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Innate lymphoid cells control early colonization resistance against intestinal pathogens through Id2–dependent regulation of microbiota

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Summary

Microbiota–mediated effects on the host immune response facilitate pathogen colonization resistance. However, it is unclear whether and how the host immune response can regulate the microbiota to mediate colonization resistance. Id2, an essential transcriptional regulator for the development of innate lymphoid cell (ILC) progenitors, remains highly expressed in differentiated ILCs with unknown function. Using conditionally deficient mice that delete Id2 in differentiated ILC3s, we observed that these mutant mice exhibited greatly impaired gut colonization resistance against *Citrobacter rodentium*. Utilizing gnotobiotic hosts, we showed that the Id2-dependent early colonization resistance was mediated by interleukin-22 (IL-22) regulation of microbiota. In addition to regulating development, Id2 maintained homeostasis of ILC3s, and controlled IL-22 production through an aryl hydrocarbon receptor (Ahr) and IL-23 receptor pathway. Thus, ILC3s can mediate immune surveillance that constantly maintains proper microbiota to mediate early colonization resistance through an Id2–dependent regulation of IL-22.

Introduction

Pathogen colonization resistance is dependent on direct inhibition by host microbiota (van der Waaij et al., 1971). Microbiota can also regulate the host immune response to mediate indirect pathogen colonization resistance (Buffie and Pamer, 2013). In contrast, various host genetic factors including immune factors also contribute to the varying level of individual susceptibility to pathogen infections (Chapman and Hill, 2012; Wlodarska et al., 2014). However, it is unclear whether and how such host genetic factors contribute to pathogen colonization resistance through shaping of the microbiota. *Citrobacter rodentium* (*C. rodentium*) is a natural mouse intestinal pathogen that mimics human Enterohaemorrhagic

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E. coli and Enteropathogenic *E. coli*, which cause severe diarrhea (Crim et al., 2014; Ochoa et al., 2008). Both innate and adaptive immune components, including IL-22 producing innate lymphoid cells (ILCs), $CD4^+$ T cells, B cells, and *C. rodentium*-specific antibodies, are essential for controlling and eradicating the infection (Bry and Brenner, 2004; Bry et al., 2006; Guo et al., 2014; Zheng et al., 2008). Gut flora has been shown to be important in protection against infection. C3H/HeOuJ mice suffer 100% mortality after *C. rodentium*-induced colitis (Vallance et al., 2003), whereas microbiota transfer from C57BL/6 mice led to a complete rescue of C3H/HeOuJ from death (Ghosh et al., 2011).

IL-22 is induced by IL-23 through the IL23R–STAT3 pathway in the early phase of infection and is essential for host defense against *C. rodentium* infection (Guo et al., 2014; Zheng et al., 2008). The major function of IL-22 is to promote mucosal epithelial cell survival and proliferation, and to trigger the secretion of antimicrobial peptides, such as RegIIIγ (Pickert et al., 2009). Previous studies also show that exogenous RegIIIγ can partially rescue IL-22 deficient mice from death (Zheng et al., 2008). Interestingly, in vitro assay suggests that RegIIIγ can only kill some Gram-positive bacteria but not the Gram–negative bacteria *C. rodentium* (Cash et al., 2006). Therefore, it is still unknown how IL-22–induced RegIIIγ controls *C. rodentium* infection. Multiple studies also show that IL-22 can shape the gut microflora, which contributes to protection or exacerbation of inflammatory bowel disease or infections (Behnsen et al., 2014; Qiu et al., 2013; Zelante et al., 2013). However, it is not known whether IL-22 shapes the microbiota to mediate early *C. rodentium* colonization resistance.

Group 3 innate lymphoid cells (ILC3s) are the major producer of IL-22 in the naive gut (Guo et al., 2014; Qiu et al., 2011). Innate lymphoid cells (ILCs) are newly defined immune cells that protect the host from various infections and include group 1 ILCs, group 2 ILCs and $ROR\gamma t^+$ ILC3s (including $CD4^+$LTi, NCR^- ILC3s and NCR^+ILC3s) (Spits et al., 2013). To date, the developmental and functional program of ILC3s is known to involve the transcription factors, such as RORγt (Eberl and Littman, 2003; Eberl et al., 2004), Ahr (Kiss et al., 2011; Lee et al., 2011; Qiu et al., 2011), and STAT3 (Guo et al., 2014). Recent data suggest that NCR^+ILC3s ($NKp46^+$ $ROR\gamma t^+$ ILCs) may originate from NCR^-ILC3s (Rankin et al., 2013; Vonarbourg et al., 2010). IL7R signaling is critical for the survival of ILC3s, but it also maintains RORγt expression in mature NCR^+ILC3s (Schmutz et al., 2009; Vonarbourg et al., 2010).

E proteins belong to bHLH transcription factor family that contains a basic DNA-binding region and a helix–loop–helix (HLH) dimerization domain. They can form homodimers or heterodimers with other HLH proteins and function as transcription activators or repressors. Inhibitor of DNA binding (ID) proteins are HLH proteins that lack a basic region and can prevent E proteins from binding to DNA. Both E and ID proteins play important roles in the lymphoid cell development (Kee, 2009). In particular, Id2 is thought to be required for the development of the ILC precursor since $Id2^{-/-}$ mice lack all the currently known ILCs, including group 1, 2 and 3 ILCs (Boos et al., 2007; Hoyler et al., 2012). Id2 is continuously and highly expressed in all ILCs, including differentiated ILCs (Hoyler et al., 2012). However, the function of Id2 in these well-differentiated ILCs is still unclear, as $Id2^{-/-}$ mice lack ILCs from the earliest identifiable stage. Here, through conditional deletion of *Id2* after

RORγt expression in the ILC3 lineage, we demonstrated that continuous Id2 expression is required for the homeostasis and function of ILC3. Using this system we showed that ILC3s were essential for regulating the microbiota to mediate early colonization resistance against intestinal pathogen.

Results

Id2 is continuously expressed in intestinal innate lymphoid cells

To test whether Id2 could function in differentiated ILCs, we first analyzed Id2 protein expression in different ILCs population with *Id2*$^{gfp/+}$ mice. As previously reported (Cherrier et al., 2012; Hoyler et al., 2012), Id2 was expressed in differentiated ILCs, including NK, ILC1, ILC2 and ILC3 (**Figure S1A**). Id2 was not homogeneously expressed in the different subsets of ILC3s. NCR^{+}ILC3 expressed higher concentrations of Id2 compared with LTi and NCR^{-}ILC3 (**Figure S1B**). Since NCR^{+}ILC3 could be differentiated from NCR^{-}ILC3 (Rankin et al., 2013; Vonarbourg et al., 2010), our findings raise the possibility that Id2 may continue to play a role in the development and function of ILC3 after their formation.

To study the requirements for Id2 in the homeostasis and function of differentiated ILC3s, *Id2*-floxed mice were crossed with *Rorc*-cre transgenic mice to achieve specific deletion of *Id2* after RORγt expression in ILC3s (*Rorc*^{cre}*Id2*$^{fl/fl}$). Because RORγt is transiently expressed at high levels at the double positive stage of T cell development, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice not only lack Id2 expression in RORγt^{+} ILC3s, but also in most αβ T cells (**Figure S1C**).

Id2 is essential for early colonization resistance and protection against *C. rodentium* infection

Previous studies have shown that ILC3s are essential for host protection against *C. rodentium* infection (Guo et al., 2014; Qiu et al., 2011). We next investigated the importance of Id2 for ILC3 function in this infection model. After high doses of *C. rodentium* infection, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice rapidly lost body weight and died around day 10, whereas no weight loss or death was observed in their littermate control *Id2*$^{fl/fl}$ mice (Figure 1A and 1B). Consistent with the increased morbidity and mortality, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice had 10–100 times higher bacterial titers in the feces compared to *Id2*$^{fl/fl}$ mice at day 5 post-infection (Figure 1C). Systemic dissemination of *C. rodentium* in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice was also observed by increased bacterial titers in the blood, liver and spleen (Figure 1D and 1E). The *Rorc*^{cre}*Id2*$^{fl/fl}$ mice also exhibited severe diarrhea, in ammation, and colon pathology upon *C. rodentium* challenge (data not shown and Figure 1F). Collectively, these data demonstrate that continued Id2 expression in RORγt^{+} cells is required for host defense against *C. rodentium* infection.

Since disease signs, including diarrhea and body weight loss, appeared in Id2 deficient mice before day 5 post infection, we hypothesized that an Id2–dependent mechanism influenced the intestinal environment to limit early colonization even before the innate response was initiated. To test our hypothesis, we challenged both *Rorc*^{cre}*Id2*$^{fl/fl}$ and *Id2*$^{fl/fl}$ littermate mice with a low dose of *C. rodentium* to determine whether *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were more

readily colonized. Interestingly, there was significantly more colonization of *C. rodentium* in the *Rorc*^{cre}*Id2*$^{fl/fl}$ mice in the first few days, even as early as day 1, after infection compared with *Id2*$^{fl/fl}$ mice (Figure 1G). Moreover, *C. rodentium* could be detected in the whole intestine of *Rorc*^{cre}*Id2*$^{fl/fl}$ mice even at day 1 post infection, compared with only in the cecum of *Id2*$^{fl/fl}$ mice (**Figure S1D**). As in the high dose infection, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice infected with a low dose of *C. rodentium* had severe diarrhea, rapidly lost body weight and died around day 10 whereas *Id2*$^{fl/fl}$ mice were not affected (Figure 1H and 1I). Together, our data indicate that Id2 is essential in RORγt^{+} cells for host defense against a mucosal bacterial pathogen and is required for maintenance of early colonization resistance.

Since it takes a few days for an effective innate immune response to control pathogen, the impaired early colonization resistance against *C. rodentium* suggested there was a preexisting defect in the *Rorc*^{cre}*Id2*$^{fl/fl}$ mice. Previous studies have shown that either increased inflammation or reduced mucus layer could result in increased colonization of intestinal pathogen (Bergstrom et al., 2010; Wlodarska et al., 2011). However, colonic mRNA analysis showed that there was no increased expression of pro-inflammatory cytokines or reduction of mucin in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice (**Figure S1E and S1F**). Moreover, HE and PAS staining showed that there were no obvious alteration of histopathology, mucus layer and goblet cells (**Figure S1G**). Together, these data suggest that the increased *C. rodentium* colonization in our Id2 deficient mice is unlikely due to changed colonic inflammation or mucus environment.

Id2 dependent microbiota controls colonization resistance against *C. rodentium* infection

Host microbiota has been recognized as a direct mediator for pathogen colonization resistance (Buffie and Pamer, 2013). To test whether Id2 regulated early *C. rodentium* colonization through the microbiota, we first treated both *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice with antibiotics for 1 week, then infected these mice with a low dose of *C. rodentium* after 1 day of rest. As shown in Figure 2A, fecal *C. rodentium* titers in antibiotic treated *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were much higher than in *Id2*$^{fl/fl}$ mice at day 1 and 3 post infection. However, bacterial titers were increased in both the *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice after antibiotic treatment during first few days after infection (Figure 2A). In addition, both *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ antibiotic treated mice rapidly lost more body weight than untreated mice (Figure 2B). However, only a little systemic dissemination of *C. rodentium* was observed in the blood and liver of *Id2*$^{fl/fl}$ antibiotic treated mice, despite high pathogen titers in the feces (Figure 2A, S2A and S2B). Together, these results suggest that the microbiota are critical for early colonization resistance against *C. rodentium*, but with limited effect on systemic dissemination of the pathogen.

Our data led us to hypothesize Id2 controlled the microbiota to maintain colonization resistance. To test this hypothesis we reconstituted gnotobiotic C57BL/6 mice with cecal content from *Id2*$^{fl/fl}$ or *Rorc*^{cre}*Id2*$^{fl/fl}$ mice by oral inoculation. One day later, the reconstituted mice were challenged with a low dose of *C. rodentium*. As shown in Figure 2D, germ free mice that did not receive a microbiota transplantation exhibited very high fecal *C. rodentium* titers even at day 1, while the mice reconstituted with *Id2*$^{fl/fl}$ microbiota showed markedly reduced *C. rodentium* titers. Importantly, germ free mice inoculated with

Rorc^{cre}*Id2*$^{fl/fl}$ cecal content showed higher *C. rodentium* titers than *Id2*$^{fl/fl}$ mice (Figure 2D). These data demonstrate that the Id2 dependent microbiota controls colonization resistance against *C. rodentium*.

Microbiota can also indirectly mediate colonization resistance through regulation of host immune response (Buffie and Pamer, 2013). Reconstitution of microbiota in germ free mice will induce the host immune response, which may result in increased indirect colonization resistance against *C. rodentium*. We examined whether the different microbiota from *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice induce different host immune response of germ free mice. As shown in **Figure S2C**, reconstitution of *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ microflora in germ free mice induced similar amount of expression of pro-inflammatory cytokines and antimicrobial proteins. Together, these data indicate that the early colonization resistance mediated by Id2 dependent microbiota might not be through the regulation of host immune response.

Ltbr$^{-/-}$ mice have multiple defects in innate and adaptive immune responses, including reduced IL-22 and antibody production, which are both essential for the host protection against *C. rodentium* infection (Kang et al., 2002; Spahn et al., 2004; Tumanov et al., 2011; Wang et al., 2010). To further determine whether the Id2 dependent microbiota could directly mediate colonization resistance, we use *Ltbr*$^{-/-}$ germ free mice as the recipient. As observed in the C57BL/6 germ free recipient mice, the *Ltbr*$^{-/-}$ germ free mice repopulated with *Id2*$^{fl/fl}$ microbiota showed reduced *C. rodentium* colonization, whereas *Rorc*^{cre}*Id2*$^{fl/fl}$ microbiota repopulated *Ltbr*$^{-/-}$ mice showed more *C. rodentium* colonization (Figure 2E). Moreover, *Id2*$^{fl/fl}$ microbiota transplantation improved the survival of *Ltbr*$^{-/-}$ mice after *C. rodentium* challenge, while *Ltbr*$^{-/-}$ germ free mice transferred with *Rorc*^{cre}*Id2*$^{fl/fl}$ cecal content showed a similar mortality to the untreated *Ltbr*$^{-/-}$ germ free mice (Figure 2F). All together, these data indicate that microbiota mediate colonization resistance, which is dependent on continued expression of Id2 in RORγt^{+} cells.

To further understand how Id2 dependent microbiota regulated the pathogen colonization, we examined the microbiome by bacterial 16S rRNA gene pyrosequencing. Analysis of 16S rRNA genes revealed that although there were no obvious difference of the bacterial diversity and compositions at the phyla level between *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice (**Figure S2D** and **S2E**), we observed several operational taxonomic units (OTUs) were over- and underpresented in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice (**Figure S2F**). Furthermore, quantitative PCR with primers specific for different bacteria demonstrated that Segmented filamentous bacteria (SFB) was overgrown in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice (**Figure S2G**), which has been shown could induce the development of Th17 cells and are regulated by the ILC3s (Ivanov et al., 2009; Qiu et al., 2013). All together, our data suggest that Id2 in in RORγt^{+} cells regulates the intestinal microbiota.

Id2 mediates the colonization resistance against *C. rodentium* through IL-22 dependent regulation of microbiota

IL-22, mainly produced by RORγt^{+} cells, is not only required for protection against pathogen infection, but also regulates the homeostasis of microflora in the intestine (Qiu et al., 2013; Qiu et al., 2011). We tested whether Id2 mediated colonization resistance through IL-22 dependent regulation of the microbiota. *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were infected

with *C. rodentium* and the expression of *Il22* mRNA and mRNA for two antimicrobial proteins dependent on IL-22, RegIIIγ, RegIIIβ, were examined in both naive and infected colon tissues. As shown in Figure 3A, compared with *Id2*$^{fl/fl}$ mice, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice showed significant reduction of *Il22*, *Reg3g* and *Reg3b* mRNA in both naive and infected states, indicating that continued Id2 expression in RORγt^{+} cells was essential for the IL-22 production in the intestine. To test whether IL-22 regulated the microbiota to mediate early colonization resistance, *Id2*$^{fl/fl}$ mice were treated with anti-IL-22 neutralization antibody at weaning and challenged with *C. rodentium* when they were 7 weeks old. Similar to the *Rorc*^{cre}*Id2*$^{fl/fl}$ mice, there was increased fecal *C. rodentium* amounts and decreased survival in anti-IL-22 treated *Id2*$^{fl/fl}$ mice (Figure 3B and 3C), indicating that blocking IL-22 function in early life destroyed colonization resistance in adulthood. To further determine whether the early colonization resistance was mediated by IL-22 dependent microbiota, but not *C. rodentium* induced immune response, *Id2*$^{fl/fl}$ mice were treated with anti-IL-22 antibody 7 days before or at the same day of infection. As shown in **Figure S3A–S3C**, although only early anti-IL-22 treatment resulted in increased colonization of *C. rodentium* in feces, both anti-IL-22 treated groups revealed increased *C. rodentium* CFU in blood, spleen and liver. Our earlier data in this study showed that antibiotic treated microbiota dramatically reduced the early colonization resistance against *C. rodentium*, but not the systemic dissemination (**Figure S3A** and **S3B**). Thus, our data indicate that IL-22 not only mediates the colonization resistance against intestinal pathogen through regulation of microbiota, but also controls the systemic dissemination of pathogen, which is less microbiota dependent. Consistent with this, *Rorc*^{cre}*Stat3*$^{fl/fl}$ mice, which lack IL-22 production from RORγt^{+} cells, also exhibited impaired early colonization resistance (**Figure S3D**). To further test the requirement for IL-22 to maintain a protective microbiota, we transplanted germ free C57BL/6 and *Ltbr*$^{-/-}$ mice with microflora from the anti-IL-22 or control treated mice and challenged them with *C. rodentium*. As observed with germ free mice repopulated with microbiota from *Rorc*^{cre}*Id2*$^{fl/fl}$ mice, microbiota from anti-IL-22 treated mice failed to prevent colonization by *C. rodentium* (Figure 3D and 3E). Collectively, our results suggest that Id2 mediates colonization resistance against *C. rodentium* through IL-22 dependent regulation of microbiota.

IL-22 producing innate lymphoid cells are necessary and sufficient to mediate the colonization resistance against *C. rodentium*

Although IL-22 can be produced by both ILC3s and T cells, we observed that ILC3s are the main producer of IL-22 in the naive state (Figure 4A and 4B) and that innate IL-22 production is markedly reduced in Id2 deficient mice (Figure 4A). This IL-22 expression profile suggests that ILC3s may be responsible for colonization resistance. To test our hypothesis, ILCs were depleted with anti-CD90 antibody in *Rag1*$^{-/-}$ mice one week before infection. Compared with control treated mice, ILC depleted *Rag1*$^{-/-}$ mice showed increased early *C. rodentium* colonization (Figure 4C) and decreased life span post infection (Figure 4D). Furthermore, innate cells from the intestine of *Rag1*$^{-/-}$ mice were isolated and transferred into *Rorc*^{cre}*Id2*$^{fl/fl}$ mice at weaning. Consistent with our hypothesis, innate cells restored the expression of IL-22 and the colonization resistance against *C. rodentium* in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice and completely rescued the *Rorc*^{cre}*Id2*$^{fl/fl}$ mice from the death (Figure

4E, 4F and **S4A**). Together, these results demonstrated that IL-22 producing ILCs mediate the early colonization resistance against an intestinal pathogen.

Continued expression of Id2 is required for the development and maintenance of group 3 ILCs

Since Id2 expression in ILC3s is essential for IL-22 dependent regulation of microbiota against pathogen colonization, we next determined how Id2 regulated IL22 production by ILC3s, and whether Id2 regulated ILC3s' homeostasis or functionality. Id2 is required for the development of all ILC precursors, thus first we tested whether Id2 was still important for the further development of ILC3s. *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were crossed with *Rorc*$^{gfp/+}$ mice and ILC3s were examined in the intestinal LPLs. RORγt expressing ILC3s were dramatically reduced in both the large and small intestine in *Rorc*$^{gfp/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ mice, and the remaining ILC3s are mostly CD4^{+} LTi cells and NCR^{-} ILC3s (Figure 5A–C and **S5A**–**B**). Previous studies have shown that NCR^{+} ILC3s can develop from NCR^{-} ILC3s, and that IL7R signaling is required for this further development as well as for the survival and proliferation of ILCs (Schmutz et al., 2009; Vonarbourg et al., 2010). We found that Id2 deficient ILC3s has diminished IL7Rα expression at both the protein and mRNA level (Figure 5D and 5E).

To further determine the role of Id2 in ILC3 maintenance, both WT and *Rorc*^{cre}*Id2*$^{fl/fl}$ ILC3s were sorted from CD45.1^{+} C57BL/6 and CD45.2^{+} *Rorc*^{cre}*Id2*$^{fl/fl}$ mice and injected into *Rag2*$^{-/-}$ *Il2*γ*c*$^{-/-}$ mice at 1:1 ratio. The gut LPL were isolated 4 weeks later and flow cytometry analysis revealed that WT ILC3s were 4 folds more than *Rorc*^{cre}*Id2*$^{fl/fl}$ cells (**Figure S5C)**. Moreover, we found that Id2 deficient ILC3 exhibited increased apoptosis and reduced Bcl2 expression, which is considered as an important anti-apoptotic protein (data not shown and **Figure S5D**). Since Id2 is required for the development of all the ILCs and early defect of development may also result in dramatic reduction of adult ILC3s (Cherrier et al., 2012), we further isolated the fetal liver cells to examine whether Id2 was required for the development rather than the maintenance of ILC3s. As shown in Figure 5F and 5G, there was no reduction of fetal LTi cells in the liver of *Rorc*^{cre}*Id2*$^{fl/fl}$ mice. However, the same as the adult ILC3s, Id2 deficient fetal ILC3s also exhibited reduced expression of IL7Rα (Figure 5H and 5I). Together, our results clearly demonstrate that Id2 expression continues to be required in ILC3 after expression of RORγt for proper expression of IL7Rα and ILC3 maintenance.

Id2 regulates IL-22 production by group 3 ILCs through IL-23R pathway

Multiple transcription factors, such as RORγt, Ahr and GATA-3 regulate ILC3 development as well as ILC3 functions (Qiu et al., 2011; Serafini et al., 2014). To further test whether Id2 also regulated the function of ILC3s, both *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ LPLs were isolated from the intestine and IL-22 production was examined. Interestingly, the residual Id2 deficient ILC3s were unable to produce IL-22 after IL-23 stimulation (Figure 6A and 6B). This is also confirmed by the marked reduction of IL-22 production in LTi and NCR^{-} ILC3s sorted from *Rorc*$^{gfp/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ mice compared with *Rorc*$^{gfp/+}$*Id2*$^{fl/fl}$ ILC3s (Figure 6C and S6A). Together, these data suggest that Id2 also plays an essential role in regulating the function of ILC3s.

Previous studies showed that IL-23 can interact with the IL-23 receptor (IL23R) and activate STAT3, RORγt, , Ahr and STAT3 can bind to the *Il22* locus and directly promote IL-22 production (Guo et al., 2014; Qiu et al., 2011). To determine how Id2 regulated ILC3s producing IL-22, we first examined the expression of these transcription factors and cytokine receptor. LTi and NCR$^-$ ILC3s were purified from both *Rorc*$^{gfp/+}$ and *Rorc*$^{gfp/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ mice. Consistent with a previous study (Zhang et al., 2014), Id2 deficient LTi cells showed increased RORγt expression. The expression of Ahr was reduced in both Id2 deficient resting LTi and NCR$^-$ ILC3s, but not IL-23 activated LTi cells. Interestingly, the expression of IL23R and STAT3 were reduced in both Id2 deficient LTi and NCR$^-$ ILC3s (Figure 6D and **S6A**). Moreover, Id2 deficient fetal liver LTi cells also showed significant reduction of IL-22 and IL-23 receptor (**Figure S6B**), suggesting that early Id2 regulation of IL-22 may be through IL23R–STAT3 pathway.

ID proteins are transcription factors that inhibit the function of the E protein transcription factors by preventing them from binding to DNA. Previous studies have shown that Id2 regulates the development of NK and LTi cells through suppression of E2A (Boos et al., 2007). To test whether Id2 also regulated IL-22 production through suppression of E2A, we infected EL4 cells with either Id2 or E47 (one E protein encoded by *E2A* gene) expressing retrovirus and examined IL-22 production. Since RORγt is the master regulator for IL-22 production, we also infected the EL4 cells with or without RORγt expressing retrovirus. As shown in Figure 6E and 6F, compared with empty MigR retrovirus control group, Id2 retrovirus infected EL4 cells produced more IL-22, while the E47 group showed reduced IL-22 production with or without RORγt expression. Moreover, increased expression of Id2 also causes increased IL23R and STAT3 expression in EL4 cells, while over-expression of E47 inhibited IL23R expression. Together, our data indicate that E2A may regulate IL-22 production through suppression of the IL23R pathway, while Id2 promotes IL-22 production through suppression of E2A activity.

Next we wanted to determine how E2A inhibited IL-22 production. Previous studies have shown that Ahr deficient ILC3s, like Id2 deficient ILC3s, showed reduced IL7Rα and IL23R expression (Qiu et al., 2011). In addition, Ahr is also a member of the bHLH transcription factor family. Although there was slight reduction of Ahr expression in Id2 deficient ILC3s, we also considered the possibility that E2A could interact with Ahr to prevent its transcriptional activity, resulting in a reduction of IL-22 in Id2 deficient ILC3s. Indeed, using EL4 cells expressing the double flag-tagged Ahr (DFTC-Ahr) with or without E47, we detected an interaction between Ahr and E2A by coimmunoprecipitation (Figure 6G). Furthermore, a chromatin immunoprecipitation (ChIP) assay revealed that E2A could suppress Ahr binding to the *Il22* locus in EL4 cell lines (Figure 6H). Together, these data suggest that E2A may regulate IL-22 production by directly binding to Ahr and preventing its transcription activity at *Il22* locus.

Discussion

Host sequential responses from innate and adaptive immune cells are essential for late stage colonization resistance and clearance of pathogens. Here, our study demonstrates that microbiota is important for early colonization resistance ahead of innate and adaptive

responses. Fecal microbiota transplantation has been successfully used in some patients with *Clostridium difficile* infection (Austin et al., 2014). However, since the stability of the gut microbiota is dependent on many host and environment factors (Lozupone et al., 2012), it remains unclear how a stable healthy microbial community should be introduced to prevent recurrent infection. Our results further demonstrate that baseline IL-22 production by ILC3s can regulate gut microbial homeostasis promoting pathogen colonization resistance. Therefore, innate immunity contributes to colonization resistance not only by rapid responses after invasion but also by maintaining the proper microbiota that limit colonization before invasion. Our study suggests that a combination of microbiota transplantation with immune molecule treatment may restore a stable microbial community to prevent intestinal pathogen colonization.

Commensals have been shown to utilize several different mechanisms to directly mediate colonization resistance against pathogen, including competition for niches and nutrients, altering host environmental conditions (for example, pH), producing bacteriocins, and affecting pathogen virulence by O2 consumption and production of specific metabolites such as the short-chain fatty acid (Kamada et al., 2013). Previous studies have shown that *E. coli* can directly compete with *C. rodentium* for nutritional resources and help the host to clear the pathogen (Kamada et al., 2012). Repopulation of Segmented filamentous bacterium (SFB) partially protects the Jackson B6 mice from *C. rodentium* infection (Ivanov et al., 2009). However, we detected very few *E. coli* in the mice housed in our SPF facility and the SFB level was greatly increased in conditional Id2 deficient mice, which is consistent with other IL-22 deficient mice (Qiu et al., 2013; Upadhyay et al., 2012). Thus, IL-22 dependent colonization resistance is not mediated through *E. coli* and SFB. It remains to be determined which commensals are regulated by innate IL-22 for colonization resistance and how commensals suppress *C. rodentium* colonization, which may leads to discovery of novel therapeutic probiotics and prebiotics.

Notch, Id2, and RORγt sequentially orchestrate the development of ILC3s (Cherrier et al., 2012). However, the function of the highly expressed Id2 in differentiated ILCs is unclear. Our data clearly demonstrate that the maintenance of Id2 expression is required for the further development and function of ILC3s. Consistent with the role of Id2 on IL-7Rαp regulation in $CD4^+$ thymocytes (Jones-Mason et al., 2012), we found that Id2 controls IL-7Rα expression in ILC3s but how Id2 regulates IL-7Rα remains to be determined. Since IL7R signaling is also essential for the homeostasis of the other ILCs (Hoyler et al., 2012), our results suggest Id2 may globally and constantly regulate the ILC lineage from progenitor to effector ILCs.

After IL-23 stimulation, STAT3, Ahr and RORγt can be recruited to the *Il22* locus to promote IL-22 production. It has been shown that Ahr requires interaction with RORγt to bind to the *Il22* locus. Without RORγt, there is only a little recruitment of Ahr to *Il22* locus (Guo et al., 2014; Qiu et al., 2011). Consistently, our data confirm that Ahr weakly binds to *Il22* locus without over-expressing RORγt. However, E2A physically interacted with Ahr and completely blocked the binding of Ahr to the *Il22* locus. Our studies suggest that two bHLH transcription factors, E2A and Ahr, may form a heterodimer that either fails to bind DNA or has a DNA binding specificity different with the Ahr–RORγt dimer. Therefore, an

essential role of Id2 may be to liberate Ahr from E2A and allow Ahr bind to other transcriptional activators, such as RORγt. Whether E2A also interrupts the interaction between Ahr and RORγt remain to be examined. We also showed that Id2 was required for expression of IL7Rα and IL23R. Since Ahr deficient ILC3s also exhibit impaired expression of these two cytokine receptors (Qiu et al., 2011), E2A may also suppress their expression through its interaction with Ahr; however, an complete understanding of the molecular mechanism by which E2A affects gene expression in ILC3s requires further investigation.

Taken together, our studies demonstrated several important findings. (1) Id2 is essential not only for ILC lineage specification but also for the maintenance and further development of RORγt^{+}ILC3s. (2) The Id2–E2A interaction regulates IL-22 production function in ILC3 through the Ahr and IL23R pathway. (3) IL-22 producing ILC3s are essential for the maintenance of the proper microbiota to mediate early pathogen colonization resistance. Proper addition of immune molecule treatment to the fecal microbiota transplantation may help the host to reestablish a more stable and healthier microbial community in patients with recurrent gut infection.

Materials and Methods

Mice

C57BL/6 and *Rag1*$^{-/-}$ mice were purchased from Harland Teklad. *Rag2*$^{-/-}$ *Il2γc*$^{-/-}$, *Rorc*$^{gfp/+}$ (Eberl et al., 2004) and *Id2*$^{gfp/+}$ mice (Rawlins et al., 2009) were purchased from The Jackson Lab. *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were generated by crossing *Id2-floxed* mice (Niola et al., 2012) with *Rorc-cre* transgenic mice (Eberl and Littman, 2004). All mice are on C57BL/6 background. Germ free *Ltbr*$^{-/-}$ mice were rederived in Taconic. All the germ free mice are maintained in the gnotobiotic facility at the University of Chicago. Animal care and use were in accordance with institutional and National Institutes of Health guidelines and all studies were approved by the Animal Care and Use Committee of the University of Chicago.

Infection with *Citrobacter rodentium* and Treatment

Mice were orally gavaged with *C. rodentium* strain DBS100 (ATCC 51459; American Type Culture Collection) and body weight, survival, CFU counts, tissue histology, PAS staining were assessed as previously described (Tumanov et al., 2011; Wang et al., 2010). Where indicated, mice were treated antibiotics (1 g/L Ampicillin, 1 g/L neomycin, 1 g/L metronidazole, 0.5 g/L vancomycin) in drinking water for 1 week, then infected with *C. rodentium* after 1 day's rest. Where indicated, mice were injected intraperitoneally with either anti-IL-22 antibody (8E11.9) or mouse IgG1 as isotype control (100 μg per mouse) by the age of 3, 4 and 5 weeks or indicated day. Then the mice were infected with *C. rodentium* at 7 weeks old. For depletion of innate lymphoid cells, *Rag1*$^{-/-}$ mice were injected intraperitoneally with anti-CD90 antibody (30H12, 100 μg per mouse) or Rat IgG 10 days before *C. rodentium* infection. Where indicated, innate cells were isolated from the intestine of *Rag1*$^{-/-}$ mice and transferred by i.v. injection (1×10^6 innate cells or 2×10^5 purified CD45^{low}CD90^{high} ILC3s per mouse) into 3–4 weeks old *Rorc*^{cre}*Id2*$^{fl/fl}$ mice.

Germ free experiments

C57BL/6 or *Ltbr*$^{-/-}$ germ-free mice were transferred to specific pathogen free environment and immediately gavaged with fresh cecal contents from *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ littermate donors, or anti-IL-22 antibody and isotype control treated mice. One day later, these microflora reconstituted mice were infected with *C. rodentium* (Ahern et al., 2014).

Isolation of Intestinal LPLs and Fetal Liver Cells

The intestinal lamina propria leukocytes were isolated by Lamina Propria Dissociation Kit (Miltenyl Biotec) according to the manufacturer's recommendations. Fetal liver were dissociated by mechanical shearing in PBS containing 0.5% bovine serum and then filtered through a 70-μM mesh.

Flow Cytometry, Antibodies and ELISA

Antibodies against lineage marker, CD3, CD4, CD45, CD90, Nkp46, CD117, CD127, α4β7, RORγt and Streptavidin-APC were purchased from BioLegend or eBioscience. Anti-IL-22 antibody was a gift from Genentech. For nuclear staining, cells were fixed and permeabilized using a Transcription Factor Staining Buffer Set (eBioscience). For cytokine production, cells were stimulated ex vivo by IL-23 (25 ng/ml, R&D) for 4 hrs. IC Fixation Buffer and Permeabilization Buffer (eBioscience) were used for intracellular cytokine staining. Flow cytometry was performed on LSR-Fortessa (BD Biosciences) instruments and analyzed with FlowJo software (Tree Star Inc.). ILC3s were sorted from the intestine LPLs and fetal liver cells on a FACS Aria III instrument (BD Bioscience). IL-22 in supernatants was measured by ELISA according to the manufacturer's recommendations (R&D Systems).

Quantitative Real-Time RT-PCR

RNA isolation and Real-time PCR was performed as previously described (Guo et al., 2014) with different primer sets (Table S1).

Retroviral Transduction of Cell Lines

The MigR, Id2, E47 and RORγt expression retrovirus were made as previously described (Qiu et al., 2011). EL4 cells were infected with2 ml of virus supernatant (MigR, Id2 or E47) and 48 hours later, the GFP^{+} infected cells were sorted on Avalon Cell Sorter (Propel Labs). The sorted EL4 cells were then infected with either MigR or RORγt expression retrovirus. Cell culture supernatant and cell RNA were analyzed by ELISA and Realtime PCR separately.

Co-immunoprecipitation and Chromatin Immunoprecipitation

EL4 cells were infected with DFTC (double flag epitope tagged)–Ahr retroviral vector and selected in the presence of 7 μg/ml puromycin. Ahr stably expressing EL4 cells were then infected either MigR or E47 retrovirus. The GFP^{+} infected cells were sorted and the whole cell lysate supernatant was immunoprecipitated with EZview™ Red anti-flag affinity gel (Sigma). Western blotting was performed with anti-E2A rabbit antibody (Thermo Scientific)

and anti-GAPDH antibody (Sigma). ChIP assays with EL4 cells were performed as previously described (Qiu et al., 2011).

Statistical Methods

Statistical analysis was performed by two-tailed Student's *t* test using GraphPad Prism 5.0 program. Data from such experiments are presented as mean values ± SEM; $p < 0.05$ was considered significant. For survival curves, statistics were done using the log rank (Mantel-Cox) test.

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Highlights

- Id2 is essential to mediate the colonization resistance against *C. rodentium*.
- Continued expression of Id2 is required for the homeostasis of ILC3s.
- Id2-E2A interaction regulates ILC3 production of IL-22 through Ahr and IL23R pathway.
- IL-22 from ILC3s controls the colonization resistance through regulating microbiota.

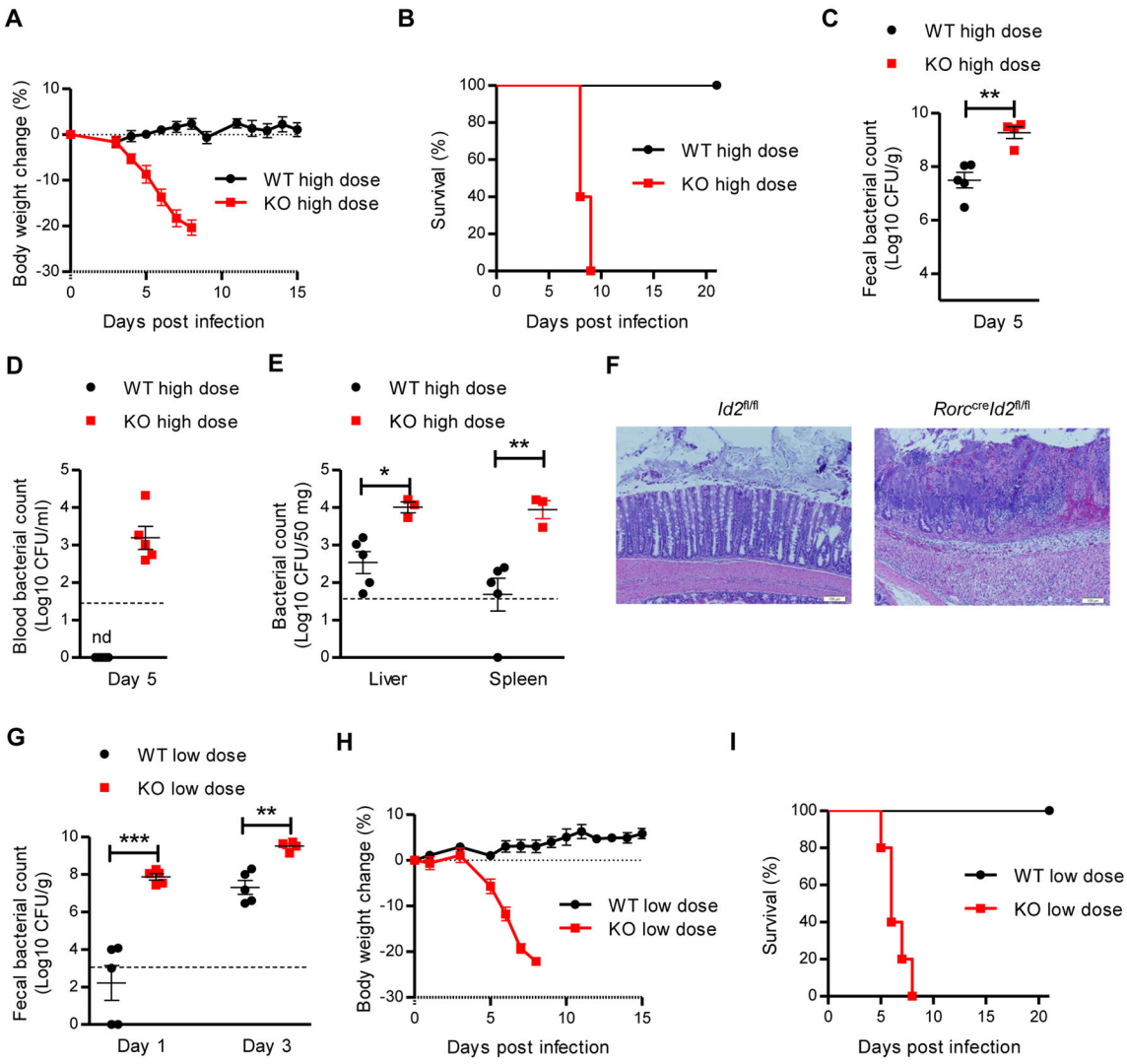

Figure 1. Id2 is essential to mediate the colonization resistance and protection against *C. rodentium* infection

(**A**–**F**) 7 weeks old *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO, n=5) and their littermate wild type *Id2*$^{fl/fl}$ mice (WT, n=5) were orally inoculated with high dose (2 × 10^9 CFU) of *C. rodentium*. Body weight change (**A**) and survival rates (**B**) are shown. Fecal and blood *C. rodentium* titers at indicated day post infection (**C**, **D**) and *C. rodentium* titers from spleen and liver homogenate cultures at day 8 post infection (**E**)are shown. Dash line, limit of detection. (**F**) Histological analysis of representative colons from WT and KO mice at day 8 after infection. Scale bars, 100 μm.

(**G**–**I**) 7 weeks old KO (n=5) and their littermate WT (n=5) mice were orally inoculated with low dose (5 × 10^6 CFU) of *C. rodentium*. (**G**) Fecal *C. rodentium* titers at indicated day post infection are shown. Body weight change (**H**) and survival rates (**I**) are shown.

Each dot represents one individual mouse (**C**, **D**, **E** and **G**). Error bars represent SEM. *P<0.05, **P<0.01, ***P<0.001 (Student's *t*-test). nd, nondetectable. Data are

representative of three independent experiments (**A**–**D**, **G**–**I**) or two independent experiments (**E**, **F**). See also Figure S1.

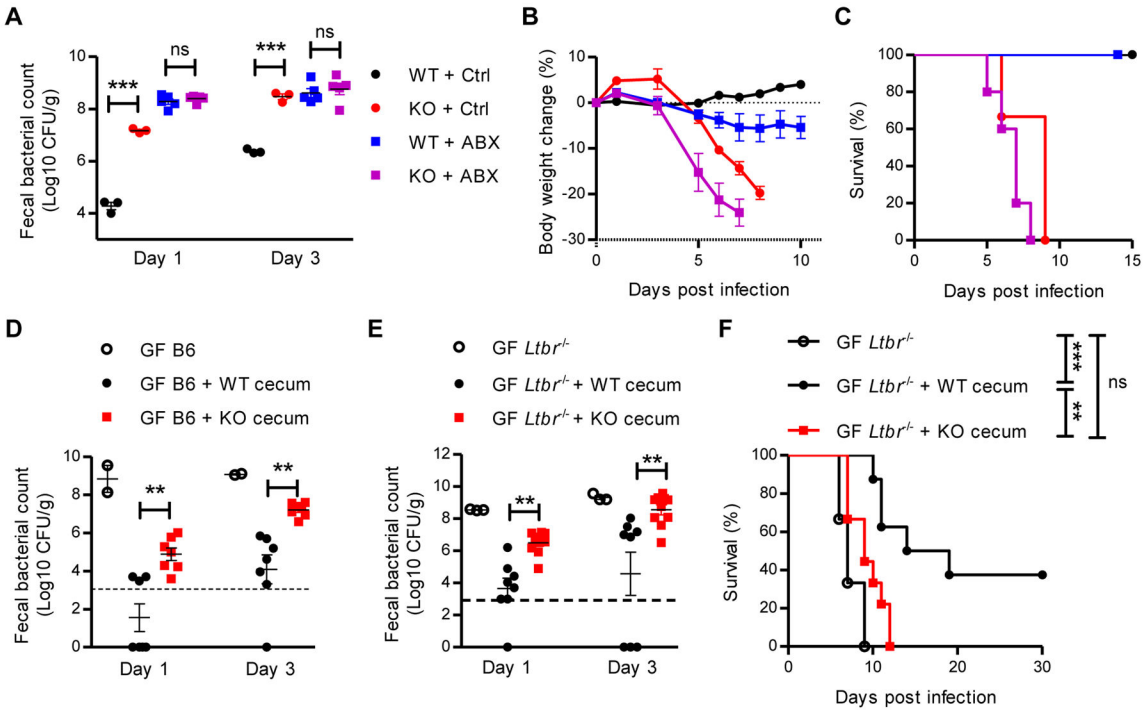

Figure 2. Id2 dependent microbiota mediates the colonization resistance against *C. rodentium* infection

(**A**–**C**) 7 weeks old *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) and their littermate *Id2*$^{fl/fl}$ mice (WT) were treated with either antibiotic (n=5) or control (n=3) in drinking water for one week. One day later, WT and KO mice were orally inoculated with 5 × 10^6 CFU of *C. rodentium*. (**A**) Fecal *C. rodentium* titers at indicated day post infection. Body weight change (**B**) and survival rates (**C**) are shown. Data are representative of two independent experiments.

(**D**–**F**) Germ free (GF) wild type B6 mice (**D**) or GF *Ltbr*$^{-/-}$ mice (**E**, **F**) were colonized with the microbiota from either WT or KO littermate mice by gavage of cecal material. One day later, these mice were orally inoculated with 5 × 10^6 CFU (**D**) or 1 × 10^7 CFU (**E**, **F**) of *C. rodentium* and fecal *C. rodentium* titers were examined at indicated day post infection (**D**–**E**). (**F**) Survival rates were monitored every day. Data are pooled from three independent experiments.

Each dot represents one individual mouse (**A**, **D**, **E**). Dash line, limit of detection. Error bars represent SEM. *P<0.05, **P<0.01, ***P<0.001; ns, no significant difference (Student's *t*-test). See also Figure S2.

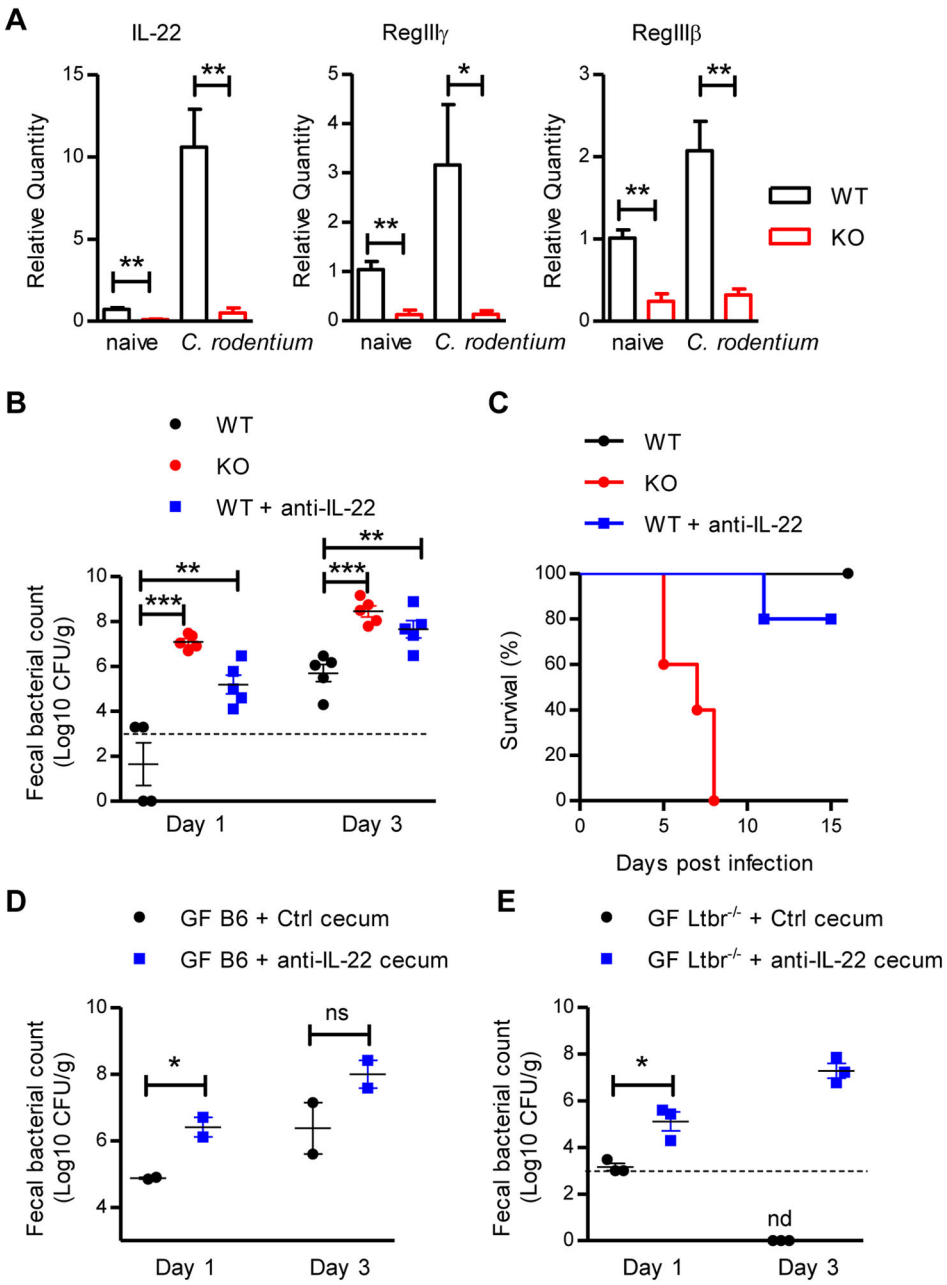

Figure 3. Id2 mediates the colonization resistance against *C. rodentium* through IL-22 dependent regulation of microbiota

(**A**) *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) and their littermate *Id2*$^{fl/fl}$ (WT) mice were infected with 2 × 10^9 CFU of *C. rodentium*. The mRNA expression of IL-22, RegIIIγ and RegIIIβ antimicrobial proteins in the colon of naive or 5 days infected mice were measured by real-time PCR. Data are representative of two independent experiments (n = 3 to 5 per group; mean ± s.e.m.). (**B**, **C**) WT mice were injected intraperitoneally with either anti-IL-22 antibody (8E11.9, 100 μg per mouse per week, n=5) or mouse IgG control (n=5) at 3, 4, 5 weeks old. Two weeks later, 7 weeks old WT and KO mice were orally infected with low dose (5 × 10^6 CFU) of *C. rodentium*. (**B**) Fecal *C. rodentium* titers at indicated day post infection. (**C**) Survival rates

are shown. Dash line, limit of detection. Data are representative of two independent experiments.

(**D**) Germ free (GF) wild type B6 mice or

(**E**) GF *Ltbr*$^{-/-}$ mice were colonized with the microbiota from either anti-IL-22 or mouse IgG treated WT mice by gavage of cecal material. One day later, these mice were orally inoculated with low dose (5×10^6 CFU, **D**; 1×10^7 CFU, **E**) of *C. rodentium* and fecal *C. rodentium* titers were examined at indicated day post infection. Data are representative of two (**D**) or three (**E**) independent experiments.

Each dot represents one individual mouse (**B**, **D**, **E**). Error bars represent SEM. **P<0.01, ***P<0.001; ns, no significant difference (Student's *t*-test). nd, nondetectable. See also Figure S3.

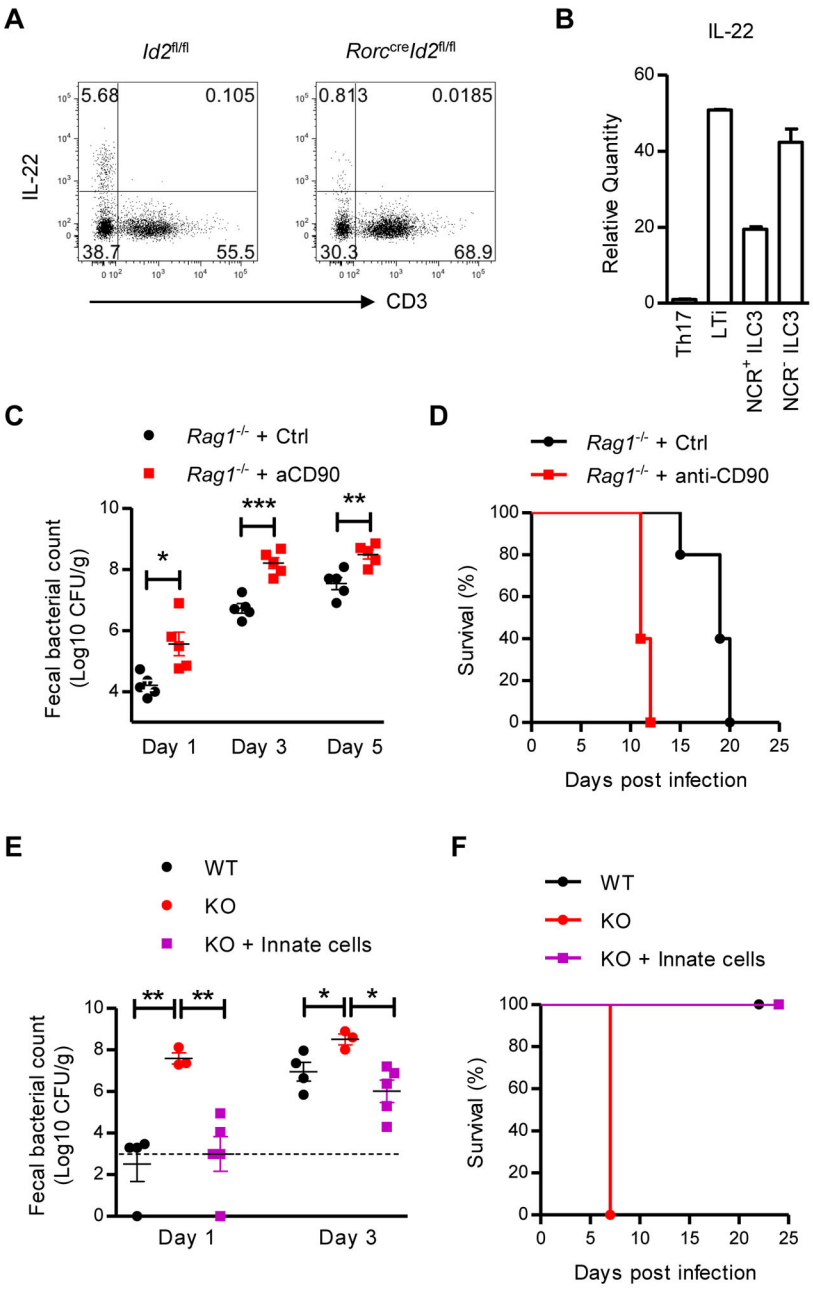

Figure 4. IL-22 producing innate lymphoid cells mediates the colonization resistance against *C. rodentium*

(**A**) IL-22 expression in $CD3^-$ and $CD3^+$ cells were analyzed by intracellular cytokine staining. Intestinal LPLs were isolated from the colons of naive $Id2^{fl/fl}$ (WT) or $Rorc^{cre}Id2^{fl/fl}$ (KO) mice, were stimulated with IL-23 (25 ng/ml) for 4 hours and gated in $Thy1^+$ lymphocytes. Data are representative of at least five independent experiments. (**B**) $ROR\gamma t^+$ ILC3s and T helper cells were purified by flow cytometric sorting from intestinal LPLs of $Rorc^{gfp/+}$ mice. The mRNA expression of IL-22 was measured by real-time PCR. Data are representative of two independent experiments (mean ± s.e.m. of triplicate samples of real-time PCR).

(**C**, **D**) *Rag1*$^{-/-}$ mice were injected intraperitoneally with either anti-CD90 antibody (30H12, 100 μg per mouse each time) or Rat IgG control at day -10 and day -5 before infection for depletion of ILCs (n=5). Ten days later, mice were orally infected with 1×10^7 CFU of *C. rodentium*. (**C**) Fecal *C. rodentium* titers at indicated day post infection. (**D**) Survival rates are shown. Data are representative of two independent experiments. Each dot represents one individual mouse (**C**).

(**E**, **F**) KO mice were injected with innate cells at weaning. Four weeks later, WT and KO mice were orally infected with 5×10^6 CFU of *C. rodentium*. (**E**) Fecal *C. rodentium* titers at indicated day post infection. (**F**) Survival rates are shown. Dash line, limit of detection. Data are representative of two independent experiments.

Error bars represent SEM. *P<0.05, **P<0.01, ***P<0.001 (Student's *t*-test). See also Figure S4.

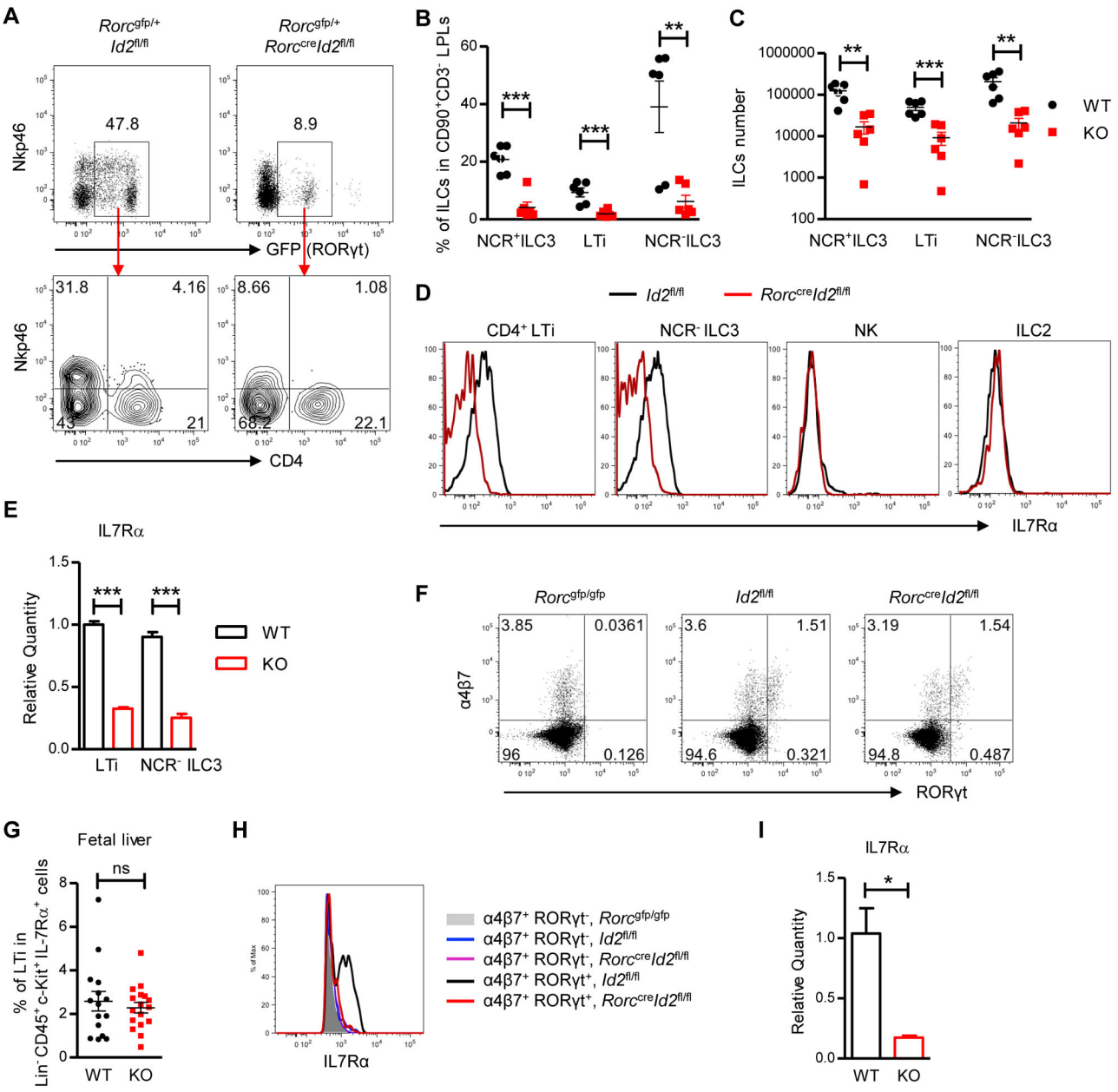

Figure 5. Continued expression of Id2 is required for the further development and maintenance of group 3 ILCs

(**A, D, E**) Small intestinal LPLs were isolated from 4 weeks old *Rorc*$^{gfp/+}$*Id2*$^{fl/fl}$ (WT) and *Rorc*$^{gfp/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice and gated in live CD90$^+$ CD3$^-$ ILCs. (**A**) Different populations of ILCs were analyzed by flow cytometry. Numbers adjacent to outlined areas indicate percent cells in each gate. (**D**) The expression of IL-7Rα on different ILCs was analyzed by flow cytometry. Data are representative of three independent experiments. NCR$^+$ILC3, Nkp46$^+$CD4$^-$RORγt$^+$ILCs; LTi, Nkp46$^-$CD4$^+$RORγt$^+$ILCs; NCR$^-$ILC3, Nkp46$^-$CD4$^-$RORγt$^+$ ILCs; NK, Nkp46$^+$RORγt$^-$ ILCs; ILC2, RORγt$^-$Nkp46$^-$ILCs. (**B**, **C**) Percentage of different ILC3s in the CD90$^+$ CD3$^-$ ILCs, as well as the absolute numbers of RORγt$^+$ ILC3s in the small intestine of *Id2*$^{fl/fl}$ (WT) and *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO)

mice are shown. Each dot represents one individual mouse. Data are pooled from two independent experiments (mean ± s.e.m.).

(**E**) LTi and NCR^{-} ILC3 were purified by flow cytometric sorting. The mRNA expression of IL-7Rα was measured by real-time PCR. Data are representative of two independent experiments (mean ± s.e.m. of triplicate samples of real-time PCR).

(**F, G**) LTi cells in fetal liver were analyzed by flow cytometry. E14-E15 fetuses were isolated and genotyped by PCR. Fetal liver cells were isolated from *Rorc*$^{gfp/gfp}$, *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ fetus and gated in Lineage^{-}CD45^{+}c-Kit^{+}IL7Rα^{+} ILCs. Numbers adjacent to outlined areas indicate percent cells in each gate. Data are representative of four independent experiments. (**G**) Percentage of LTi cells (α4β7^{+}RORγt^{+}) in the Lineage^{-}CD45^{+}c-Kit^{+}IL7Rα^{+} cells were shown. Each dot represents one individual mouse. Data are pooled from four independent experiments (mean ± s.e.m.).

(**H**) The expression of IL-7Rα on different fetal liver cells was analysis by flow cytometry. The fetal liver cells were gated in Lineage^{-}CD45^{+}c-Kit^{+}IL7Rα^{+} cells first and then gated in either α4β 7^{+}RORγt^{+} LTi cells or α4β7^{+}RORγt^{-} LTi precursor cells. Data are representative of four independent experiments.

(**I**) Fetal LTi were purified by flow cytometric sorting from fetal liver of *Rorc*$^{gfp/+}$*Id2*$^{fl/fl}$ and *Rorc*$^{gfp/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ mice. The mRNA expression of IL-7Rα was measured by real-time PCR. Data are representative of two independent experiments (mean ± s.e.m. of triplicate samples of real-time PCR).

**P<0.01, ***P<0.001; ns, no significant difference (Student's *t*-test). See also Figure S5.

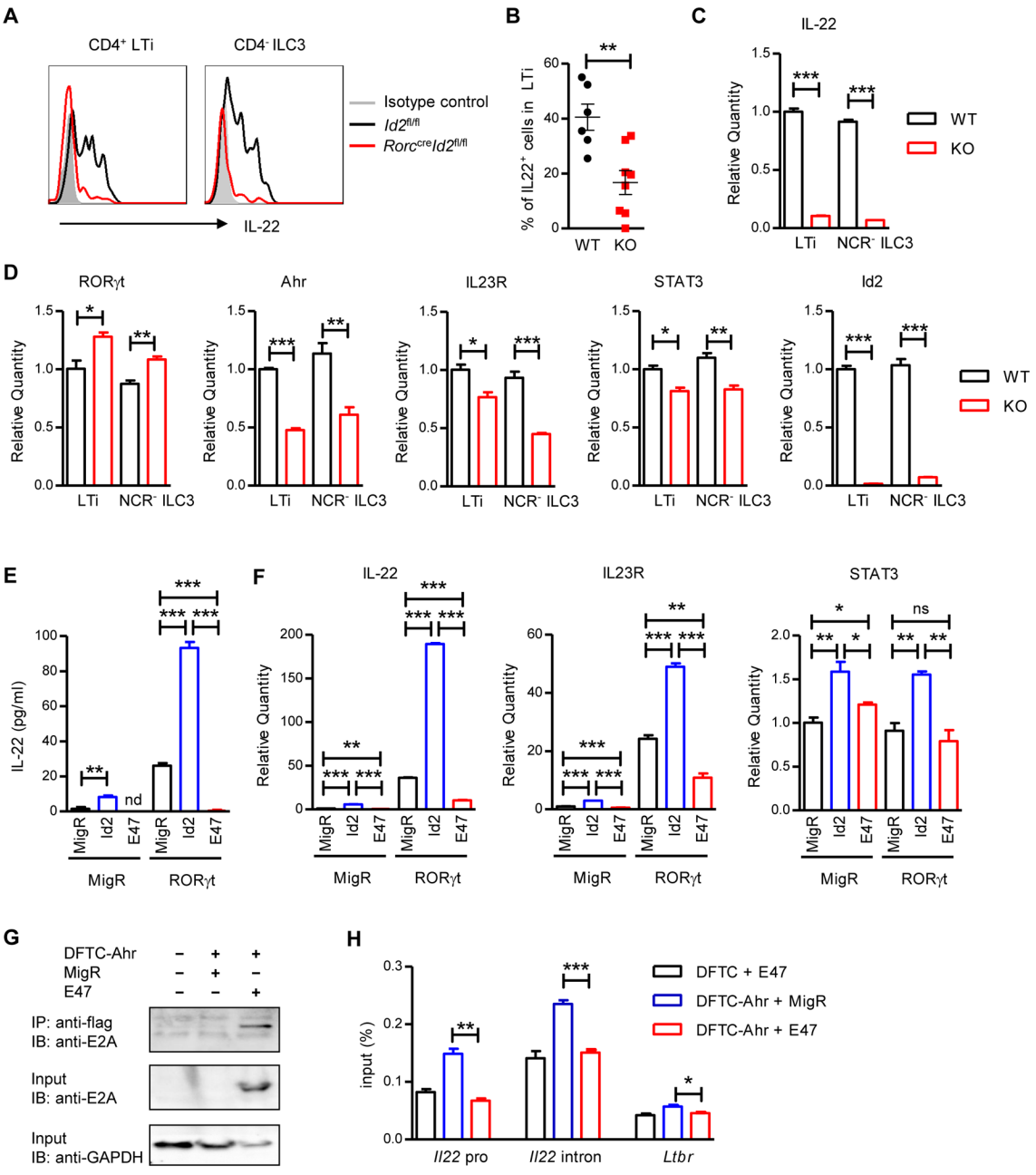

Figure 6. Id2 regulates IL-22 production by group 3 ILCs through IL-23R pathway

(**A**) Intestinal LPLs were isolated from naive *Id2*$^{fl/fl}$ (WT) or *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice and stimulated with IL-23 (25 ng/ml) for 4 hours. IL-22 expression in CD3^{-}CD90^{high}CD45^{low}CD4^{+} LTi or CD3^{-}CD90^{high}CD45^{low}CD4^{-} ILC3s was analyzed by intracellular cytokine staining followed by flow cytometry. Data are representative of three independent experiments.

(**B**) Percentages of IL-22 producing cell in LTi cells are shown. Colonic LPLs were isolated from *C. rodentium* infected WT or KO mice at day 5 post infection, restimulated with IL-23

and gated in $CD3^{-}CD90^{high}CD45^{low}CD4^{+}$ LTi cells. Each dot represents one individual mouse. Data are pooled from two independent experiments (mean ± s.e.m.).

(**C**, **D**) LTi and NCR^{-} ILC3 were purified by flow cytometric sorting from intestinal LPLs of $Rorc^{gfp/+}Id2^{fl/fl}$ or $Rorc^{gfp/+}Rorc^{cre}Id2^{fl/fl}$ mice and lysed directly for RNA extraction. The mRNA expression of IL-22 (**C**), RORγt, Ahr, IL23R and STAT3 (**D**) were measured by real-time PCR. Data are representative of two independent experiments (**C**, **D**; mean ± s.e.m. of triplicate samples of real-time PCR).

(**E**, **F**) EL4 cells were infected with Id2, E47 expression or control retrovirus with or without RORγt expression retrovirus and stimulated with IL-23 for 2 days. (**E**) The production of IL-22 in the cell culture supernatant was measured by ELISA. (**F**) The mRNA expression of IL-22, IL23R and STAT3 were measured by real-time PCR. Error bars represent SEM of triplicate samples. Data are representative of two independent experiments.

(**G**) E2A physically interacted with Ahr. EL4 cells were stably infected with the indicated expression constructs. Whole cell extracts were immunoprecipitated with anti-flag beads and subsequently immunoblotted with anti-E2A antibodies. Data are representative of three independent experiments.

(**H**) Empty MigR or E47 were coexpressed by retroviral transduction in EL4 cell lines stably expressing either DFTC or DFTC-Ahr. Ahr binding at the *Il22* locus was detected by ChIP assay. *P<0.05, **P<0.01, ***P<0.001; ns, no significant difference (Student's *t*-test). nd, nondetectable. See also Figure S6.