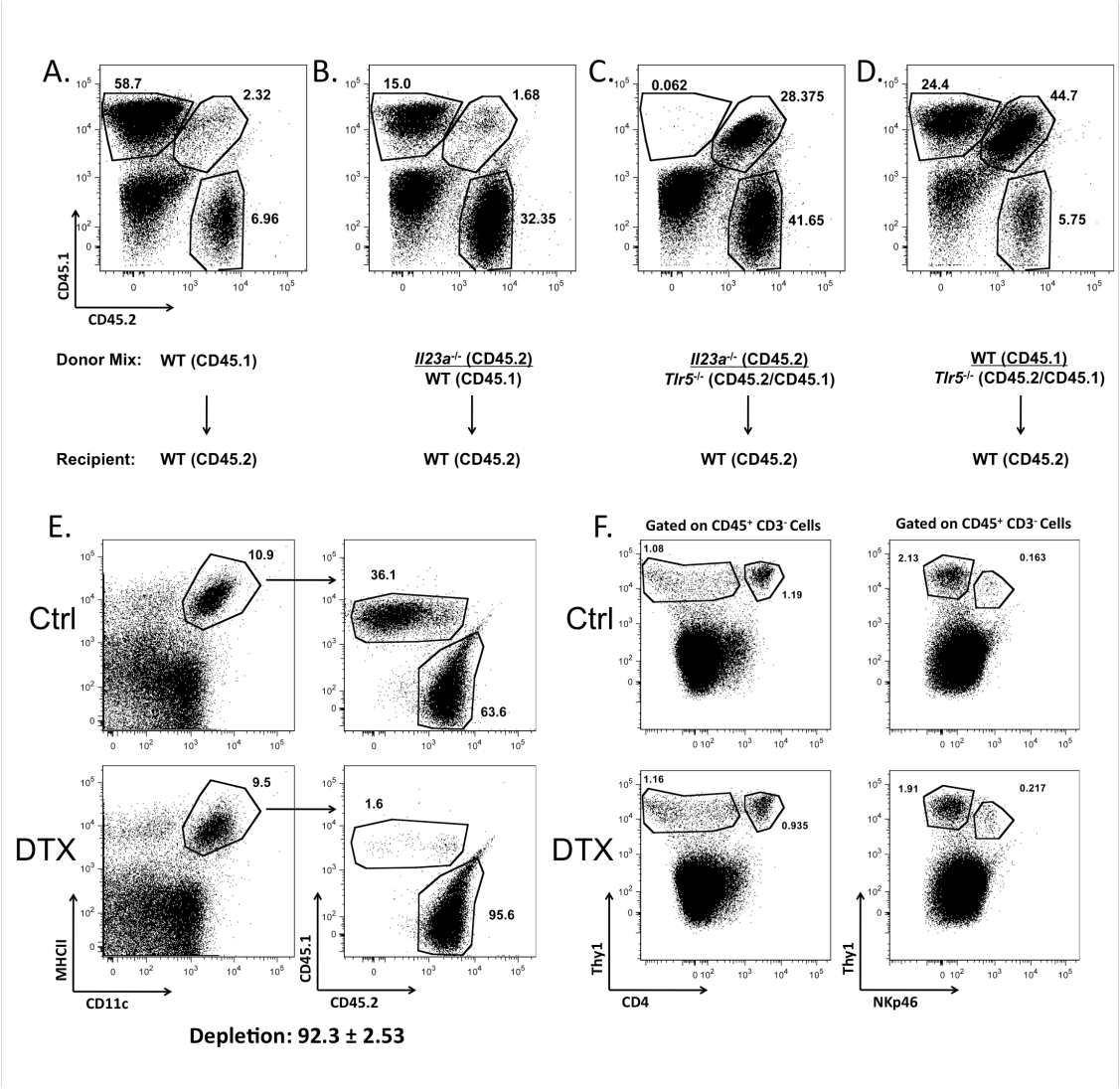

Figure S4. IL-23p19-deficient bone marrow cells efficiently reconstitute the hematopoietic compartment.

Equal mixtures (1:1) of *Il23a*$^{-/-}$(IL-23p19-deficient):WT, *Il23a*$^{-/-}$:*Tlr5*$^{-/-}$, *Tlr5*$^{-/-}$:WT, *Il23a*$^{-/-}$:CD11c.DTR, or WT:CD11c.DTR bone marrow cells were used to reconstitute lethally irradiated wild-type mice. Reconstitution was assessed 7 weeks following BM transplant. Lamina propria cells were isolated from the small intestine and analyzed by flow cytometry.

(A – D) *Tlr5*$^{-/-}$ BM-derived cells were distinguished by CD45.1 and CD45.2 double-positive staining. WT BM-derived cells were identified by CD45.1^{+} staining, and *Il23a*$^{-/-}$ BM-derived cells were identified by CD45.2^{+} staining. (A) Roughly 6% of CD45^{+} cells in the lamina propria are radioresistant after lethal irradiation and bone marrow reconstitution; however, these cells do not contribute to flagellin-mediated IL-22 induction since lethally irradiated WT mice reconstituted with *Tlr5*$^{-/-}$ BM fail to respond to flagellin (shown in Figure 1E). Numbers on dot plots are the percentage of gated cells out of CD45^{+} cells.

(E) Following treatment with diphtheria toxin (DTX) as described in Methods, the composition of CD11c^{+} MHCII^{+} dendritic cells in the lamina propria of mice reconstituted with equal parts *Il23a*$^{-/-}$ BM-derived cells and CD11c.DTR BM-derived cells was assessed by CD45.1 (CD11c.DTR) and CD45.2 (*Il23a*$^{-/-}$) congenic markers.

(F) Frequency of innate immune cells, gated by CD45^{+} CD3ε^{-}, after DTX treatment. Numbers on dot plots are the percentage of gated cells out of CD45^{+} cells. Data are representative of 2 independent experiments (n = 2 – 4 mice per group).

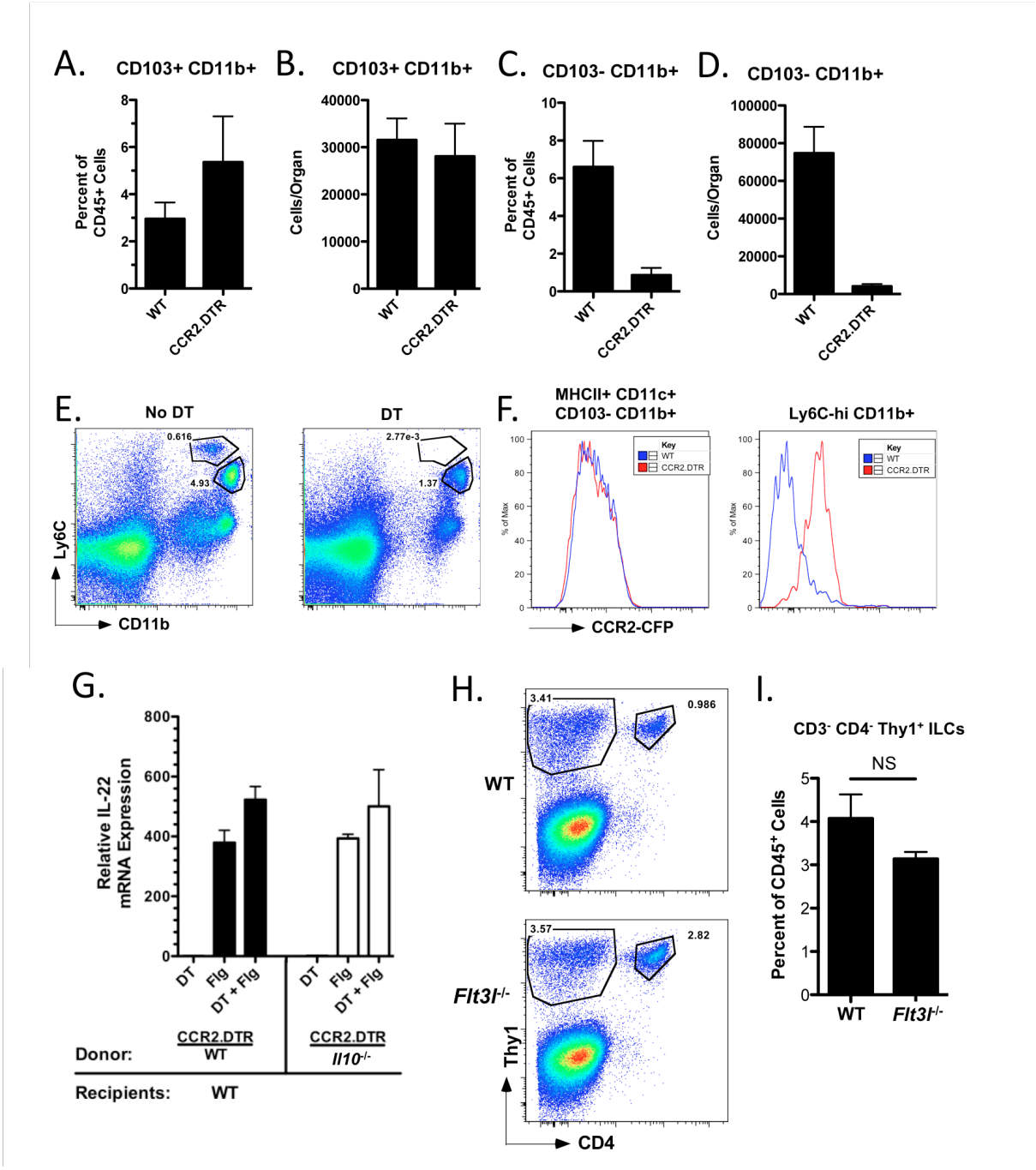

Figure S5. Enhanced flagellin-mediated IL-23 induction in monocyte-depleted mice is not due to proliferation of CD103+ CD11b+ LPDCs and loss of flagellin-mediated IL-22 expression in Flt3L-deficient mice is not due to impaired development of innate lymphoid cells

(A – F) Lethally irradiated WT mice were reconstituted with CCR2.DTR or WT bone marrow. Following a reconstitution period, mice received 3 doses of DTX administered every other day. One day after the last dose of DTX, mice were sacrificed. Lamina propria cells were purified and analyzed by flow cytometry to assess the degree of depletion of dendritic cell subsets. Dendritic cells were gated by positive staining of MHCII and CD11c surface markers. Frequencies of $CD103^+$ $CD11b^+$ (A) and $CD103^-$ $CD11b^+$ (C) DCs are expressed as percentage out of $CD45^+$ cells. Total cell counts of $CD103^+$ $CD11b^+$ (B) and $CD103^-$ $CD11b^+$ (D) DCs in the small intestine lamina propria are shown. Depletion of $Ly6C^{hi}$ monocytes is shown in dot plots (E), which are pre-gated on $CD45^+$ cells. CCR2.DTR mice were trangenically alter to express CFP under control of the CCR2 promoter. CCR2 driven CFP expression (F) is detected in $Ly6C^{hi}$ monocytes but not $CD103^-$ $CD11b^+$ monocyte-derived DCs.

(G) Mixed BM chimeric mice were generated by combining CCR2.DTR BM cells and WT BM cells or CCR2.DTR BM cells and *Il10*$^{-/-}$ BM cells in a 1:1 ratio and transferring the mixture into lethally irradiated mice. After the reconstitution period, mice received 1 μg of flagellin by i.v. injection and were sacrificed 2 hours later. Graph shows fold induction relative to CCR2.DTR:WT mixed BM chimeric mice that only received DTX (n = 2 – 3 mice per group). Error bars denote ± s.e.m.

(H-I) Small intestine lamina propria cells were harvested from wild-type and *Flt3l*$^{-/-}$ mice and analyzed by flow cytometry. (H) Cells were gated on $CD45^+$ Lin^- (CD11b, CD11c, B220) $CD3\varepsilon^-$ cells. No significant differences were observed between frequency of $CD3\varepsilon^-$ $Thy1^+$ $CD4^-$ innate lymphoid cells among total $CD45^+$ cells (I) in WT and *Flt3l*$^{-/-}$ mice (*n* = 2 – 3 mice per group). Error bars denote ± s.e.m.

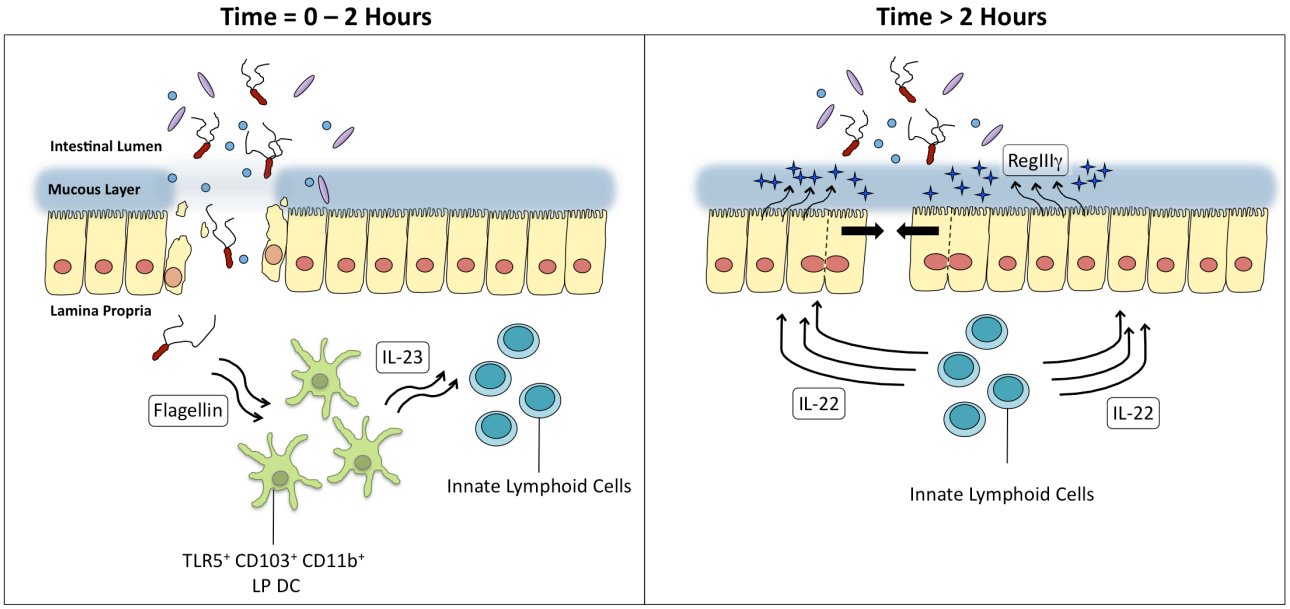

Figure S6. Model for TLR5-Mediated RegIIIγ Expression

When the intestinal mucosal barrier is compromised, microbes are able to cross the damaged epithelium and enter the intestinal lamina propria. $CD103^+$ $CD11b^+$ lamina propria dendritic cells (LPDC) detect the presence of flagellated bacteria via activation of the TLR5 receptor expressed on the surface of these dendritic cells. Within 1 hour, TLR5 signaling induces $CD103^+$ $CD11b^+$ LPDCs to secrete IL-23. $CD3\varepsilon^-$ $CD127^+$ innate lymphoid cells (ILCs) that reside in the lamina propria express the IL-23R and thus, immediately respond to IL-23 by secreting IL-22 (Van Maele et al., 2010). Three hours after the initial detection of microbes in the lamina propria, intestinal epithelial cells begin to express RegIIIγ in response to stimulation with IL-22. RegIIIγ is secreted into the mucous layer to form a protective barrier in which Gram-positive bacteria are killed. IL-22 also activates STAT3-mediated epithelial cell proliferation and protection against apoptosis, which serves to repair damage to the intestinal epithelial layer (Pickert et al., 2009). Together IL-22-induced antimicrobial protein secretion and wound healing help to restore the intestinal mucosal barrier injured during infection or trauma.

Reg3g	Mm00441127_m1
Il22	Mm00444241_m1
Il1b	Mm01336189_m1
Il6	Mm00446190_m1
Il23a	Mm00518984_m1
Il12b	Mm00434174_m1
Il12a	Mm00434165_m1
Tnfa	Mm00443258_m1
Hprt1	Mm00446968_m1

Table S1. Taqman Expression Assay (Applied Biosystems)

Pickert, G., Neufert, C., Leppkes, M., Zheng, Y., Wittkopf, N., Warntjen, M., Lehr, H.-A., Hirth, S., Weigmann, B., Wirtz, S., *et al*. (2009). STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. J. Exp. Med. *206*, 1465-1472.

Van Maele, L., Carnoy, C., Cayet, D., Songhet, P., Dumoutier, L., Ferrero, I., Janot, L., Erard, F., Bertout, J., Leger, H., *et al*. (2010). TLR5 signaling stimulates the innate production of IL-17 and IL-22 by CD3(neg)CD127+ immune cells in spleen and mucosa. J. Immunol. *185*, 1177-1185.