Immunity
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The Aryl Hydrocarbon Receptor Regulates Gut Immunity through Modulation of Innate Lymphoid Cells

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DOI 10.1016/j.immuni.2011.11.011

SUMMARY

Innate lymphoid cells (ILCs) expressing the nuclear receptor RORγt are essential for gut immunity presumably through production of interleukin-22 (IL-22). The molecular mechanism underlying the development of RORγt$^+$ ILCs is poorly understood. Here, we have shown that the aryl hydrocarbon receptor (Ahr) plays an essential role in RORγt$^+$ ILC maintenance and function. Expression of Ahr in the hematopoietic compartment was important for accumulation of adult but not fetal intestinal RORγt$^+$ ILCs. Without Ahr, RORγt$^+$ ILCs had increased apoptosis and less production of IL-22. RORγt interacted with Ahr and promoted Ahr binding at the *Il22* locus. Upon IL-23 stimulation, Ahr-deficient RORγt$^+$ ILCs had reduced IL-22 expression, consistent with downregulation of IL-23R in those cells. Ahr-deficient mice succumbed to *Citrobacter rodentium* infection, whereas ectopic expression of IL-22 protected animals from early mortality. Our data uncover a previously unrecognized physiological role for Ahr in promoting innate gut immunity by regulating RORγt$^+$ ILCs.

INTRODUCTION

The aryl hydrocarbon receptor (Ahr) is a ligand-dependent transcription factor. Upon ligand binding, Ahr translocates into the nucleus and dimerizes with its partner, the aryl hydrocarbon receptor nuclear translocator (Arnt), to induce target gene transcription. Ahr is best known to mediate the effects of environmental toxins (e.g., dioxin), yet its physiological role and endogenous ligands remain elusive (Stevens et al., 2009). Ahr has been previously shown to be involved in T cell differentiation and function (Apetoh et al., 2010; Gandhi et al., 2010; Kimura et al., 2008; Quintana et al., 2008; Veldhoen et al., 2008). For example, Ahr activation enhances the differentiation of T helper 17 (Th17) cells, a subset of CD4$^+$ T cells that express the cytokines interleukin-17 (IL-17) (also known as IL-17A), IL-17F, and IL-22, thereby exacerbating in vivo Th17 cell-mediated autoimmunity (e.g., EAE) (Veldhoen et al., 2008).

IL-17, a signature cytokine secreted by Th17 cells, is generally considered to play a pathogenic role in autoimmunity (Korn et al., 2009). However, in the gut, IL-17 can be either detrimental or protective, presumably depending on the different mouse models of disease (O'Connor et al., 2010). IL-22, a member of the IL-10 family of cytokines, works exclusively on nonhematopoietic cells to exert its biological functions (Ouyang et al., 2011). IL-22 has been shown to induce the production of antimicrobial peptides and mucins by epithelial cells, thus playing an important role in maintaining mucosal immunity and integrity (Aujla et al., 2008; Sugimoto et al., 2008; Zheng et al., 2008).

Recent studies suggest that IL-22 can be produced not only by Th17 cells but also by certain innate immune cells in the gut (Colonna, 2009). Among them is a recently identified subset of nonconventional natural killer (NK) cells that are present in the intestinal lamina propria and that secrete large amounts of IL-22. These cells have cell surface expression of NKp46 and low amounts of NK1.1 and have been given different names (e.g., NK-22, NCR22, NKR-LTi, or NKp46$^+$ ILC) (Sawa et al., 2010; Spits and Di Santo, 2011). In this paper, we use the term NK-22 to denote these cells. Lineage marker-negative lymphoid tissue inducer (LTi) cells, originally described to promote lymphoid organogenesis, have also been found to secrete IL-22 and IL-17 in adult mice and humans (Crellin et al., 2010; Cupedo et al., 2009; Eberl et al., 2004; Mebius, 2003; Takatori et al., 2009). LTi cells in the gut represent a heterogeneous population of cells either with (LTi$_4$) or without (LTi$_0$) CD4 expression (Sawa et al., 2010). A common characteristic of the innate lymphoid cells (ILCs) (e.g., NK-22 and LTi cells) is the expression of RORγt, a transcription factor critical for the developmental programs of these cells. RORγt$^+$ ILCs are abundantly present in the intestinal lamina propria and produce IL-22 and/or IL-17 that are important for mucosal immunity against certain extracellular pathogens such as *Citrobacter rodentium* (Mangan et al., 2006; Ota et al., 2011; Tumanov et al., 2011; Wang et al., 2010; Zheng et al., 2008). Transferring wild-type LTi$_4$ cells has been recently shown to provide innate immunity to *C. rodentium* infection in mice, highlighting the crucial role of RORγt$^+$ ILCs in innate immune responses (Sonnenberg et al., 2011). Although it is clear that RORγt$^+$ ILCs play an important role in gut immunity (Colonna, 2009; Cua and Tato, 2010), transcriptional regulation of RORγt$^+$ ILCs remains to be determined.

Ahr is expressed in NK-22 cells and LTi cells (Colonna, 2009; Cua and Tato, 2010); however, there is little known about the

role of Ahr in the development and/or function of RORγt+ ILCs. In this report, we have discovered that Ahr regulates the accumulation of RORγt+ ILCs in the gut. RORγt+ ILCs had increased apoptosis and their number was markedly reduced in adult but not fetal intestines of Ahr-deficient mice. Ahr was also required for the function of RORγt+ ILCs, as shown by the fact that it regulated IL-22 expression. RORγt interacted with Ahr and facilitated the recruitment of Ahr to the *Il22* locus. *Il23r* expression in RORγt+ ILCs was downregulated in the absence of Ahr, consistent with the decreased production of IL-22 by Ahr-deficient RORγt+ ILCs upon stimulation with IL-23. Because of impaired maintenance and function of RORγt+ ILCs, Ahr-deficient mice succumbed to *C. rodentium* infection. Exogenous expression of IL-22 protected Ahr-deficient mice from infection. Thus, our data reveal a previously unknown protective function of Ahr in orchestrating the gut innate immunity.

RESULTS

RORγt+CD3− Non-T Cells Are a Major Source of Intestinal IL-22 that Is Regulated by Ahr

We examined cytokine expression in the intestinal lamina propria leukocytes (LPLs) isolated from Ahr-deficient ($Ahr^{-/-}$) mice and their littermate control wild-type ($Ahr^{+/+}$) mice. In $Ahr^{+/+}$ mice, innate-like lymphocytes (ILCs) (CD3 negative) produced more IL-22 on a per-cell basis compared to CD3+ cells (Figure 1A). Furthermore, IL-22 was mostly produced by RORγt+ cells, consistent with the important role of RORγt in regulating IL-22 expression (Ouyang et al., 2011). Compared to those in $Ahr^{+/+}$ mice, intestinal LPLs in $Ahr^{-/-}$ mice had markedly decreased expression of IL-22, especially CD3− non-T cells, indicating that Ahr is important for gut IL-22 expression (Figure 1A). Although IL-17 production by CD3+ T cells is not dependent on Ahr, a reduction of IL-17 produced by CD3− intestinal LPLs in $Ahr^{-/-}$ mice was consistently observed (Figure 1B). Of note, wild-type CD3+ T cells in the small intestine produce more IL-22 and IL-17 than those in the large intestine (Figures 1A and 1B), in agreement with the notion that Th17 cells are more abundant in the small intestine (Ivanov et al., 2006, 2009). A decrease in RORγt+CD3− cells in $Ahr^{-/-}$ mice (Figures 1A and 1B) prompted us to examine the direct regulation of cytokine expression by Ahr. We gated on RORγt+ cells in the intestines and examined IL-22 and IL-17 production by intracellular staining. IL-22 expression was consistently decreased in RORγt+ cells in the absence of Ahr, indicating that Ahr may directly regulate IL-22 production by RORγt+ cells in the gut (Figure 1C). In contrast, the absence of Ahr had less effect on IL-17 expression when gating on RORγt+ cells, suggesting that the reduction of IL-17 expression by CD3− cells in $Ahr^{-/-}$ mice is mainly due to fewer RORγt+ cells (Figure 1D). These data indicate that RORγt+ ILCs in the gut are main producers of IL-22 and are sensitive to genetic loss of Ahr. We therefore focus hereafter on the role of Ahr in RORγt+ ILCs.

Ahr Deficiency Leads to Decreased Number of RORγt+ ILCs in the Gut

To examine RORγt expression in different LPL populations, we generated $Rorc^{gfp/+}Ahr^{-/-}$ and $Rorc^{gfp/+}Ahr^{+/+}$ littermate mice in which RORγt expression was marked by GFP (Eberl et al., 2004). We stained both the large and small intestinal LPLs with CD3, CD4, and NKp46 antibodies to examine RORγt+ ILCs (Figure 2A). Both the percentage and number of NK-22 cells ($CD3^-NKp46^+GFP(ROR\gamma t)^+$), LTi_4 cells ($CD3^-NKp46^-CD4^+GFP(ROR\gamma t)^+$), and LTi_0 cells ($CD3^-NKp46^-CD4^-GFP(ROR\gamma t)^+$) in the intestines were significantly decreased in $Ahr^{-/-}$ mice (Figures 2A–2C). When intracellular staining with RORγt-specific antibody was performed, there was a similar reduction of RORγt+ ILCs (i.e., NK-22, LTi_4, and LTi_0 cells) in $Ahr^{-/-}$ mice compared to $Ahr^{+/+}$ mice (data not shown). Together, these data suggest that Ahr is important for the accumulation of RORγt+ ILCs in the gut.

To determine the developmental stage at which Ahr is important for promoting RORγt+ ILCs, we next examined the effect of Ahr deletion on RORγt+ ILC populations during ontogeny. There were no $ROR\gamma t^+NKp46^+$ cells in E17.5 fetal intestines, consistent with the observation that NK-22 cells appear late after birth (Figure 2D; Figure S1 available online; Sawa et al., 2010), yet abundant LTi_4 and LTi_0 cells were found in the fetal intestines (Figure 2D). In contrast to the significant decrease of LTi cells in the LPLs of adult $Ahr^{-/-}$ mice, the frequency of LTi cells in the fetal intestines isolated from $Ahr^{-/-}$ embryos was similar to those from littermate control embryos, suggesting that Ahr is dispensable for the development of LTi cells in the fetal intestines (Figures 2D and 2E). A decrease in RORγt+ ILCs (NK-22, LTi_4, and LTi_0 cells) from $Ahr^{-/-}$ mice was not evident until the weaning age of week 3 (Figures S1A and S1B), suggesting that environmental stimuli (e.g., gut flora or food) contribute to the development of RORγt+ ILCs via the action of Ahr. Although the endogenous Ahr ligands remain elusive, 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan photoproduct, has been proposed to be a physiological agonist for Ahr (Nguyen and Bradfield, 2008). In vivo treatment of FICZ significantly increased the accumulation of RORγt+ ILCs in $Ahr^{+/-}$ or $Ahr^{+/+}$ mice but not in $Ahr^{-/-}$ mice, consistent with the important role of Ahr signaling in RORγt+ ILC development (Figures 2F and 2G).

Deficiency of Ahr in the Hematopoietic Compartment Affects the Development of RORγt+ ILCs in the Gut

Real-time reverse transcription PCR (RT-PCR) analysis indicated that similar to intestinal Th17 cells, RORγt+ ILCs expressed high amounts of Ahr (Figure S1C). However, because Ahr is broadly expressed by many cell types (Harper et al., 2006), it remained to be determined whether the decrease of RORγt+ ILCs in the gut of $Ahr^{-/-}$ mice was due to defects in hematopoietic cells or those in other cell types. It has been recently reported that intestinal RORγt+ ILCs are derived from hematopoietic precursor cells in the bone marrow (Sawa et al., 2010). Therefore, we developed chimeras by transferring bone marrow cells isolated from either $Rorc^{gfp/+}Ahr^{+/+}$ or $Rorc^{gfp/+}Ahr^{-/-}$ littermate mice into lethally irradiated wild-type C57BL/6 mice. Consistent with the published data (Sawa et al., 2010), bone marrow cells isolated from either $Rorc^{gfp/+}Ahr^{+/+}$ or $Rorc^{gfp/+}Ahr^{-/-}$ donor mice did not express GFP (data not shown). After bone marrow transfer, however, donor-derived RORγt+ cells (marked by GFP expression) were readily detectable in the intestinal lamina propria of C57BL/6 hosts (Figures 3A and 3B). Donor-derived NK-22, LTi_4, and LTi_0 cells were all

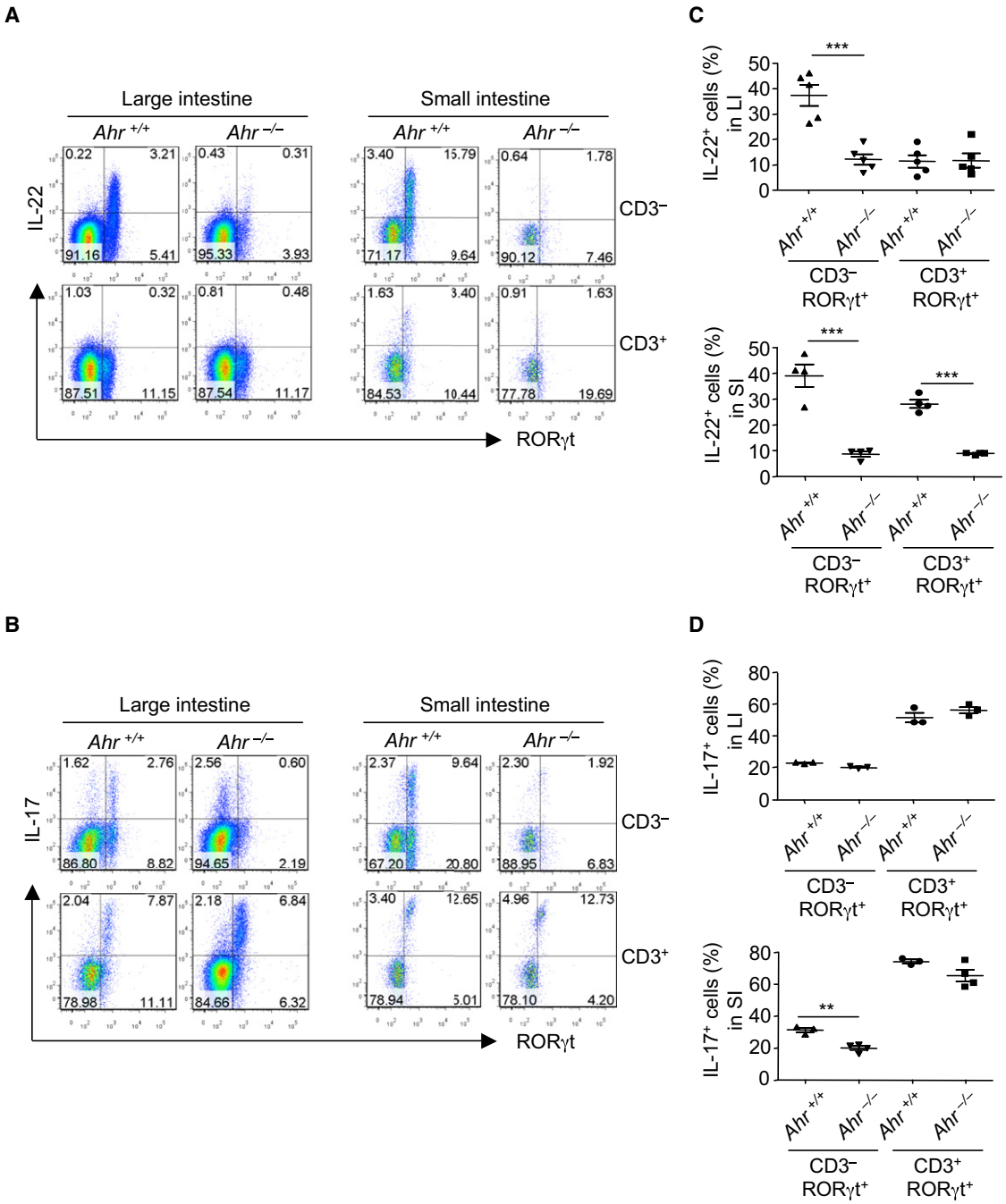


Figure 1. RORγt+ ILCs Are the Major Source of Intestinal IL-22 that Is Dependent on Ahr

Intestinal LPLs were stimulated by PMA and ionomycin for 4 hr.

(A and B) RORγt, IL-22, and IL-17 expression in $CD3^-$ or $CD3^+$ cells were analyzed by flow cytometry.

(C and D) Frequencies of IL-22- and IL-17-expressing cells in the $ROR\gamma t^+$ gated cell populations of the large (LI) and small (SI) intestines are shown. Horizontal lines represent the mean. $^{**}p < 0.01$; $^{***}p < 0.001$. Error bars represent SEM. Experiments were independently repeated at least three times.

decreased in the mice receiving $Rorc^{gfp/+}Ahr^{-/-}$ bone marrow cells compared to the mice receiving $Rorc^{gfp/+}Ahr^{+/+}$ bone marrow cells, whereas the number of donor-derived GFP(RORγt)+ $CD3^+$ T cells was less affected (Figures 3A and S2A). On the contrary, $Rorc^{gfp/+}Ahr^{+/+}$ bone marrow cells developed equally well into $ROR\gamma t^+$ ILCs and produced comparable amount of IL-22 when transferred into either $Ahr^{+/+}$ or $Ahr^{-/-}$ littermate hosts (Figures 3B and S2B and data not shown). Together, these data indicate that the impaired accumulation of $ROR\gamma t^+$ ILCs in the gut of $Ahr^{-/-}$ mice is due to defects caused by the deficiency of Ahr in the hematopoietic compartment.

Although Ahr was required for RORγt+ ILC development as examined with *Ahr*+/+ and *Ahr*−/− mice that were littermate controlled and cohoused, loss of Ahr expression in hematopoietic cells might affect the composition of gut microbiota. The difference in microbiota might in turn contribute to the developmental defect of ILCs in *Ahr*−/− mice. To address these possibilities, we performed bacterial 16S rRNA gene sequencing of fecal pellets collected from *Ahr*+/+ or *Ahr*−/− mice. Our data showed that the major composition of intestinal flora of *Ahr*−/− mice was similar to that of *Ahr*+/+ littermate mice (Figure S2C). We further examined the development of ILCs in *Ahr*−/− and *Ahr*+/+ littermate mice upon antibiotic treatment. As expected, IL-17 expression was reduced upon antibiotic treatment (Figure S2D). However, the defective accumulation of RORγt+ ILCs was evident in *Ahr*−/− mice with or without antibiotic treatment (Figure 3C). Together, these data suggest that the defect of RORγt+ ILCs in *Ahr*−/− mice is less likely due to perturbation of the gut flora.

To examine whether Ahr in other cells (e.g., T cells) indirectly affects the development of RORγt+ ILCs, we generated *Ahr*−/−*Rag1*−/− mice in which T and B cells are absent because of Rag deficiency. Lack of Ahr led to marked reduction of intestinal RORγt+ ILCs in *Ahr*−/−*Rag1*−/− mice, suggesting that Ahr plays a direct role in the development of RORγt+ ILCs independently of adaptive immune cells (e.g., Th17 cells) (Figure 3D).

Ahr Is Important for the Survival of Intestinal RORγt+ ILCs

To determine the mechanism by which Ahr regulates the development of RORγt+ ILCs in the gut, we stained LPLs with 7-AAD and Annexin V to examine apoptosis. In contrast to other cell populations in the gut, CD3−RORγt+ innate cells isolated from *Rorc*gfp/+*Ahr*−/− mice consistently showed higher Annexin V staining (Figures 4A and S3A), indicating that intestinal RORγt+ ILCs are more prone to apoptosis without Ahr. Consistently, the expression of antiapoptotic genes (e.g., *Bcl2* and *Bcl2l1*) was decreased in *Ahr*−/− ILCs as shown by real-time RT-PCR (Figure 4B). To determine whether the loss of RORγt+ ILCs in *Ahr*−/− mice is due to decreased cell expansion, we examined the proliferation of RORγt+ ILCs by Ki67 staining. RORγt+ ILCs from *Ahr*−/− mice displayed a similar rate of proliferation compared to wild-type cells (Figure S3B). Together, these data suggest that survival defect rather than impaired cell expansion accounts for the decrease of RORγt+ ILCs in *Ahr*−/− mice.

IL-7 signaling pathway has been shown to stabilize RORγt expression in ILCs and may be required for survival and maintenance of RORγt+ ILCs (Meier et al., 2007; Vonarbourg et al., 2010). Consistent with enhanced apoptosis of RORγt+ ILCs in the absence of Ahr, we observed a reduction of IL-7 in the large intestine of *Ahr*−/− mice (Figure S3C). IL-7R mRNA and protein (CD127) expression were also decreased in Ahr-deficient ILCs (Figures S3D and S3E). These data suggest that although engagement of IL-7R sustains RORγt+ ILCs in wild-type mice when IL-7 is generated in the presence of microflora (Vonarbourg et al., 2010), the defect in the maintenance of Ahr-deficient RORγt+ ILCs may be in part due to the compromised IL-7 and IL-7R expression.

Ahr Is Important for Production of IL-22 by RORγt+ ILCs under Steady-State Conditions

Fewer RORγt+ ILCs in *Ahr*−/− mice led to an overall reduction of IL-22 expression (Figure 1). To determine whether Ahr directly regulates IL-22 expression in each individual RORγt+ ILC population, we examined IL-22 protein in the remaining RORγt+ ILCs in *Ahr*−/− mice by intracellular staining (Figure 5A). Production of IL-22 by NK-22 cells, LTi_4 cells, or LTi_0 cells was impaired in *Ahr*−/− mice compared to *Ahr*+/+ mice (Figure 5A), suggesting that Ahr regulates the ability of those cells to produce IL-22. Intestinal CD3−NKp46+NK1.1lo cells but not CD3−NKp46+NK1.1hi cells contain NK-22 cells and produce large amounts of IL-22 that was also greatly reduced in *Ahr*−/− mice (Figures S4A and S4B), consistent with the role of Ahr in regulating IL-22 expression in NK-22 cells. Accordingly, the induction of RegIIIγ, an antimicrobial peptide produced by IECs and involved in mucosal homeostasis, was significantly reduced in *Ahr*−/− mice, in agreement with the role for IL-22 in the regulation of RegIIIγ expression (Figure S4C).

In contrast to Th17 cells in which Ahr promotes IL-17 expression (Kimura et al., 2008; Veldhoen et al., 2008), IL-17 produced by residual RORγt+ ILCs in the large intestine of *Ahr*−/− mice was not significantly changed upon phorbol myristate acetate (PMA) plus ionomycin activation, indicating that the expression of IL-17 in ILCs is less dependent on Ahr in the gut (Figure 5A). CCR6, a target gene of RORγt, is highly expressed by RORγt+ cells and presumably regulates the migration of these cells via CCL20 (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Bouskra et al., 2008; Hirota et al., 2007; Martin et al., 2009; Takatori et al., 2009). The absence of Ahr caused a slight but significant decrease of CCR6 expression by LTi_0 cells, suggesting a role for Ahr in modulation of CCR6 (Figure 5A). Nevertheless, the expression of CCL20, the ligand for CCR6, was normal in the intestinal epithelial cells (IECs) of *Ahr*−/− mice (Figure S4D). Consistent with the protein data (Figure 5A), marked reduction of IL-22 but not IL-17 mRNA was observed in Ahr-deficient RORγt+ cell populations freshly isolated from the gut under steady-state conditions (Figure 5B). Together, these data demonstrate that Ahr selectively regulates IL-22 expression in RORγt+ ILCs.

RORγt Interacts with Ahr and Enhances the Recruitment of Ahr to the *Il22* Locus

After searching for transcription factor binding sites at the *Il22* locus, we found multiple Ahr-responsive elements (AhRE) that are clustered with ROR-responsive elements (RORE), among which are those at the promoter and intron 1 of the *Il22* gene, suggesting that *Il22* may be a direct target gene of Ahr and/or RORγt (Figure 5C). To examine transcription factor binding at the *Il22* locus, we took advantage of EL4 cells expressing the double flag peptide (DTFC), double flag-tagged RORγt (DFTC-RORγt), or double flag-tagged constitutively active Ahr (DFTC-CA-Ahr). CA-Ahr lacks the PAS-B domain, enabling it to constitutively dimerize with Arnt, bind to DNA, and activate transcription in a ligand-independent manner (McGuire et al., 2001). A chromatin immunoprecipitation (ChIP) assay with flag antibodies

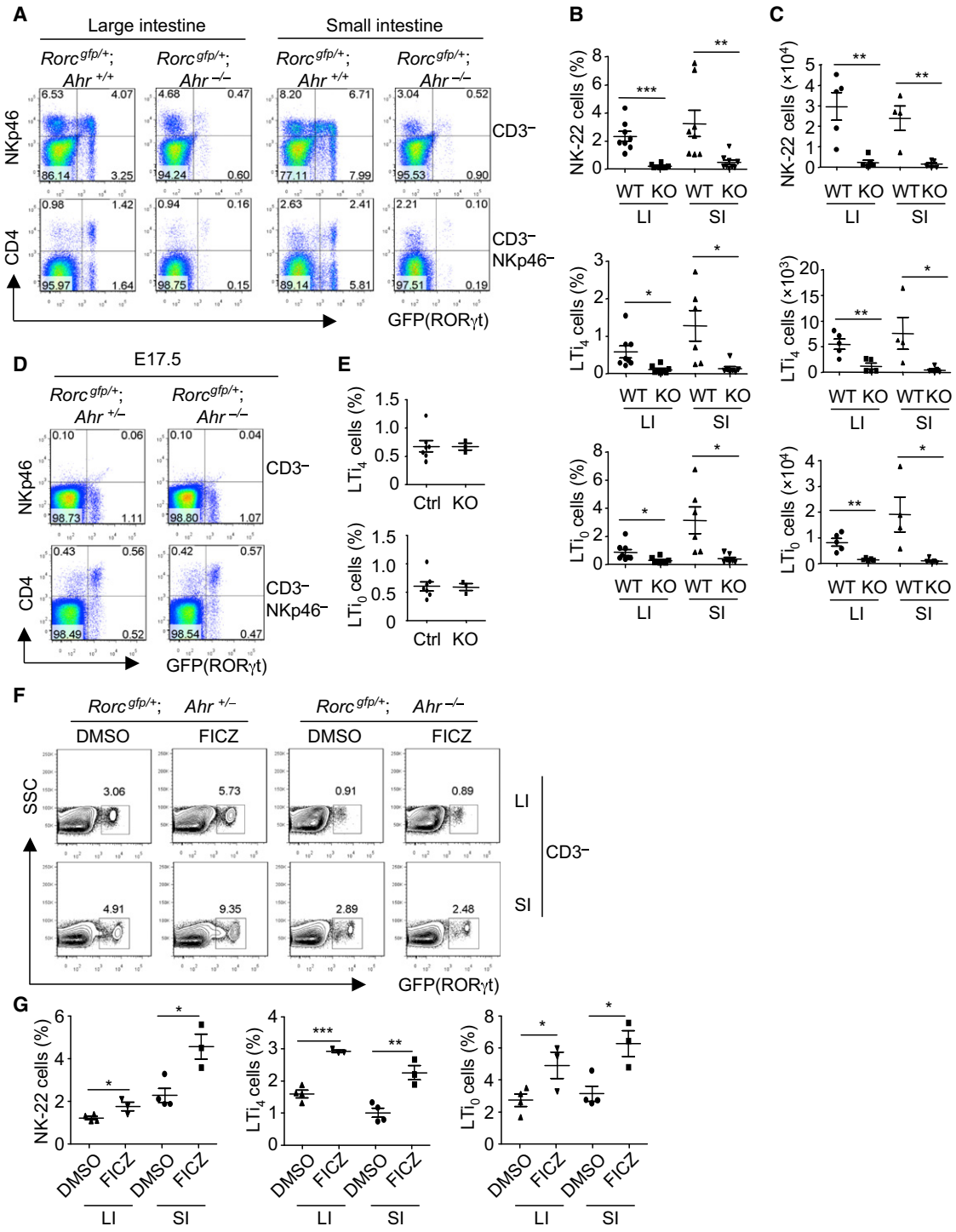


Figure 2. Ahr Is Important for the Accumulation of RORγt+ ILCs in the Gut

(A) GFP(RORγt) and NKp46 expression were analyzed in intestinal LPLs by flow cytometry after gating on CD3$^-$ cells. GFP(RORγt) and CD4 expression were analyzed after gating on CD3$^-$NKp46$^-$ cells. Data are representative of at least three independent experiments.

(B) Percentage of NK-22 cells (CD3$^-$NKp46$^+$GFP(RORγt)$^+$) in the CD3$^-$ population, as well as percentages of LTi$_4$ cells (CD3$^-$NK46$^-$CD4$^+$GFP(RORγt)$^+$) and LTi$_0$ cells (CD3$^-$NKp46$^-$CD4$^-$GFP(RORγt)$^+$) in the CD3$^-$NKp46$^-$ cell population of the large (LI) and small (SI) intestinal LPLs isolated from *Rorc*$^{gfp/+}$*Ahr*$^{+/+}$ (WT) and *Rorc*$^{gfp/+}$*Ahr*$^{-/-}$ (KO) mice are shown.

(C) Absolute numbers of NK-22, LTi$_4$, and LTi$_0$ cells in the LI and SI LPLs of WT and KO mice are shown.

(D) Intestinal LPLs were isolated from 17.5 day of embryos (E17.5) of the indicated genotypes. NK-22, LTi$_4$, and LTi$_0$ cells were analyzed by flow cytometry. Data are representative of two independent experiments.

showed enhanced binding of RORγt at the *Il22* locus in cell lines expressing DFTC-RORγt, suggesting that *Il22* is a direct target gene of RORγt (Figure 5D). In contrast, no consistent enhancement of Ahr binding at the *Il22* locus was detected by ChIP analysis with DFTC-CA-Ahr-expressing cell lines, whereas binding of Ahr at a known target gene (i.e., *Cyp1a1*) (Hankinson, 2005) could be readily detected (Figure 5E). The recruitment of CA-Ahr to the *Il22* locus but not the *Cyp1a1* locus was markedly enhanced when RORγt was coexpressed in DFTC-CA-Ahr-expressing cells, indicating that RORγt facilitates Ahr binding at the *Il22* locus (Figure 5E). By using a coimmunoprecipitation assay, we detected the interaction between Ahr or CA-Ahr and RORγt (Figure 5F). Furthermore, individual expression of Ahr or RORγt induced IL-22, but coexpression of Ahr and RORγt synergistically upregulated IL-22 mRNA (Figure 5G). Together, these data suggest that the cooperativity between Ahr and RORγt promotes IL-22 expression.

IL-23-Induced IL-22 and IL-17 Expression in RORγt+ ILCs Are Impaired in *Ahr*$^{-/-}$ Mice

Given that IL-23 is shown to promote the production of IL-22 by RORγt+ cells (Cella et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008), we sorted ILCs (CD3$^-$GFP(RORγt)$^+$) from *Ahr*$^{+/+}$ or *Ahr*$^{-/-}$ littermate mice and examined IL-22 expression by ELISA. Although Ahr-deficient RORγt+ ILCs appeared to be responsive to IL-23 stimulation, they had a marked reduction in IL-22 expression compared to wild-type cells upon IL-23 stimulation (Figure 6A), consistent with the intracellular staining data with total LPLs (Figure 6B). Although IL-17 production by RORγt+ ILCs under these culture conditions was low, the expression was augmented by in vitro activation with PMA plus ionomycin and further enhanced by IL-23 treatment. Similar to the reduction of IL-22, IL-17 production by Ahr-deficient ILCs was decreased upon IL-23 stimulation compared to that by wild-type ILCs (Figure S5). Together, these data suggest that Ahr is important for IL-23-stimulated IL-22 and IL-17 expression in RORγt+ ILCs. To determine whether IL-23 signaling is defective in the absence of Ahr, we sorted RORγt+ cells from the intestinal lamina propria and examined the expression of IL-23R by real-time RT-PCR. IL-23R expression was markedly reduced in NK-22 cells and LTi cells isolated from *Ahr*$^{-/-}$ mice, suggesting that Ahr regulates IL-23R expression in RORγt+ ILCs (Figure 6C). Recent data suggest that RORγt+ innate cells (e.g., LTi cells) in the spleen produce IL-22 (Sonnenberg et al., 2011; Takatori et al., 2009). Indeed, CD4$^+$CD11c$^-$ innate lymphoid cells in the spleen of *Rag1*$^{-/-}$ mice expressed IL-22 that was further enhanced by IL-23 treatment. IL-22 production by CD4$^+$CD11c$^-$ splenocytes was greatly reduced in *Rag1*$^{-/-}$;*Ahr*$^{-/-}$ mice (Figure 6D), suggesting that the production of IL-22 by ILCs outside the intestines is also dependent on Ahr.

Ahr Regulates Host Immunity to *C. rodentium* in an IL-22-Dependent Manner

Controlling *C. rodentium* is dependent on IL-23 signaling and requires early production of IL-22 in the host (Ota et al., 2011; Sonnenberg et al., 2011; Tumanov et al., 2011; Zheng et al., 2008). Our data showed that Ahr-deficient RORγt+ ILCs made less IL-22 upon IL-23 stimulation. Therefore, we used the model of *C. rodentium* infection to study the impact of Ahr on the host responses during inflammation. When infected with *C. rodentium*, *Ahr*$^{-/-}$ mice rapidly lost body weight and died by day 10 postinfection, whereas no weight loss or death was observed in the control mice (Figures 7A and 7B). Accordingly, on day 5, *Ahr*$^{-/-}$ mice had higher *C. rodentium* colony counts in their feces than did *Ahr*$^{+/+}$ littermate mice, indicating that Ahr is crucial for controlling the bacterial infection (Figure 7C). We next examined whether ectopic expression of IL-22 could rescue *Ahr*$^{-/-}$ mice from death. To this end, we infected both wild-type and *Ahr*$^{-/-}$ mice with *C. rodentium* and administered a plasmid expressing IL-22 or a control empty plasmid to animals by hydrodynamic injection. 60% of *Ahr*$^{-/-}$ mice receiving IL-22 expression plasmid via hydrodynamic injection survived infection beyond day 17 and had much less weight loss compared to *Ahr*$^{-/-}$ mice receiving control plasmid (Figures 7D and 7E). Both wild-type and *Ahr*$^{-/-}$ mice receiving IL-22 plasmid had reduced bacterial counts in their feces compared to mice receiving the control plasmid, consistent with the crucial role of IL-22 in controlling infection (Figure 7F). Collectively, these data demonstrate that impaired IL-22 production is the main reason for the high bacterial load and death of *Ahr*$^{-/-}$ mice during *C. rodentium* infection.

DISCUSSION

The development of RORγt+ innate lymphoid cells (e.g., LTi cells) is dependent on several transcription factors such as RORγt (Eberl et al., 2004), TOX (Aliahmad et al., 2010), Ikaros (Wang et al., 1996), and the E-protein inhibitor Id2 (Boos et al., 2007; Yokota et al., 1999). Unlike the aforementioned transcription factors, Ahr is unique in that it is not required for the development of fetal LTi cells, consistent with the normal development of secondary lymphoid organs (i.e., lymph nodes) and tertiary lymphoid aggregates (e.g., Peyer's patches) in the intestines of *Ahr*$^{-/-}$ mice (data not shown). Instead, adult *Ahr*$^{-/-}$ mice have markedly reduced numbers of RORγt+ ILCs in both the small and the large intestines.

Although the development of Th17 cells in the small intestine can be promoted by specific commensal gut flora (e.g., segmented filamentous bacteria) (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009), it is still under debate whether the development of NK-22 cells requires intestinal symbionts (Reynders et al., 2011; Sanos et al., 2009; Satoh-Takayama et al., 2008; Sawa et al., 2010; Vonarbourg et al., 2010). It has

(E) Percentages of LTi_4 cells and LTi_0 cells isolated from the E17.5 intestines of *Rorc*$^{gfp/+}$*Ahr*$^{+/-}$ (Ctrl) or *Rorc*$^{gfp/+}$*Ahr*$^{-/-}$ (KO) mice are shown.
(F) RORγt+ cells in CD3$^-$ population of LI and SI LPLs isolated from littermate mice of the indicated genotypes after DMSO or FICZ injection were analyzed by flow cytometry. Data are representative of two independent experiments.
(G) Percentages of NK-22, LTi_4, and LTi_0 cells from LPLs isolated from *Rorc*$^{gfp/+}$*Ahr*$^{+/+}$ or *Rorc*$^{gfp/+}$*Ahr*$^{+/-}$ mice after DMSO or FICZ injection are shown.
(B, C, E, and G) Horizontal lines show the mean. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Error bars represent SEM.
See also Figure S1.

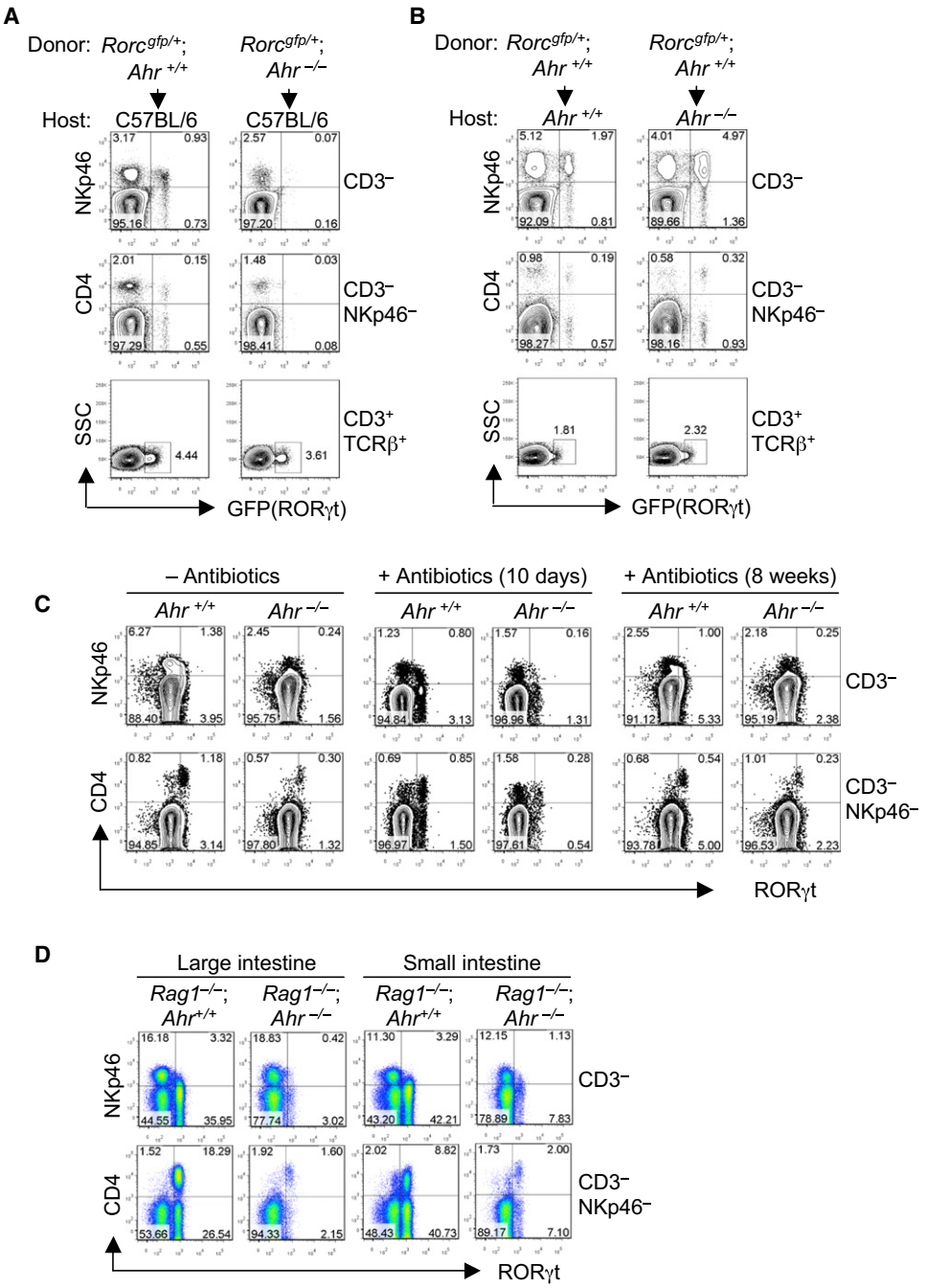


Figure 3. Reduction of RORγt+ ILCs in *Ahr*−/− Mice Is Attributed to a Hematopoietic Defect

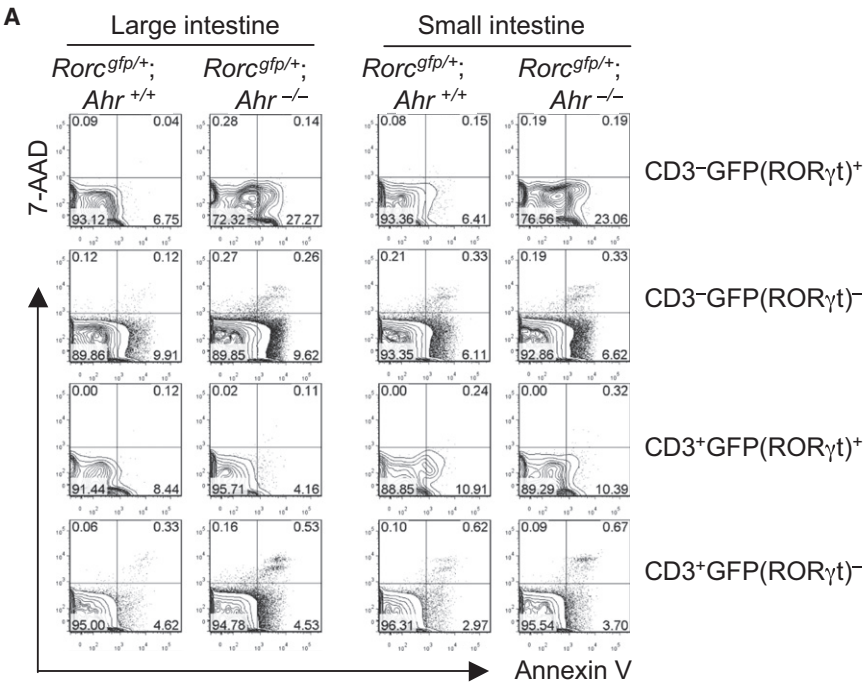
(A and B) Bone marrow cells from mice of the indicated genotypes were transferred into lethally irradiated recipient mice. NK-22 ($CD3^-NKp46^+GFP(ROR\gamma t)^+$), LTi_4 ($CD3^-NK46^-CD4^+GFP(ROR\gamma t)^+$), LTi_0 ($CD3^-NKp46^-CD4^-GFP(ROR\gamma t)^+$), and RORγt+ T ($CD3^+TCR\text{-}\beta^+GFP(ROR\gamma t)^+$) cells were analyzed by flow cytometry. Data are representative of three independent experiments.

(C) Large intestinal LPLs were isolated from mice untreated or treated with antibiotics for 10 days or 8 weeks. Percentages of NK-22, LTi_4, and LTi_0 cells were analyzed by flow cytometry. Data are representative of two experiments.

(D) Percentages of NK-22, LTi_4, and LTi_0 cells were analyzed by flow cytometry. Data are representative of three independent experiments.

See also Figure S2.

been recently reported that the development of at least some RORγt+ ILCs is independent of gut microbiota, because germ-free mice or mice treated with antibiotics after birth have normal development of RORγt+ LTi cells (Sawa et al., 2010). The absence of Ahr might cause changes in the gut microbiota, which in turn could influence the development of RORγt+ ILCs



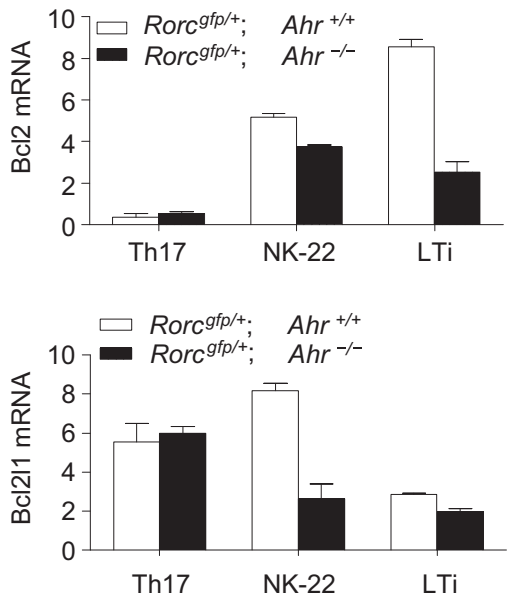


Figure 4. Ahr-Deficient RORγt+ ILCs Have Enhanced Apoptosis

(A) Intestinal LPLs were examined by flow cytometry for CD3, GFP, and apoptosis markers (Annexin V and 7-AAD). Data are representative of three independent experiments.

(B) Th17 ($CD3^+TCR\beta^+CD4^+GFP(ROR\gamma t)^+$), NK-22 ($CD3^-TCR\beta^-NKp46^+GFP(ROR\gamma t)^+$), and LTi ($CD3^-TCR\beta^-NKp46^-GFP(ROR\gamma t)^+$) cells were sorted from large intestinal LPLs by flow cytometry. Bcl2 and Bcl2l1 mRNA expression in each cell population were analyzed by real-time RT-PCR. RNA samples were pooled from three to four mice in each experiment. Data are representative of two independent experiments. Error bars represent SEM of triplicate samples of real-time RT-PCR.

See also Figure S3.

in a cell-extrinsic manner. However, we discovered that the development of RORγt+ ILCs was impaired when Ahr-deficient bone marrow cells were transferred to wild-type mice, whereas transfer of wild-type bone marrow cells completely restored the number of RORγt+ ILCs in the gut of *Ahr*$^{-/-}$ mice. Fecal bacterial 16S rRNA gene analysis further revealed no major difference in bacterial composition between wild-type and *Ahr*$^{-/-}$ mice. These data argue that Ahr regulates intestinal RORγt+ ILCs in a cell-autonomous manner and that cell-extrinsic effects (e.g., the potential differences in gut flora between *Ahr*$^{-/-}$ mice and wild-type mice) are less likely to contribute to the reduction of RORγt+ ILCs in the gut of *Ahr*$^{-/-}$ mice. Of note, the decrease of RORγt+ ILCs in *Ahr*$^{-/-}$ mice became evident when mice were close to weaning age, suggesting that the colonization of gut microbiota may regulate the function of Ahr. It is tempting to speculate that endogenous ligands generated by gut-resident bacteria activate Ahr and promote or sustain RORγt+ ILC development in wild-type mice but fail to do so in *Ahr*$^{-/-}$ mice. Indeed, it has been shown that certain bacteria in the gut generate metabolites from the amino acid tryptophan to activate Ahr in vitro (Perdew and Babbs, 1991). Consistently, RORγt+ ILCs were increased in wild-type mice but not in *Ahr*$^{-/-}$ mice upon exogenous agonist FICZ administration. However, ligands generated independently of gut flora by metabolizing food product(s) and/or other nondietary components (Nguyen and Bradfield, 2008; Nguyen et al., 2009) may also activate Ahr to promote ILC development in vivo. Consistent with this hypothesis, the defective accumulation of RORγt+ ILCs was still apparent in *Ahr*$^{-/-}$ mice upon antibiotic treatment. Thus, a definitive answer awaits generation of germ-free *Ahr*$^{-/-}$ mice, detailed characterization of gut flora, and identification and characterization of Ahr ligand(s).

CCL20, a chemokine produced by IECs, controls the migration of cells expressing CCR6, a homing receptor important for RORγt+ ILC migration to certain intestinal microenvironments (Cook et al., 2000; Tanaka et al., 1999). Our data showing relatively normal expression of CCL20 and CCR6 makes it unlikely

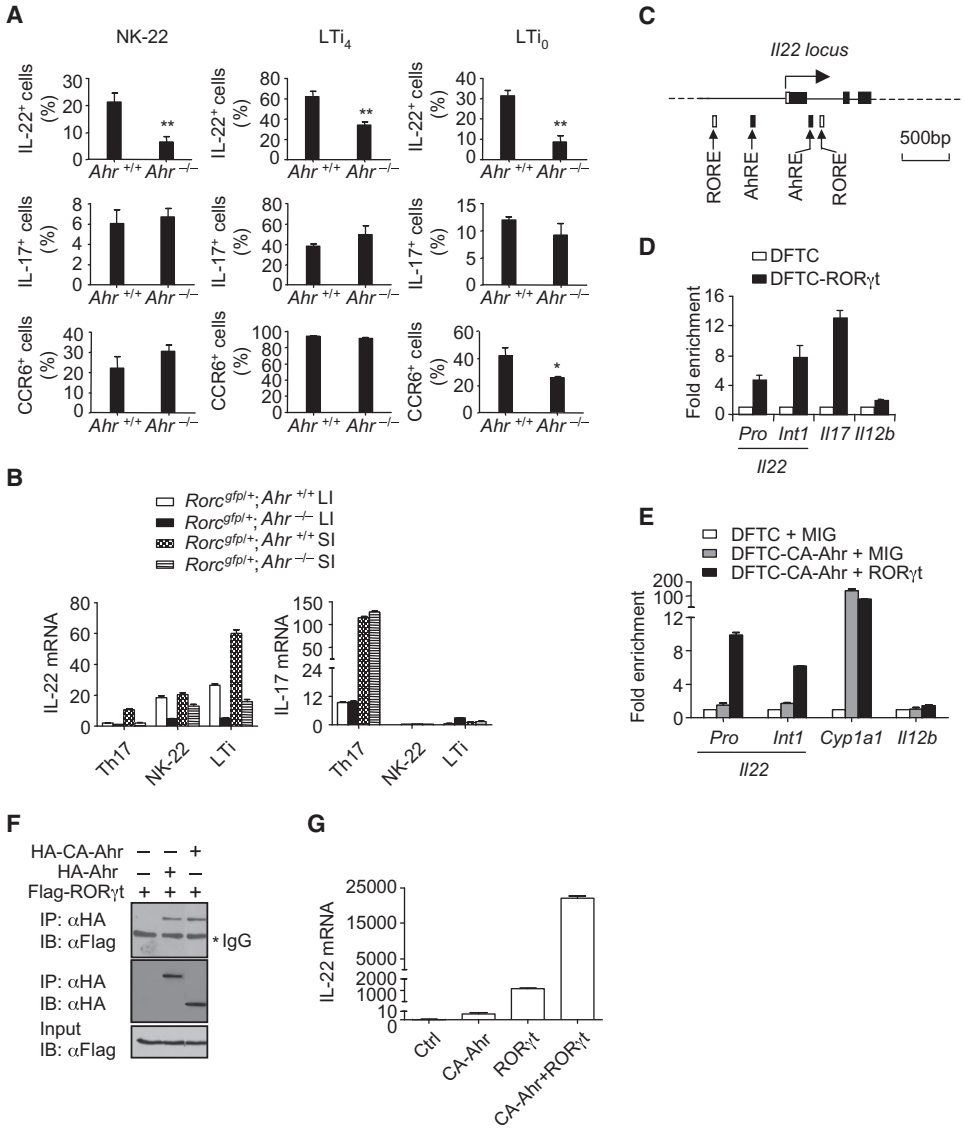


Figure 5. Ahr Regulates the Function of NK-22 and LTi Cells to Produce IL-22

(A) IL-22, IL-17, and CCR6 expression in large intestinal NK-22, LTi_4, and LTi_0 cells were analyzed by flow cytometry. For IL-22 and IL-17 staining, cells were stimulated for 4 hr with PMA and ionomycin. Data were derived from three to four pairs of littermate mice. *$p < 0.05$; **$p < 0.01$. Error bars represent SEM. Experiments were repeated at least three times.

(B) Th17 ($CD3^+TCR\beta^+CD4^+GFP(ROR\gamma t)^+$), NK-22 ($CD3^-TCR\beta^-NKp46^+GFP(ROR\gamma t)^+$), and LTi ($CD3^-TCR\beta^-NKp46^-GFP(ROR\gamma t)^+$) cells were sorted from LI and SI LPLs by flow cytometry. IL-22 and IL-17 mRNA expression in each cell population were directly analyzed by real-time RT-PCR. RNA samples were pooled from three to four mice in each experiment. Data are representative of two independent experiments. Error bars represent SEM of triplicate samples of real-time RT-PCR.

(C) AhREs and ROREs at the *Il22* locus are shown.

(D) RORγt binding at the *Il22* locus in EL4 stable cell lines expressing either flag peptide (DFTC) or flag-tagged RORγt (DFTC-RORγt) was monitored by flag ChIP assay. The fold enrichment of RORγt binding at each locus was normalized to DFTC-empty EL4 cells. The *Il12b* and *Il17* loci were used as negative and positive controls, respectively. Error bars represent SEM of triplicate samples of real-time PCR. Data are representative of three independent experiments.

(E) Empty MIG (MIG) or RORγt-MIG were coexpressed by retroviral transduction in EL4 cell lines stably expressing either flag peptide (DFTC) or flag-tagged CA-Ahr (DFTC-CA-Ahr). Ahr binding at the *Il22* locus was monitored by flag ChIP assay. Data are representative of three independent experiments.

(F) Ahr physically interacted with RORγt. HEK293T cells were transiently transfected with the indicated expression constructs. Whole cell extracts were made and subjected to anti-HA immunoprecipitation and subsequently immunoblotted with flag or HA antibodies. Data are representative of three independent experiments.

(G) EL4 cells were transduced with retroviruses encoding RORγt and/or CA-Ahr. IL-22 mRNA expression was analyzed by real-time RT-PCR. Data are representative of two independent experiments. Error bars represent SEM of triplicate samples of real-time RT-PCR.

See also Figure S4.

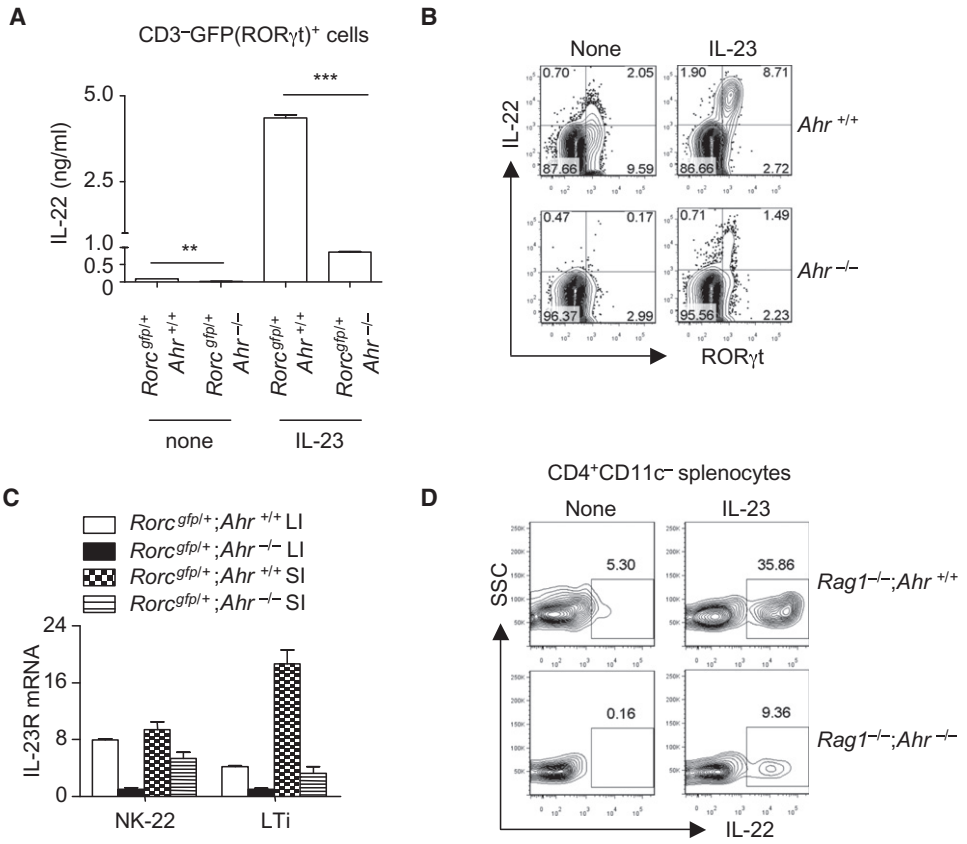


Figure 6. IL-23-Induced IL-22 Expression Is Impaired in Ahr-Deficient Mice

(A) CD3$^-$GFP(RORγt)$^+$ cells were sorted from large intestinal LPLs by flow cytometry. Equal numbers of cells (1×10^4) were stimulated with or without IL-23 for 24 hr without restimulation by PMA and ionomycin. The concentration of IL-22 in the supernatant was examined by ELISA. Error bars represented SEM of triplicate samples. Data are representative of two independent experiments.

(B) Large intestinal LPLs were stimulated with or without IL-23 overnight without restimulation by PMA and ionomycin. Expression of IL-22 and RORγt in CD3$^-$ cells were analyzed by flow cytometry. Data are representative of three independent experiments.

(C) IL-23R mRNA expression in flow cytometry-sorted NK-22 (CD3$^-$TCRβ^-NKp46$^+$GFP(RORγt)$^+$) and LTi (CD3$^-$TCRβ^-NKp46$^-$GFP(RORγt)$^+$) cells from LI and SI LPLs was directly analyzed by real-time RT-PCR. RNA samples were pooled from three to four mice for each genotype. Data are representative of two independent experiments.

(D) Splenocytes of the indicate genotypes were stimulated with or without IL-23 overnight without restimulation by PMA and ionomycin. Expression of IL-22 in CD4$^+$CD11c$^-$ cells was analyzed by flow cytometry. Data are representative of two independent experiments.

See also Figure S5.

that a migrational defect of RORγt$^+$ ILCs to the lamina propria could account for the reduction of intestinal RORγt$^+$ ILCs in *Ahr*$^{-/-}$ mice. Consistently, it has been reported that CCR6 is not required for the development of RORγt$^+$ ILCs but is important for the bacteria-induced generation of isolated lymphoid follicles (ILFs) from cryptopatches, where most of the RORγt$^+$ ILCs are clustered (e.g., LTi cells) (Bouskra et al., 2008).

Although Ahr has been shown to be required for IL-22 expression, it was unknown whether the regulation is through a transcriptional mechanism. Our data suggested that binding of Ahr at the *Il22* locus appeared to be weak without the aid of RORγt. The recruitment of Ahr to the *Il22* locus was markedly enhanced by coexpression of RORγt, underscoring the essential function of RORγt for the Ahr-directed IL-22 gene expression. Our data further suggested that the cooperativity between Ahr and RORγt to promote *Il22* transcription may be mediated through protein-protein interaction. Facilitated by RORγt, Ahr may achieve enhanced DNA binding activity, whereby directly binding to the AhREs at the *Il22* locus induces transcription. Alternatively, Ahr may indirectly bind to ROREs through interaction with RORγt. The detailed molecular mechanism by which Ahr orchestrates with RORγt to regulate IL-22 expression in RORγt$^+$ ILCs remains to be determined. IL-23 signaling is important for the function of ILCs during colitis in mice and in humans (Buonocore et al., 2010; Geremia et al., 2011), presumably when large amounts of IL-23 are produced by antigen-presenting cells because of activation of pattern recognition receptors. Ahr-deficient RORγt$^+$ ILCs had decreased expression of IL-23R and produced less IL-22 upon IL-23 stimulation. Thus, regulation of *Il23r* transcription by Ahr in RORγt$^+$ ILCs may have an important implication in IL-22 expression during inflammation.

Our data showing Ahr deficiency had a greater in vivo impact on gut ILCs than Th17 cells suggested that Ahr played a more important role in intestinal RORγt$^+$ ILC maintenance or survival. In

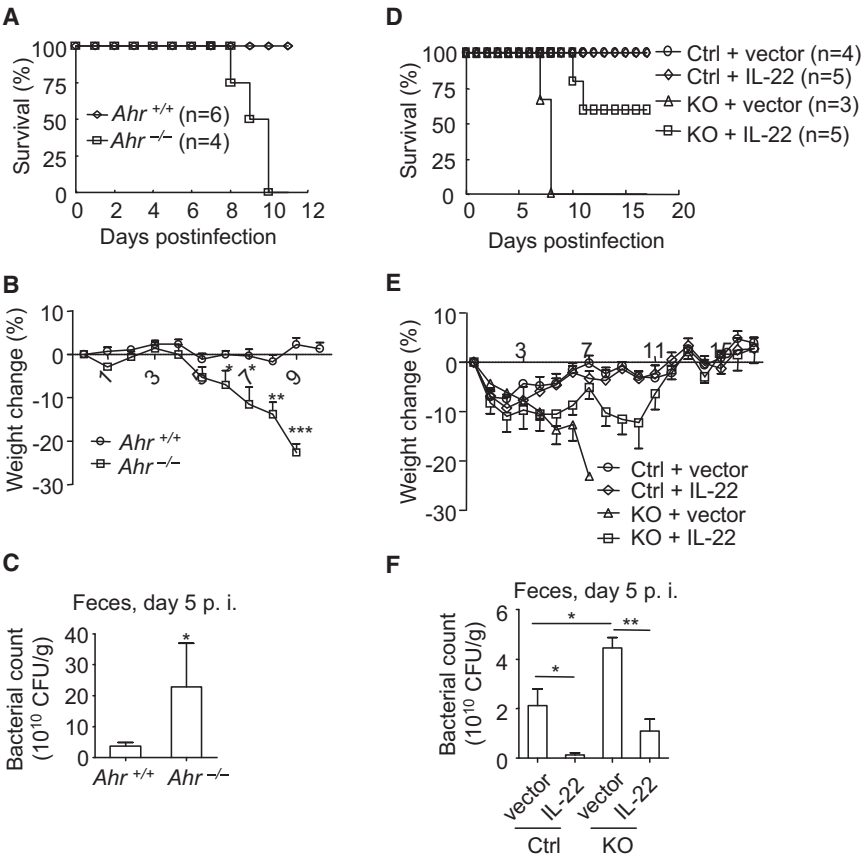


Figure 7. Ectopic Expression of IL-22 Protects *Ahr*$^{-/-}$ Mice from *C. rodentium* Infection

(A–C) Littermate mice of the indicated genotypes were infected with *C. rodentium*.

(D–F) *Ahr*$^{-/-}$ (KO) mice and *Ahr*$^{+/-}$ or *Ahr*$^{+/+}$ (Ctrl) littermate mice were infected with *C. rodentium*. Six hours after infection, IL-22-expressing plasmid (pRK-mIL-22, Genentech) or control vector (pRK) was administered into the mice via hydrodynamic injection.

(A and D) Survival rates were monitored at the indicated time points.

(B and E) Body mass was monitored at the indicated time points. Results are shown as mean percentage of body weight change ± SEM. Data were pooled from three to six mice for each genotype (B) or for each treatment group (E). Mice were excluded from analyses after the time of death.

(C and F) Colony-forming unit (CFU) counts of *C. rodentium* in the fecal pellets on day 5 after infection were shown. Data were pooled from three to six mice for each genotype (C) or for each treatment group (F). Statistical analyses were performed by Mann-Whitney test (C).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Error bars represent SEM. Data are representative of two independent experiments.

accordance with the notion, although Ahr is required for IL-22 production by in-vitro-differentiated Th17 cells in the presence of Ahr ligand, it is not required for the expression of IL-17, a hallmark cytokine for Th17 cells (Veldhoen et al., 2008). Additional T cell-restricted factors may thus compensate for the loss of Ahr to drive RORγt-directed Th17 cell differentiation. It has been shown that inactivation of Ahr enhances IL-6 production in macrophages (Kimura et al., 2009), which may signal through Stat3 to promote Th17 cell differentiation in vivo. It is also possible that in vivo Th17 cell differentiation can be indirectly affected by regulatory T (Treg) cells in which Ahr seems to play a complex role (Quintana et al., 2008). Thus, the function of Ahr in steady-state intestinal Th17 cell differentiation is yet to be determined. Of note, although our data suggest that T cells make less IL-22 than RORγt$^+$ ILCs on a per-cell basis under the steady-state conditions in the gut, they can secrete high amounts of IL-22 under certain inflammatory conditions (e.g., *C. rodentium* infection). Indeed, in addition to the defects of RORγt$^+$ ILCs, *Ahr*$^{-/-}$ mice cannot mount effective Th17 cell responses in the gut at the later stage of sublethal *C. rodentium* infection (unpublished data). Ectopic expression of IL-22 rescued *Ahr*$^{-/-}$ mice from early death during infection, highlighting IL-22 regulation as an important mediator of Ahr's protective role in gut immunity.

EXPERIMENTAL PROCEDURES

Mice

All mice used in this study were maintained in specific-pathogen-free facilities at Northwestern University. The mice were littermate controlled and were 6–10 weeks old unless otherwise indicated in the text. The generation of mice was described in the Supplemental Information. All studies with mice were approved by the Animal Care and Use Committee of Northwestern University.

C. rodentium Infection and CFU Counts

C. rodentium infection was done by gavage and CFU counts of fecal bacteria after infection was determined as described in the Supplemental Information.

In Vivo Ahr Agonist Treatment and Hydrodynamic Gene Delivery of IL-22

12-day-old mice were injected with 0.5 μg FICZ or vehicle DMSO per day intraperitoneally for 6 days before analysis. Plasmid DNA was introduced into mice via a hydrodynamic tail vein injection-based gene transfer technique as described in the Supplemental Information.

Isolation of LPLs and IECs

The isolation of fetal, 1-week-old, and adult (≥3 weeks) intestinal LPLs and IECs was done as described in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures (methods for bone marrow transfer, antibiotic treatment, flow cytometry, antibodies and ELISA, quantitative real-time RT-PCR, plasmids and retroviral transduction of cell lines, ChIP assays, coimmunoprecipitation, western blot, 16S rRNA gene analysis, and statistical analysis), five figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2011.11.011.

ACKNOWLEDGMENTS

We thank entire L.Z. laboratory for their assistance and J. Marvin at Flow Cytometry Facility (Northwestern University) for cell sorting support. We are grateful to D. Littman, G. Diehl, F. Gonzalez, W. Ouyang, Q. Zhou, and

W. Muller for reagents, technical support, and discussion. Our special thanks to P. Stein for advice. We thank P. Stein and C.-R. Wang for critical reading of the manuscript. The work was supported by the National Institutes of Health (AI 089954 and AI091962 to L.Z., CA141975 and AI090392 to Y.-X.F.). This work was also supported by a Pew Scholarship and a Cancer Research Institute Investigator Award to L.Z.

Received: June 6, 2011
Revised: September 5, 2011
Accepted: November 29, 2011
Published online: December 15, 2011

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