Immunity
Article

Regulated Expression of Nuclear Receptor RORγt Confers Distinct Functional Fates to NK Cell Receptor-Expressing RORγt$^+$ Innate Lymphocytes

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DOI 10.1016/j.immuni.2010.10.017

SUMMARY

Whether the recently identified innate lymphocyte population coexpressing natural killer cell receptors (NKRs) and the nuclear receptor RORγt is part of the NK or lymphoid tissue inducer (LTi) cell lineage remains unclear. By using adoptive transfer of genetically tagged LTi-like cells, we demonstrate that NKR$^-$RORγt$^+$ innate lymphocytes but not NK cells were direct progenitors to NKR$^+$RORγt$^+$ cells in vivo. Genetic lineage tracing revealed that the differentiation of LTi-like cells was characterized by the stable upregulation of NKRs and a progressive loss of RORγt expression. Whereas interleukin-7 (IL-7) and intestinal microbiota stabilized RORγt expression within such NKR-LTi cells, IL-12 and IL-15 accelerated RORγt loss. RORγt$^+$ NKR-LTi cells produced IL-22, whereas RORγt$^-$ NKR-LTi cells released IFN-γ and were potent inducers of colitis. Thus, the RORγt gradient in NKR-LTi cells serves as a tunable rheostat for their functional program. Our data also define a previously unappreciated role of RORγt$^-$ NKR-LTi cells for the onset or maintenance of inflammatory bowel diseases.

INTRODUCTION

The evolutionarily ancient innate immune system is the first barrier against infections and tumors. It is equipped with two principal hematopoietic cell lineages: myeloid and lymphoid. In addition to natural killer (NK) cells, two additional innate lymphocyte subsets have been described: lymphoid tissue inducer (LTi) cells and natural helper cells (also known as type 2 innate lymphocytes or nuocytes) (Moro et al., 2010; Neill et al., 2010; Price et al., 2010). Development of LTi cells, NK cells, and natural helper cells depends on the transcription factor inhibitor of DNA binding 2 (Id2), suggesting that the innate lymphocyte lineages share a common transcriptional and developmental program (Moro et al., 2010; Yokota et al., 1999). Specific transcription factors and cytokines unique to LTi and NK cell fate decisions have also been determined. Commitment to the LTi lineage requires the orphan nuclear receptor RORγt and interleukin-7 (IL-7), whereas the NK cell fate is determined by the recently identified transcription factor E4BP4 (NFIL3) and IL-15 (Gascoyne et al., 2009; Kennedy et al., 2000; Luther et al., 2003; Meier et al., 2007; Sun et al., 2000).

NK cells are cytotoxic lymphocytes that express activating immunoreceptors (i.e., NKG2D; NCR1, also known as NKp46; NKR-P1C, also known as NK1.1) allowing them to eliminate infected, transformed, or stressed cells (Lanier, 2005). In addition, NK cells are potent producers of interferon-γ (IFN-γ). During embryonic development, LTi cells are indispensable for lymphorganogenesis (Mebius et al., 1997; Sun et al., 2000). Lymphocytes phenotypically resembling LTi cells can also be identified after birth but their role is not well defined. LTi-like cells within the intestinal lamina propria of adult mice serve as inducer cells of tertiary lymphoid organs such as cryptopatches and intestinal lymphoid follicles, which are required for T cell-independent IgA synthesis (Bouskra et al., 2008; Tsuji et al., 2008). LTi cells are also an important innate source of IL-22 and IL-17 (Cupedo et al., 2009; Takatori et al., 2009) and may be involved

in repairing tissue damage after viral infections (Scandella et al., 2008).

A population of IL-22-producing lymphocytes coexpressing RORγt and activating NKRs (i.e., NKp46$^+$RORγt$^+$ cells) that localized within the intestinal lamina propria was identified (Cella et al., 2009; Cupedo et al., 2009; Hughes et al., 2009; Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008). In mice, these cells are involved in safeguarding epithelial homeostasis (Sanos et al., 2009) and in protecting against various forms of experimental colitis (Satoh-Takayama et al., 2008; Zenewicz et al., 2008). Similar to LTi cells, NKp46$^+$RORγt$^+$ cells depend on RORγt for their differentiation and/or development and are potent producers of IL-22. In contrast, conventional (c) NK cells (i.e., NKp46$^+$RORγt$^-$) are independent of RORγt and do not produce IL-22 (Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008). It is unclear whether NKp46$^+$RORγt$^+$ cells are part of the NK cell or the LTi cell lineage or, alternatively, constitute a third independent lineage of innate lymphocytes (Cooper et al., 2009).

Chronic inflammatory disorders such as inflammatory bowel diseases (Crohn's disease, ulcerative colitis) are caused by lymphocytes that are inappropriately activated and destroy self-tissues. Recent data have assigned a disease-promoting role to IL-23, a cytokine of the IL-12 family (Duerr et al., 2006; Izcue et al., 2009). Such forms of autoimmunity have often been perceived to be exclusively caused by lymphocytes of the adaptive immune system, but recent evidence suggests that IL-23-responsive innate lymphoid cells may be sufficient to cause colitis in mice (Buonocore et al., 2010; Hue et al., 2006; Uhlig et al., 2006). In mice lacking all lymphocytes of the adaptive immune system (*Rag2*$^{-/-}$), experimental colitis can be triggered by CD40-mediated activation of myeloid cells or by infection with *Helicobacter hepaticus* (Buonocore et al., 2010; Hue et al., 2006; Uhlig et al., 2006). CD40-triggered colitis is dependent on IL-23, IFN-γ, and tumor necrosis factor (TNF) (Uhlig et al., 2006) and cannot be triggered in animals genetically lacking RORγt (Buonocore et al., 2010). However, the colitogenic, IL-23-responsive innate lymphocyte subset remains undefined (Diefenbach and Vonarbourg, 2010).

By using a combination of genetic lineage tracing and in vivo transfer of genetically tagged cells, we report that RORγt$^+$ innate lymphoid cells (i.e., LTi-like cells) and not cNK cells are direct progenitors to NKp46$^+$RORγt$^+$ lymphocytes in vivo. We now refer to these as NKR-LTi cells. NKR-LTi cells progressively lose RORγt expression, and the resulting gradient determines a distinct phenotype and functional program. RORγt$^+$ NKR-LTi cells produced IL-22, whereas RORγt$^-$ NKR-LTi cells released large amounts of IFN-γ. Interestingly, intestinal IFN-γ-producing RORγt$^-$ NKR-LTi cells, but not cNK cells, were potent inducers of experimental colitis. Thus, graded expression of RORγt serves as a molecular rheostat to generate functional plasticity within the NKR-LTi population.

RESULTS

NKp46$^-$RORγt$^+$ LTi-like Cells Are Progenitors of NKp46$^+$RORγt$^+$ Cells

Consistent with the available data, three models can be proposed for the differentiation of intestinal NKp46$^+$RORγt$^+$ cells (Figure S1A available online). One model (LTi lineage model) is that NKp46$^+$RORγt$^+$ cells are derived from NKR$^-$RORγt$^+$ LTi-like cells that upregulate NKRs. Another model (NK lineage model) proposes that NK cells, similar to IL-17-producing CD4 T cells (i.e., Th17 cells), upregulate RORγt expression as part of a distinct differentiation program endowing these cells with a particular functional profile (i.e., IL-22 production). NKp46$^+$RORγt$^+$ cells could also represent a distinct third innate lymphocyte lineage independent of NK and LTi-like cells. It is currently unknown which of these models is correct.

These models can be experimentally probed in cell transfer experiments employing donor cells from mice with a reporter gene (EGFP) knocked into the *Rorc* locus (encoding RORγt) (Eberl et al., 2004). As previously reported, RORγt expression serves as a faithful marker of intestinal LTi-like cells in adult mice (Eberl and Littman, 2004). Other innate lymphocyte lineages (i.e., natural helper cells) do not express RORγt (Moro et al., 2010). LTi cells and NKp46$^+$RORγt$^+$ cells were represented in the lamina propria of the small intestine, colon, and mesenteric LNs (Figure 1A; Eberl and Littman, 2004; Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008). We highly purified (purity > 98%) NK cells (NKp46$^+$RORγt$^-$) and genetically tagged LTi-like cells (NKp46$^-$RORγt$^+$) from the lamina propria of the small intestine of *Rorc*$^{gfp/+}$ mice (H-2^b) (Figures S1B and S1C) and transferred them into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (H-2^d) that lack all lymphocytes including NK cells and LTi cells. Transferred (H-2^b) NK cells did not appreciably upregulate RORγt expression and remained NKp46$^+$RORγt$^-$ cells even 6 months after transfer (Figure 1B and data not shown). In contrast, transferred NKp46$^-$RORγt$^+$ cells differentiated into NKp46$^+$ cells but a subpopulation remained negative for NKp46 even 6 months after transfer (Figure 1B and data not shown). Upregulation of NKp46 was already observed 2 weeks after transfer and the fraction of NKp46$^+$ LTi-derived cells further increased after 4 weeks (Figure 1B). Although virtually all of the donor-derived cells found in the small intestine remained RORγt$^+$, a considerable fraction of LTi-derived NKp46$^+$ cells that homed to the colonic lamina propria or the spleen turned off RORγt expression, demonstrating that the extent of RORγt downregulation was dependent on the tissue environment (Figure 1B). Very similar results were obtained when transferring colonic NK cells or LTi cells (Figure S2A).

Our in vivo data were confirmed by an in vitro culture system. Highly purified LTi-like cells (NKp46$^-$RORγt$^+$) were cultured with or without a stromal feeder cell layer for 7 days. A substantial fraction of LTi cells readily upregulated NKp46 whereas NK cells did not gain RORγt expression in vitro (Figure 1C). Although an appreciable proportion of LTi-derived cells became RORγt negative when the cells were cultured on an OP-9 stromal cell layer, LTi-derived cells cultured in the absence of stromal cells maintained RORγt expression (Figure 1C). This further corroborates the view that environmental cues influence maintenance or loss of RORγt expression by LTi-derived NKp46$^+$ cells.

Lymph Node LTi Cells Differentiate into NKR$^+$ Cells

NKp46$^+$RORγt$^+$ cells were originally identified in the intestinal immune system but it is unknown whether a similar cell type exists in other organs. Although an appreciable population of CD3$^-$CD19$^-$NKp46$^-$RORγt$^+$ cells was detectable in spleen

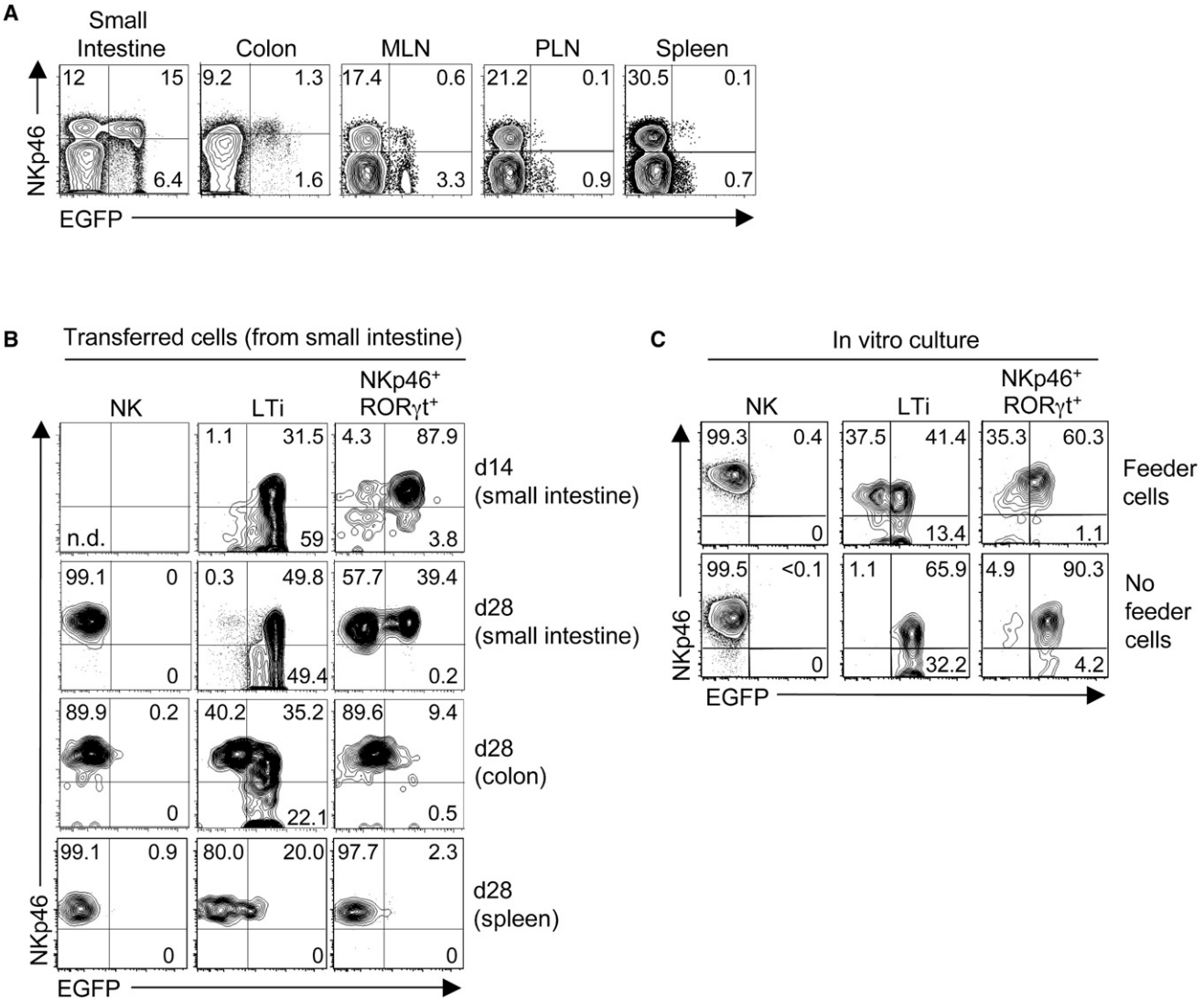

Figure 1. NKp46$^+$RORγt$^+$ Cells Are Derived from NKp46$^-$RORγt$^+$ Precursors

(A) Flow cytometry analysis of NKp46 and RORγt (EGFP) expression by CD3$^-$CD19$^-$ lymphocytes from the indicated organs of *Rorc*$^{gfp/+}$ mice. Numbers represent percent cells in quadrant.

(B) 2 × 10^4 of the indicated cell populations from the small intestine of *Rorc*$^{gfp/+}$ mice (H-2^b) were transferred into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (H-2^d). At the indicated time points, donor-derived lymphocytes were analyzed for NKp46 and RORγt (EGFP) expression. Numbers represent percent cells in quadrant. n.d.: not done.

(C) Sorted cell populations from the intestinal lamina propria of *Rorc*$^{gfp/+}$ mice were cultured with and without feeder cells for 7 days in vitro. Contour plots show NKp46 and RORγt (EGFP) expression and were electronically gated on CD45$^+$ cells. Numbers represent percent cells in quadrant.

Data are representative of three (C) or four (A, B) independent experiments.

and peripheral LNs (PLN) of RORγt reporter mice, only a very small proportion of RORγt-expressing NKp46$^+$ lymphocytes was present (Figure 1A). The NKp46$^-$RORγt$^+$ subset represented adult-type LTi-like cells as they coexpressed various LTi markers and displayed surface lymphotoxin $\alpha_1\beta_2$ (sLT$\alpha_1\beta_2$) but did not express perforin or granzyme B (Figures S2B and S2C). After transfer into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, virtually all PLN-derived LTi cells differentiated into NKp46$^+$ cells, whereas NK cells did not upregulate RORγt expression (Figure S2D). The acquisition of NKp46 by PLN LTi-like cells was confirmed by in vitro culture (Figures S2E and S2F). The differentiation of PLN-derived LTi-like cells into NKp46$^+$ cells that lost RORγt occurred more rapidly and more completely compared to intestinal LTi cells (Figures 1B and 1C; Figures S2A versus S2D–S2F). Conventional NK cells from PLN or spleen did not upregulate RORγt (Figure S2F), even under culture conditions that induce RORγt expression in CD4$^+$ T cells (Figure S2G). Thus, NKp46$^+$RORγt$^+$ cells differentiate from NKp46$^-$RORγt$^+$ precursor cells (i.e., LTi-like cells) whereas cNK cells do not acquire RORγt expression. Consequently, we will refer to these cells as RORγt$^+$ NKR-expressing LTi-like (NKR-LTi) cells.

Genetic Lineage Tracing Reveals Two Distinct NKR-Expressing Lymphocyte Lineages

Based on our in vivo transfer data (Figure 1B; Figures S2A and S2D), we considered that RORγt expression of NKp46$^+$ cells

may be transient and become undetectable in RORγt reporter mice. We used genetic lineage tracing ("fate mapping") to visualize in lymphoreplete mice all NKp46$^+$ cells derived from RORγt$^+$ progenitors, including those that had lost RORγt. Mice expressing Cre recombinase under the control of the *Rorc* locus control elements (*Rorc*-Cre^{Tg}) (Eberl and Littman, 2004) were crossed with *Rosa26*-reporter mice (*R26R*-EYFP) (Srinivas et al., 2001). The *R26R*-EYFP strain expresses a fluorescent reporter under the control of the ubiquitously active *Rosa26* promoter once the LoxP-flanked STOP cassette is excised (Figure S3A). In *Rorc*-Cre^{Tg};*R26R*-EYFP mice, all cells derived from RORγt$^+$ precursors are permanently and heritably marked by the EYFP reporter (i.e., RORγt^{fm+}), even if they subsequently lose RORγt expression. RORγt expression by RORγt^{fm+} cells can be analyzed through costaining with an RORγt-specific antibody.

Analysis of innate lymphocytes from RORγt-fate map mice revealed that a fraction of NKp46$^+$ cells in all organs tested (including the recently identified "thymic NK cells") was RORγt^{fm-}, demonstrating that these cells did not express RORγt at any time during their lineage development (Figure 2A; Figure S3L). NKp46$^+$RORγt^{fm-} cells displayed cell surface markers consistent with NK cells (Figures S3B and S3C) and expressed perforin and granzyme B, which are required for cell-mediated cytotoxicity (Figure S3D). Taking into consideration that under no conditions did NKp46$^+$RORγt$^-$ cells upregulate RORγt (Figures 1B and 1C; Figures S2A and S2D–S2G), we conclude that NKp46$^+$RORγt^{fm-} cells constitute a lymphocyte lineage developmentally distinct from RORγt-expressing lymphoid cells. We refer to NKp46$^+$RORγt^{fm-} cells as cNK cells (Figure 2A).

RORγt^{fm+} cells can be further subdivided into an NKp46$^+$ and an NKp46$^-$ subset (Figure 2A). NKp46$^-$RORγt^{fm+} cells uniformly expressed RORγt (Figure 2B) and sLTα$_1$β$_2$ (Figure S3E) but were negative for perforin and granzyme B (Figure S3D) and thus are identical to the NKp46$^-$RORγt$^+$ LTi-like cell population. This is also reflected by the absolute cell numbers of the NKp46$^-$RORγt$^+$ and the NKp46$^-$RORγt^{fm+} subsets that were virtually identical (Figure S3F). Our transfer data demonstrated that LTi-derived cells upregulate NKp46 and consecutively lost RORγt (Figure 1B; Figures S2A and S2D). Thus, the entire NKp46$^+$ LTi cell-derived population should be contained within the NKp46$^+$RORγt^{fm+} subset. Costaining of RORγt in NKp46$^+$ RORγt^{fm+} cells from various organs revealed the extent of RORγt downregulation (Figures 2B and 2C) and exactly correlated with our transfer experiments. Approximately 80% of NKp46$^+$ RORγt^{fm+} cells of the small intestine were RORγt$^+$, but <25% of cells from colon, spleen, or PLNs expressed RORγt (Figures 2B and 2C). This was corroborated by analysis of absolute cell numbers. In *Rorc*$^{gfp/+}$ mice, only the RORγt$^+$ NKR-LTi cells can be visualized, which were lower in numbers compared to the NKp46$^+$RORγt^{fm+} subset from RORγt-fate map mice (Figure S3G). This difference was most visible in organs permissive for RORγt loss (e.g., colon). Analysis of the "NK cell" population revealed the corresponding result. In *Rorc*$^{gfp/+}$ mice, the number of NKp46$^+$RORγt$^-$ cells was always larger compared to the NKp46$^+$RORγt^{fm-} population because the NKp46$^+$RORγt$^-$ population contains cNK cells and RORγt$^-$ NKR-LTi cells (Figures 2A–2C; Figure S3G).

We aimed to identify markers of NKR-LTi cells that were influenced by RORγt expression. In spleen and PLNs, CD4 was expressed by the majority of LTi cells (Kim et al., 2008; Mebius et al., 1997) and by a subpopulation of NKR-LTi cells whereas cNK cells were negative (Figure 2D). CD4$^-$ NKR-LTi cells did not express RORγt, but the CD4$^+$ subset contained all RORγt$^+$ NKR-LTi cells (Figure 2E). However, CD4 did not clearly discriminate between the RORγt$^+$ and the RORγt$^-$ NKR-LTi subsets in PLNs and only a small subpopulation of LTi cells and NKR-LTi cells in the small intestine of adult mice expressed CD4 (Figure S3H). Interestingly, CD25 (IL-2Rα chain) and CCR6 (C-C chemokine receptor 6) expression distinguished between RORγt$^+$ and RORγt$^-$ NKR-LTi cells (Figure 2F). RORγt$^+$ NKR-LTi cells also expressed high sLTα$_1$β$_2$ and CD127, which were diminished (but not absent) in RORγt$^-$ NKR-LTi cells (Figures S3E and S3I). RORγt$^-$ NKR-LTi cells expressed perforin and granzyme B, indicating that they may mediate cell-mediated cytotoxicity (Figure S3D). Thus, RORγt expression of NKR-LTi cells profoundly influences gene expression of cell surface markers and effector molecules.

RORγt$^+$ NKR-LTi Cells Are Direct Progenitors to RORγt$^-$ NKR-LTi Cells

As noted above, transfer of intestinal LTi cells into alymphoid mice led to the generation of both RORγt$^+$ and RORγt$^-$ NKR-LTi cells. To determine whether RORγt$^+$ NKR-LTi cells are the direct progenitors to RORγt$^-$ NKR-LTi cells, we transferred highly purified RORγt$^+$ NKR-LTi cells (Figures S1B and S1C) from the small intestine of RORγt-reporter mice (H-2^b) into alymphoid mice (H-2^d). Two weeks after transfer, most of the donor-derived NKR-LTi cells in the small intestine retained RORγt expression, whereas after 4 weeks, a substantial fraction had downregulated RORγt (Figure 1B). In the environment of the colon or spleen, the same donor cells almost entirely lost RORγt expression (Figure 1B). Similarly, CD4$^+$RORγt$^+$ NKR-LTi cells from PLNs differentiated into CD4$^+$RORγt$^-$ and further into CD4$^-$RORγt$^-$ NKR-LTi cells (Figure 2G). These data indicate that the CD4$^+$ subset of NKR-LTi cells contains RORγt$^+$ NKR-LTi cells and those that have recently lost RORγt. This is further supported by data from a CD4-fate map in which most of the CD4^{fm+} NKR-LTi cells did no longer express CD4, demonstrating that CD4$^-$ NKR-LTi cells have a CD4$^+$ progenitor (Figure 2H). Thus, LTi cells differentiate in a two-step differentiation program characterized by the stable upregulation of NKRs and the progressive loss of RORγt expression: RORγt$^+$ LTi cells → RORγt$^+$ NKR-LTi cells → RORγt$^-$ NKR-LTi cells. Importantly, LTi cell differentiation was restricted to NKR$^+$ cells as shown by the fact that LTi-like cells did not differentiate into any other lymphocyte or myeloid cell population under homeostatic conditions in vivo (Figures S3J–S3L).

Commensal Microflora and IL-7 Stabilize RORγt Expression within NKR-LTi Cells

We and others had previously shown that the population of RORγt$^+$ NKR-LTi cells was diminished in germ-free mice (Sanos et al., 2009; Satoh-Takayama et al., 2008). This could reflect reduced differentiation of LTi-like cells into RORγt$^+$ NKR-LTi cells or microbiota-dependent stabilization of RORγt expression, slowing the progression to RORγt$^-$ NKR-LTi cells.

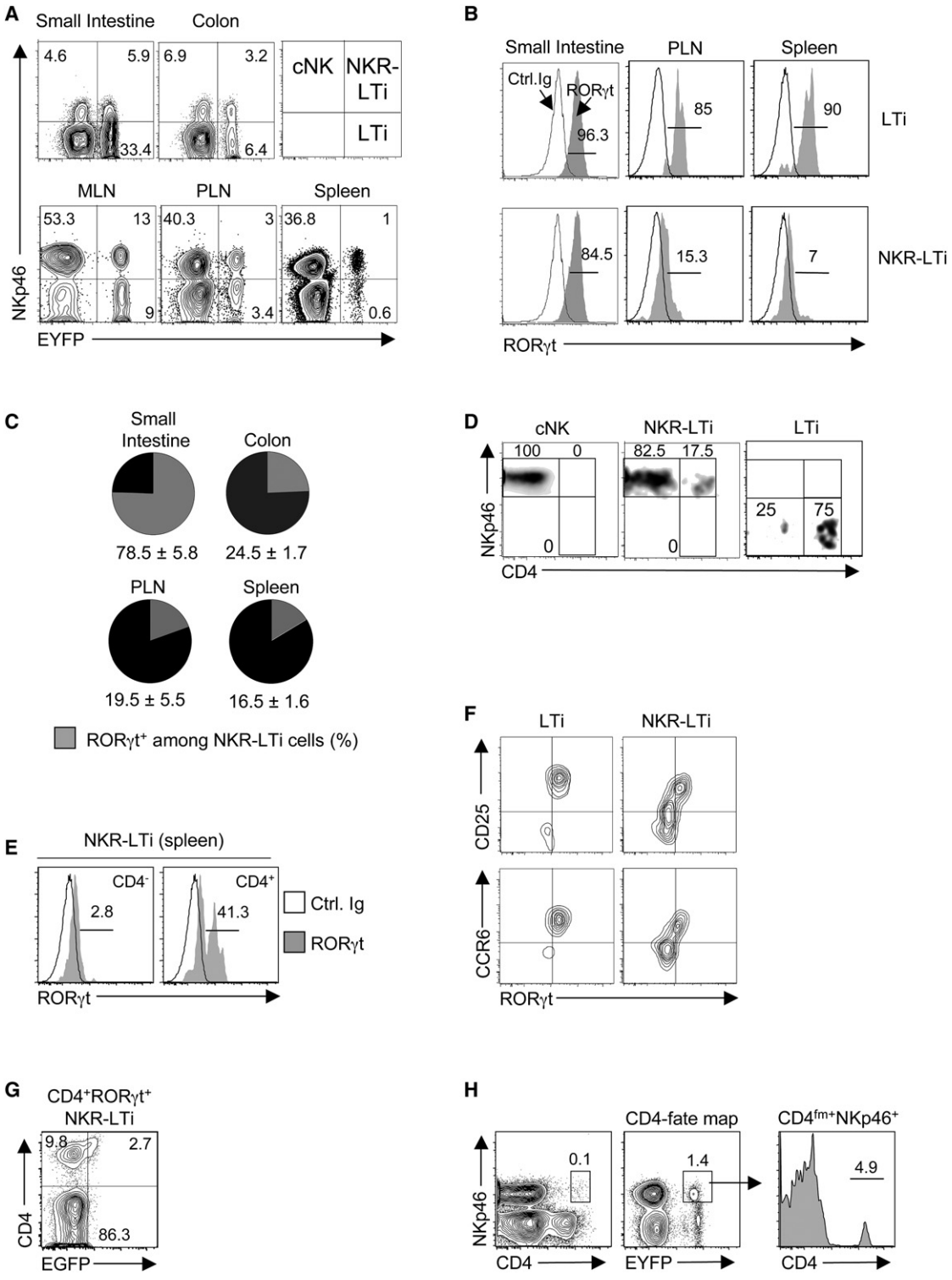

Figure 2. Genetic Lineage Tracing Reveals Progressive Loss of RORγt in NKR-LTi Cells

(A) Flow cytometry analysis of NKp46 and RORγt-fate map (EYFP) in CD3$^-$CD19$^-$ lymphocytes from the indicated organs. Numbers represent percent cells in each quadrant.

(B) Lymphocytes from the indicated organs of RORγt-fate map mice were stained with antibodies specific for CD3, CD19, NKp46, and RORγt or isotype control antibody. Histograms are electronically gated on CD3$^-$CD19$^-$ cells and represent staining of the indicated cell populations with RORγt (gray) or isotype control Ab (open). Numbers indicate percentages of RORγt$^+$ cells.

(C) Percentage (±SEM, n = 7) of RORγt$^+$ cells among all NKR-LTi cells in the indicated organs.

Eradication of the intestinal microflora in RORγt-fate map mice did not lead to substantial differences in cNK, LTi, or NKR-LTi cell populations both in relative and absolute numbers (Figure 3A and data not shown). However, analysis of RORγt expression within the NKR-LTi subset revealed that RORγt$^+$ NKR-LTi cells were significantly diminished in the absence of microbiota (Figures 3A and 3B). Thus, the commensal microflora contributes to the stabilization of RORγt within the NKR-LTi cell subset and not to their differentiation from LTi-like progenitors.

An important question is whether the differentiation of RORγt$^+$ into RORγt$^-$ NKR-LTi cells requires proliferation. We labeled highly purified intestinal RORγt$^+$ NKR-LTi cells with a cell proliferation dye and analyzed proliferation in the context of RORγt expression after in vitro culture. Cells that downregulated RORγt expression had proliferated, whereas RORγt^{hi} NKR-LTi cells did not (Figure 3C). RORγt$^+$ NKR-LTi cells cultured in IL-7 retained RORγt expression and proliferated less, whereas cells cultured in IL-15 proliferated more and downregulated RORγt expression (Figure 3C). Thus, loss of RORγt expression by NKR-LTi cells required proliferation and IL-7 may be an important factor for stabilizing RORγt.

IL-7 expression was reduced in the intestine of germ-free mice, suggesting a mechanism for the destabilization of RORγt expression of NKR-LTi cells (Figure 3D). We analyzed mice deficient in IL-7, IL-7Rα, and TSLPR (thymic stromal lymphopoietin receptor) for the presence of intestinal LTi-like cells, RORγt$^+$ NKR-LTi cells, and NKp46$^+$RORγt$^-$ cells. In extension of previously published data, intestinal LTi-like and RORγt$^+$ NKR-LTi cells were virtually absent in *Il7r*$^{-/-}$ mice that lack IL-7 and TSLP signaling, whereas NK cell development was not impacted (Figure 3E; Satoh-Takayama et al., 2010). In contrast, LTi-like cells developed in *Il7*$^{-/-}$ or *Tslpr*$^{-/-}$ mice, albeit at reduced numbers (Figures 3E and 3F). Thus, IL-7 and TSLP play redundant roles for development and/or survival of intestinal LTi-like cells. Despite equal representation of LTi-like cells in *Il7*$^{-/-}$ and *Tslpr*$^{-/-}$ mice, RORγt$^+$ NKR-LTi cells were significantly reduced in *Il7*$^{-/-}$ (but not in *Tslpr*$^{-/-}$) mice, whereas the numbers of NKp46$^+$RORγt$^-$ cells (composed of cNK cells and RORγt$^-$ NKR-LTi cells) were substantially increased (Figures 3E–3G). We considered that IL-7 stabilizes RORγt expression in NKR-LTi cells. We transferred RORγt$^+$ NKR-LTi cells from *Rorc*$^{gfp/+}$ mice into mice treated with a blocking antibody to the IL-7R or into mice overexpressing IL-7 (IL-7^{Tg} mice). Although the majority of donor-derived NKR-LTi cells retained RORγt expression in mice treated with control Ig, RORγt expression was virtually lost in the absence of IL-7 signaling. In contrast, the generation of RORγt$^-$ NKR-LTi cells was diminished in mice overexpressing IL-7 (Figure 3H). These data were confirmed by blocking IL-7R signaling in RORγt-fate map mice (Figure 3I; Figure S4A). Collectively, our data demonstrate that IL-7 but not TSLP maintains the population of RORγt$^+$ NKR-LTi cells.

No significant differences in absolute and relative numbers of LTi-like cells, RORγt$^+$ NKR-LTi cells, and NKp46$^+$RORγt$^-$ cells were found in *Il6*$^{-/-}$, *Il1r*$^{-/-}$, and *Il23p19*$^{-/-}$ mice (Figures S4B–S4I). Interestingly, mice deficient for *Il12a* (encoding IL-12p35) or *Il12rb2,* which is required for IL-12 but not IL-23 signaling, showed a significant increase in the fraction of intestinal RORγt$^+$ NKR-LTi cells (Figures S4F–S4I). This may imply that IL-12 destabilizes RORγt expression in NKR-LTi cells.

Human "NK-22" but Not NK Cells Developmentally Depend on IL7R Signaling

Human IL-22-producing CD56$^+$NKp44$^+$ cells isolated from tonsils expressed *IL22* and *RORC,* whereas conventional CD56$^+$NKp44$^-$ NK cells did not (Figure 4A; Cella et al., 2010). A 4-stage differentiation program for human "NK cells" from progenitor cells within secondary lymphoid organs was proposed and it was found that "stage 3 NK cells" contained IL-22-producing cells (Freud et al., 2006; Hughes et al., 2009). Although stage 1, 2, and 4 NK cells from peripheral blood and tonsils did not express *IL22* or *RORC*, "stage 3 NK cells" expressed high *RORC* and *IL22* (Figure 4B and data not shown). Because cNK cells and their progenitors did not express RORγt (Figure 2A), we reasoned that human IL-22-producing RORC$^+$ CD56$^+$ cells may represent human NKR-LTi cells. Mutations of the *IL7R* gene in humans cause severe combined immunodeficiency disease (SCID), and analysis of immune cells from these patients revealed a substantial reduction of T cells, whereas NK cells and B cells developed normally (Figure 4C; Lai et al., 1997; Puel et al., 1998). We investigated the representation of RORC$^+$ IL-22-producing "stage 3 NK cells" in humans with *IL7R* deficiency. "Stage 3 NK cells" were virtually absent in patients with *IL7R* deficiency whereas stage 1, 2, and 4 were represented normally (Figure 4D). Considering our mouse data showing that cNK cells at no time express RORγt and that they do not require IL-7 for their development, these data indicate that human IL-22-producing lymphocytes are not part of the cNK cell lineage but may be a subset of human NKR-LTi cells.

RORγt Levels Assign Distinct Functional Programs to NKR-LTi Cells

We found a previously unappreciated plasticity within RORγt$^+$ innate lymphoid cells, allowing us to identify discrete LTi

(D) Splenocytes from RORγt-fate map mice were stained for CD3, CD19, NKp46, and CD4. Density plots are electronically gated on CD3$^-$CD19$^-$ cells and depict the proportion of CD4- and NKp46-expressing cells within the indicated cell populations.
(E) Splenocytes from RORγt-fate map mice were stained for CD3, CD19, NKp46, CD4, and RORγt (gray) or with isotype control antibody (open). Histograms are gated on CD3$^-$CD19$^-$ cells and depict expression of RORγt within CD4$^+$ and CD4$^-$ NKR-LTi cells. Numbers indicate percentages of RORγt$^+$ cells.
(F) Splenocytes from RORγt-fate map mice were stained for CD3, CD19, NKp46, CD25, CCR6, and RORγt. Density plots are electronically gated on CD3$^-$CD19$^-$ cells and show coexpression of RORγt and CD25 (top) or CCR6 (bottom).
(G) 2 × 10^4 splenic CD4$^+$RORγt$^+$ NKR-LTi cells of *Rorc*$^{gfp/+}$ mice (H-2^b) were transferred into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (H-2^d). Two weeks later, CD4 and RORγt (EGFP) expression by donor-derived cells (H-2^b) was determined. Numbers represent percent cells in quadrants.
(H) Splenocytes from CD4-fate map mice were stained for CD3, CD19, NKp46, and CD4. Contour plots are gated on CD3$^-$CD19$^-$ cells and depict expression of NKp46 and CD4 or CD4-fate map (EYFP). Histogram shows the percentage of NKp46$^+$CD4^{fm+} cells expressing CD4.
Data are representative of ten (A), seven (C), four (B, D–G) or two (H) experiments.

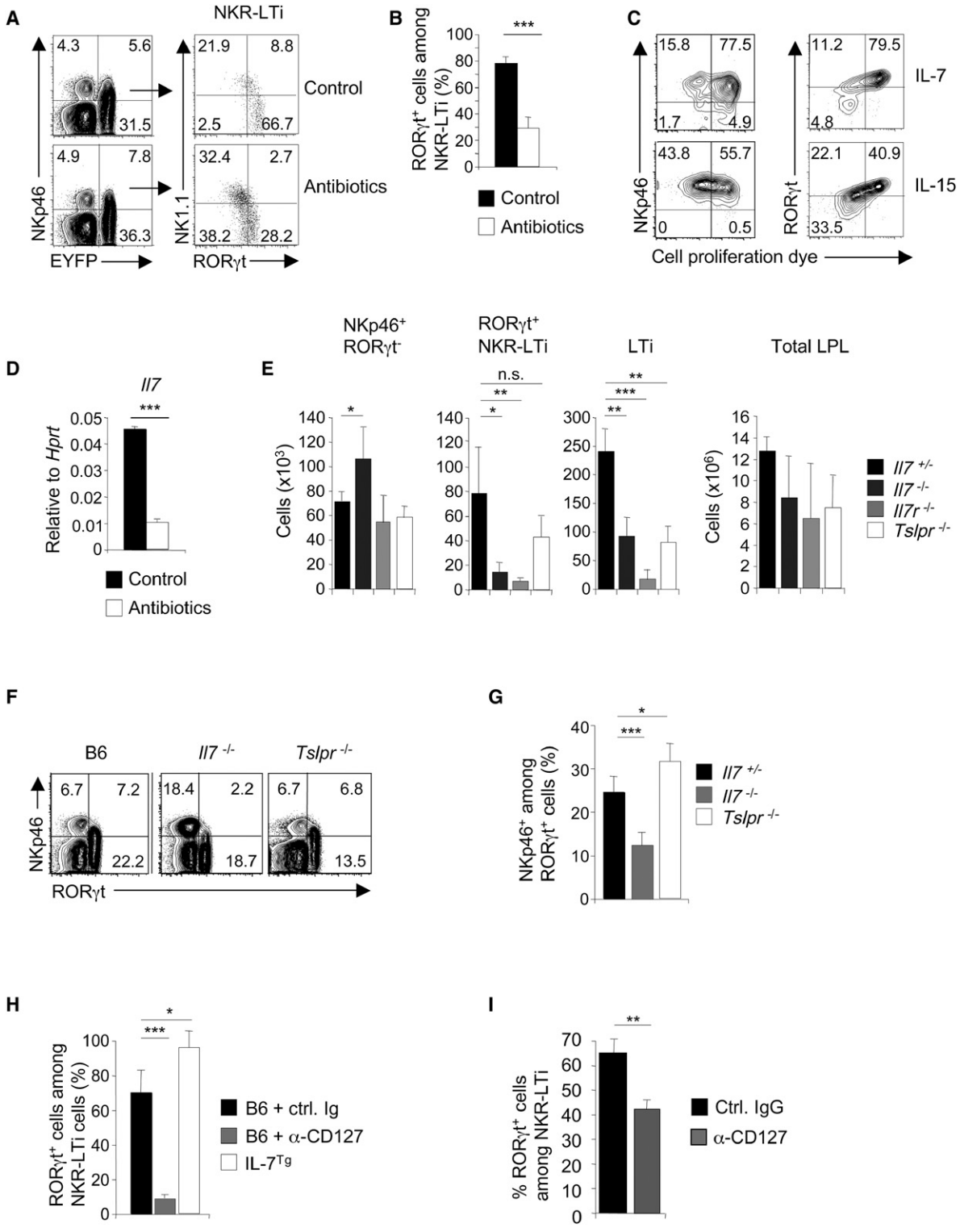

Figure 3. Commensal Microflora and IL-7 Stabilize RORγt Expression in NKR-LTi Cells

(A and B) Lymphocytes from the small intestine of conventional and antibiotic-treated RORγt-fate map mice were stained with antibodies specific for CD3, CD19, NKp46, NK1.1, and RORγt.

(A) Contour plots (left) are electronically gated on $CD3^{-}CD19^{-}$ cells and depict expression of $ROR\gamma t^{fm}$ (EYFP) and NKp46. Dot plots (right) are electronically gated on NKR-LTi cells and represent staining for RORγt and NK1.1. Numbers indicate percentages.

(B) Percentage (±SEM, n = 3) of $ROR\gamma t^{+}$ cells among NKR-LTi cells in conventional and antibiotic-treated RORγt-fate map mice.

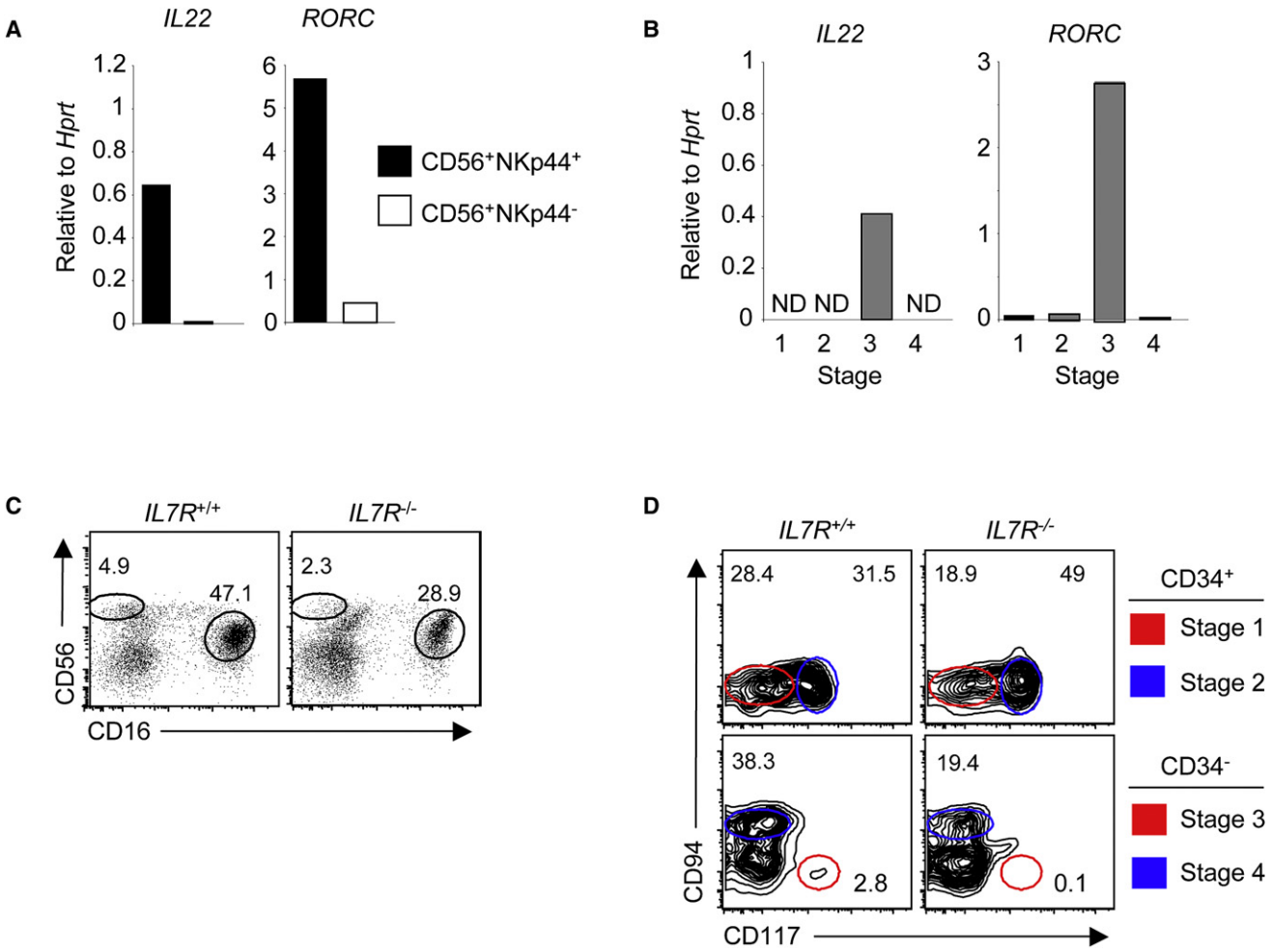

Figure 4. Human IL-22-Producing Cells Depend on IL-7 for Their Development

(A and B) Quantitative RT-PCR analysis of *IL22* and *RORC* expression by the indicated populations from human tonsils (A) or peripheral blood (B). Stage 1, $CD34^+CD117^-CD94^-$; Stage 2, $CD34^+CD117^+CD94^-$; Stage 3, $CD34^-CD117^+CD94^-$; Stage 4, $CD34^-CD117^-CD94^+$.

(C and D) Mononuclear cells were stained with antibodies specific for the indicated markers and for CD3 and CD19. Dot plots (C) were electronically gated on $CD3^-CD19^-$ cells. Contour plots (D) exclude all $CD3^+$ and $CD19^+$ cells and are electronically gated on $CD34^+$ (stage 1 and stage 2) or $CD34^-$ cells (stage 3 and stage 4). Numbers indicate percentage of cells within the adjacent gates. Data are representative of two independent experiments.

cell-derived populations that develop along a decreasing gradient of RORγt. IL-12 and IL-23 are related heterodimeric cytokines acting on NK- and LTi-derived cells that signal through related receptors (Oppmann et al., 2000). We investigated the expression of the IL-12 and IL-23 receptors (R) by cNK cells and LTi-derived cells. The IL-12R and IL-23R complexes share the IL-12 and IL-23R β1 chain that forms a heterodimer with the IL-12-specific IL-12Rβ2 chain or the IL-23-specific IL-23R.

(C) Highly purified $ROR\gamma t^+$ NKR-LTi cells from the small intestine were labeled with a cell proliferation dye and cultured in IL-7 or IL-15 for 6 days. Cells were stained for NKp46 and RORγt. Numbers indicate percentages in each quadrant.

(D) Quantitative RT-PCR analysis (± SEM, n = 3) of *Il7* expression in small intestine of control mice and germ-free mice.

(E) Absolute cell numbers (±SD, n = 5) of the indicated cell populations in the lamina propria of the small intestine.

(F and G) Lymphocytes of the small intestine were stained with antibodies specific for CD3, CD19, NKp46, and RORγt.

(F) Contour plots are electronically gated on $CD3^-CD19^-$ cells and numbers indicate percentages within quadrants.

(G) Percentage (±SD, n = 5) of $NKp46^+$ cells among all $ROR\gamma t^+$ cells.

(H) 2×10^4 highly purified $ROR\gamma t^+$ NKR-LTi cells (CD45.1) from the small intestine were transferred into groups of B6 (CD45.2) or $IL\text{-}7^{Tg}$ mice (CD45.2). B6 mice were treated with IL-7Rα antibodies or control Ig. Percentages (±SEM, n = 3) of $ROR\gamma t^+$ cells among donor-derived cells ($CD45.1^+$) were determined 2 weeks after transfer.

(I) RORγt-fate map mice were treated with either IL-7Rα antibodies or control Ig. Percentages (±SEM, n = 3) of $ROR\gamma t^+$ cells among NKR-LTi cells were determined after 6 days.

Data are representative of at least three independent experiments. n.s.: statistically not significant; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

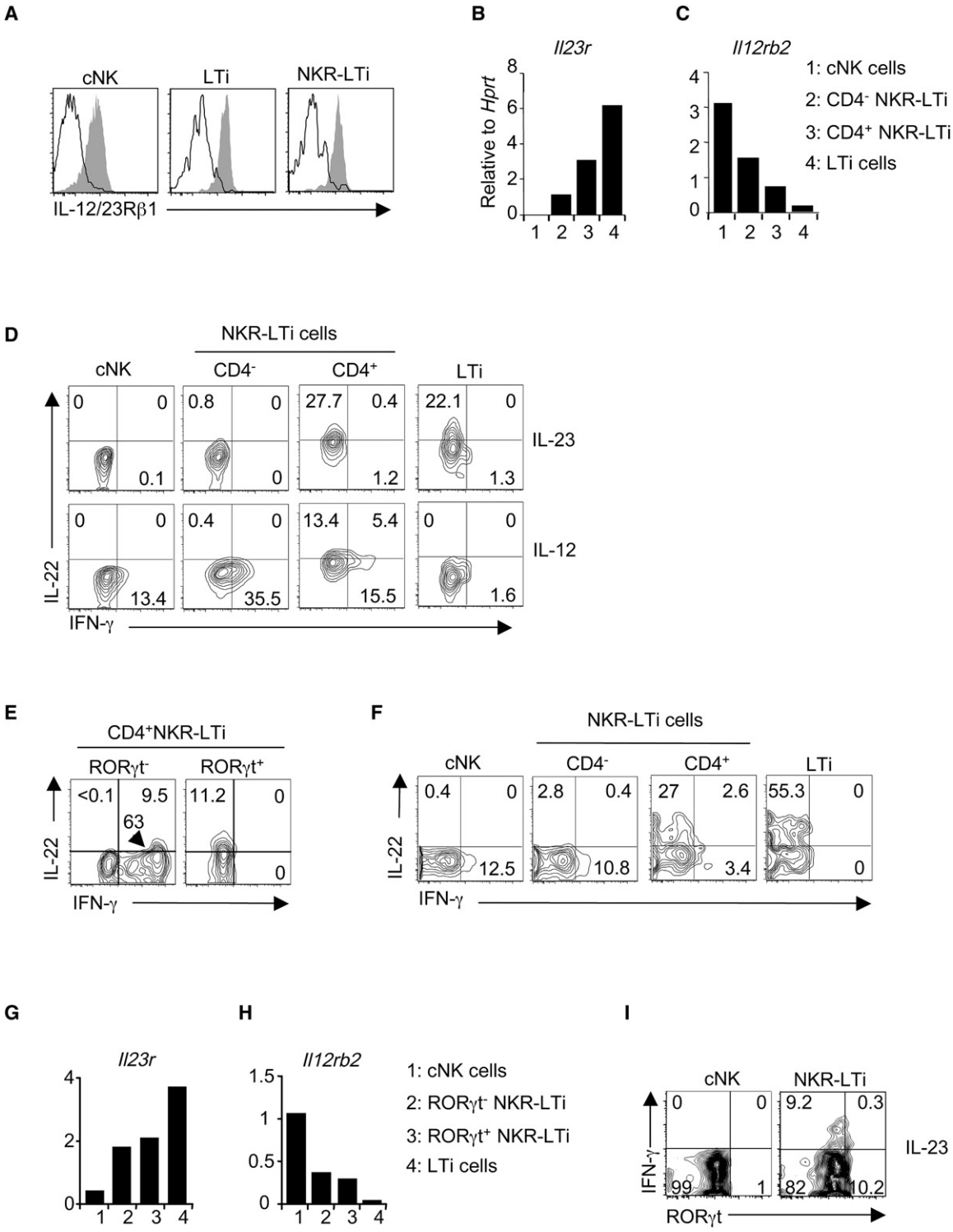

Figure 5. RORγt Expression within NKR-LTi Cells Determines Distinct Functional Fates

(A) Splenocytes from RORγt-fate map mice were stained for CD3, CD19, NKp46, IL-12/23Rβ1 (gray), or isotype control antibody (open). Histograms depict electronic gating on the indicated cell populations after exclusion of $CD3^+$ and $CD19^+$ cells.

(B, C, G, H) Quantitative RT-PCR analysis of the expression of *Il23r* (B, G) and *Il12rb2* (C, H) in sorted cell populations from spleen (B, C) or colon (G, H) of RORγt-fate map mice.

(D and E) Cytokine production of highly purified cell populations from spleens of RORγt-fate map mice (D) or $Rorc^{gfp/+}$ mice (E) stimulated for 24 hr with the indicated cytokines. Contour plots depict staining with anti-IL-22 and anti-IFN-γ. The numbers indicate the percentage of cells in each quadrant.

The β1 chain was detected on the surface of all cNK cells, LTi cells, and NKR-LTi cells from PLNs, spleen, and intestine (Figure 5A and data not shown). Expression of *Il23r* correlated with RORγt expression and was highest in LTi cells and CD4$^+$ NKR-LTi cells whereas CD4$^-$ (i.e., RORγt$^-$) NKR-LTi cells expressed very low and cNK cells had almost undetectable amounts of *Il23r* (Figure 5B). In contrast, expression of *Il12rb2* inversely correlated with RORγt expression (Figure 5C).

We stimulated highly purified lymphocytes from PLNs of RORγt-fate map mice with IL-12 or IL-23. Stimulation of LTi cells and CD4$^+$ NKR-LTi cells (containing all RORγt$^+$ NKR-LTi cells) with IL-23 led to IL-22 but not IFN-γ production (Figure 5D). CD4$^-$ (i.e., RORγt$^-$) NKR-LTi cells and cNK cells did not produce IL-22 or IFN-γ in response to IL-23, and LTi cells did not produce either cytokine in response to IL-12, which is in agreement with their very low *Il23r* or *Il12rb2* expression, respectively (Figures 5B–5D). After IL-12 stimulation, highly purified cNK cells produced IFN-γ but not IL-22 (Figure 5D). Consistent with their substantial expression of *Il12rb2*, CD4$^-$ (i.e., RORγt$^-$) NKR-LTi cells also produced IFN-γ in response to IL-12 stimulation but had lost the potential to produce IL-22 (Figure 5D). CD4$^+$ NKR-LTi cells that contain both RORγt$^+$ and RORγt$^-$ NKR-LTi subsets (Figure 2E) showed a more complex cytokine production pattern in response to IL-12. Although a substantial population of CD4$^+$ NKR-LTi cells produced IL-22, we noted a similar fraction of IFN-γ producers and even a highly reproducible subset producing both cytokines (Figure 5D). CD4$^+$ NKR-LTi cells are composed of RORγt$^+$ NKR-LTi cells and RORγt$^-$ NKR-LTi cells that have very recently lost RORγt expression (Figures 2E, 2G, and 2H). We reasoned that the cytokine production profiles may be governed by the different amounts of RORγt. Indeed, CD4$^+$RORγt$^+$ NKR-LTi cells produced IL-22, whereas CD4$^+$RORγt$^-$ NKR-LTi cells produced either IFN-γ alone or both IFN-γ and IL-22 (Figure 5E). Very similar data were obtained after in vivo stimulation of NKR-LTi cells. Splenic LTi cells and CD4$^+$ NKR-LTi cells robustly produced IL-22 (Figure 5F). Although LTi cells did not produce IFN-γ, a small fraction of IFN-γ-producing cells was detected within the population of CD4$^+$ NKR-LTi cells that contains RORγt$^-$ NKR-LTi cells. As in the in vitro experiments, cNK cells and CD4$^-$ (i.e., RORγt$^-$) NKR-LTi cells did not produce IL-22 but were instead IFN-γ producers (Figure 5F). Our data demonstrate that the gradual loss of RORγt expression within NKR-LTi cells functions as a molecular rheostat for their responsiveness to IL-12 family cytokines and for their cytokine expression program.

RORγt$^-$ NKR-LTi Cells Are Potent Initiators of Experimental Innate Colitis

RORγt$^+$ NKR-LTi cells and LTi cells are constitutive IL-22 producers and promote epithelial homeostasis by regulating expression of tissue-protective genes (e.g., *Reg3* genes) (Sanos et al., 2009). We attempted to assign function to the previously unrecognized population of RORγt$^-$ NKR-LTi cells that is the dominating subset of NKR-LTi cells in the colon (Figure 2C). In contrast to RORγt$^-$ NKR-LTi cells from PLN, colonic RORγt$^-$ NKR-LTi cells retained *Il23r* expression (Figures 5B, 5G, and 5H). Interestingly, colon RORγt$^-$ NKR-LTi cells but not RORγt$^+$ NKR-LTi, LTi, or cNK cells produced IFN-γ in response to IL-23 (Figure 5I and data not shown). IL-23-dependent IFN-γ production is required for the development of experimental colitis in *Rag2*$^{-/-}$ mice after systemic administration of CD40 antibodies but the colitogenic cell type is poorly defined (Buonocore et al., 2010; Diefenbach and Vonarbourg, 2010; Uhlig et al., 2006). *Rag2*$^{-/-}$ mice injected with anti-CD40 developed extensive colitis but *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice that lack all lymphocytes did not (Figures 6A and 6B). Colitis onset in *Rag2*$^{-/-}$ mice correlated with the presence of IFN-γ-producing cells absent in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (Figure S5A). Thus, an innate IFN-γ-producing lymphocyte subset is required for pathogenesis.

CD40 injection into *Rag2*$^{-/-}$ mice led to the induction of IFN-γ production by NKp46$^+$RORγt$^-$ cells containing cNK cells and RORγt$^-$ NKR-LTi cells (Figure 6C). RORγt$^+$ NKR-LTi cells and LTi cells did not produce IFN-γ but instead released IL-22 (Figure 6C). It should be noted that the fraction of IL-22- or IL-17-producing cells was not augmented after CD40 stimulation (Figure 6C; Figure S5B), which is in line with previous data indicating that IL-17 is not involved in the pathogenesis of CD40 colitis (Buonocore et al., 2010). To determine whether RORγt$^-$ NKR-LTi cells or cNK cells are a cellular source of IFN-γ in this model, we injected anti-CD40 into *Il15*$^{-/-}$ mice that lack all cNK cells (Kennedy et al., 2000) but normally develop LTi-like and NKR-LTi cells (Sanos et al., 2009). Anti-CD40 injection into *Il15*$^{-/-}$ mice led to IFN-γ production by NKp46$^+$ cells, indicating that RORγt$^-$ NKR-LTi cells may be the IFN-γ producers (Figure 6D). However, it remained possible that both cNK cells and RORγt$^-$ NKR-LTi cells contributed to the pool of IFN-γ-producing, colitis-promoting cells in normal mice. To definitively identify the colitogenic IFN-γ-producing cell type, we transferred highly purified cells from RORγt-fate map mice into alymphoid mice and injected CD40 antibodies 5 days later. During this time, LTi cells and RORγt$^+$ NKR-LTi cells did not appreciably differentiate into RORγt$^-$ cells (data not shown). Only RORγt$^-$ NKR-LTi cells produced IFN-γ after CD40 injection whereas cNK cells, RORγt$^+$ NKR-LTi cells, and LTi cells did not (Figure 6E). Thus, RORγt$^-$ NKR-LTi cells are the main source of IFN-γ during experimental colitis. The colitis-promoting role of RORγt$^-$ NKR-LTi cells was further confirmed in cell transfer experiments. Only mice transferred with RORγt$^-$ NKR-LTi cells developed colitis comparable to *Rag2*$^{-/-}$ mice (Figures 6F–6I). Our data extend previous work reporting that the colitogenic cells reside within a subset of Thy1$^+$Sca1$^+$ innate lymphoid cells (Buonocore et al., 2010). The Thy1$^+$Sca1$^+$ population is heterogenous and contains RORγt^{fm-} and RORγt^{fm+} cells including IFN-γ-producing RORγt$^-$ NKR-LTi cells (Figure S5C). Thus, RORγt$^-$ NKR-LTi cells but not cNK cells trigger innate colitis.

(F) Groups of RORγt-fate map mice were injected with LPS. 18 hr later, splenocyte suspensions were stained with antibodies specific for CD3, CD19, NKp46, CD4, IL-22, and IFN-γ. Contour plots are electronically gated on the indicated cell populations after exclusion of all CD3$^+$ and CD19$^+$ cells. Numbers indicate percentage of cells in each quadrant.

(I) Highly purified lamina propria lymphocytes from the colon of RORγt-fate map mice were cultured for 24 hr in IL-23. Contour plots depict expression of IFN-γ and RORγt. Numbers indicate the percentage of cells in each quadrant.

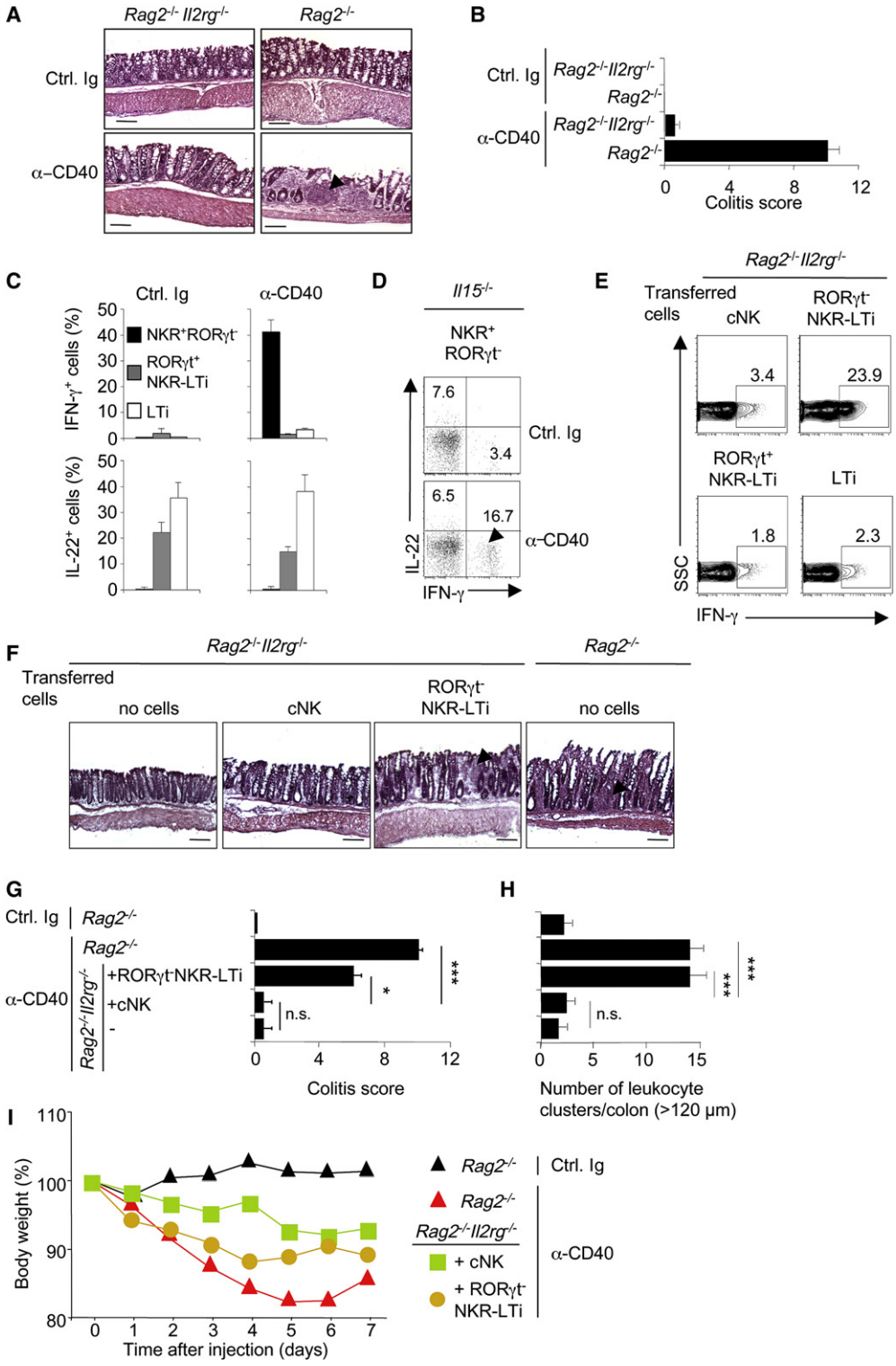

Figure 6. RORγt⁻ NKR-LTi Cells Induce CD40-Triggered Colitis

(A–D) Groups of *Rag2*$^{-/-}$, *Il15*$^{-/-}$, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were injected with control Ig or anti-CD40 and were analyzed 7 days later.

(A) Histological analysis of colon sections (H&E stain). Arrowhead points to a cluster of infiltrating leukocytes that can be found only in mice with colitis. Scale bars represent 100 μm.

(B) Clinical colitis score (±SEM; n = 5).

(C and D) Lamina propria lymphocytes from colon were stained for CD45, NKp46, RORγt, IL-22, and IFN-γ.

(C) Bar diagrams show percentages (±SEM; n = 5) of IFN-γ- or IL-22-producing cells within the indicated lymphocyte populations.

Finally, we investigated colitis development in *Rag2*$^{-/-}$ mice depleted of cNK cells or NKR-LTi cells. Application of anti-asialo-GM1 resulted in the depletion of most cNK cells, whereas NKR-LTi cells and LTi cells were largely unaffected (Figure 7A). Although almost all cNK cells were depleted, only a third of cNK cells could be stained with the asialo-GM1 antiserum (Figure 7A; Figure S6A). It is appreciated that the isolation procedure from the intestinal lamina propria involving enzymatic digests may interfere with the consecutive detection of antigens (Sanos and Diefenbach, 2010). Intestinal RORγt^{-} NKR-LTi cells and cNK cells expressed high levels of NK1.1 and were depleted after injection of anti-NK1.1, whereas RORγt^{+} NKR-LTi cells were NK1.1^{dim} and (similar to NK1.1^{-} LTi cells) were not depleted (Figure 7A; Figure S6B). NK cells, NKR-LTi cells, and LTi-like cells expressed high Thy1 (Figures S5C and S6C) and anti-Thy1 injections led to the depletion of virtually all innate lymphocyte populations (Figure 7B). Depletion of cNK cells by anti-asialo-GM1 injection did not reduce the fraction of IFN-γ- or TNF-producing cells in the colon and did not ameliorate colitis development (Figures 7B–7G). TNF was produced by myeloid cells phenotypically resembling macrophages or monocyte-derived cells (Figure S6D; Bogunovic et al., 2009; Varol et al., 2009). In contrast, depletion of NK1.1^{+} cells, including RORγt^{-} NKR-LTi cells, led to significant amelioration of the disease (Figures 7B–7G). Not surprisingly, the depletion of all innate lymphocytes by injection of Thy1 antibodies also prevented colitis development (Figures 7B–7G). Collectively, these data show that IFN-γ-producing RORγt^{-} NKR-LTi cells are potent inducers of experimental colitis.

DISCUSSION

It is an important and unresolved question where to position the recently identified population of IL-22-producing NKp46^{+} RORγt^{+} cells on hematopoietic lineage maps (Cooper et al., 2009). Because Id2-deficient mice lack both LTi-like cells and NK cells and because LTi-like cells could be differentiated into NK-like cells in vitro, it was conceivable that LTi cells might be progenitors to all NKR-expressing cells (Mebius et al., 1997; Satoh-Takayama et al., 2010; Yokota et al., 1999). Lineage tracing data via an RORγt-fate map excludes the development of cNK cells and recently identified "thymic NK cells" (Vosshenrich et al., 2006) from RORγt^{+} precursors. Thus, cNK cells and LTi-like cells are developmentally distinct and constitute separate innate lymphocyte lineages.

Do NKp46^{+}RORγt^{+} cells then differentiate from NK cell or LTi cell precursors or do they constitute a distinct third lineage of innate lymphocytes? An intriguing hypothesis was that NK cells upregulated RORγt under the influence of the intestinal micromilieu and were instructed to a particular functional fate (i.e., IL-22 production). However, we failed to detect upregulation of RORγt by cNK cells after in vivo transfer or under any in vitro conditions tested, including those known to induce RORγt expression by CD4 T cells. Thus, cNK cells are not the progenitors to NKp46^{+} RORγt^{+} cells.

We next considered that NKp46^{+}RORγt^{+} cells may represent LTi-like cells that upregulate NKRs. This view was supported by our previous data showing that LTi-like cells and NKp46^{+}RORγt^{+} cells share a similar developmental program (Sanos et al., 2009). In addition, LTi cells can upregulate NKRs when cultured under specific in vitro conditions (Cupedo et al., 2009; Mebius et al., 1997). However, these conclusions relied on in vitro experiments and on the definition of LTi cells by a combination of cell surface markers, some of which (e.g., CD127, c-kit, CD25) have now been shown to be also expressed by natural helper cells (Moro et al., 2010). RORγt is the lineage-defining transcription factor of LTi-like cells and is not expressed by any other of the recognized innate lymphocyte lineages (Moro et al., 2010). Our lineage tracing data also show that LTi-like cells permanently express RORγt. Therefore, RORγt can be used to genetically mark LTi-like cells (Eberl et al., 2004; Moro et al., 2010). By using a combination of genetic lineage tracing for RORγt and cell transfer experiments of genetically tagged LTi cells, we demonstrated that NKR^{-}RORγt^{+} cells are direct progenitors to NKR^{+}RORγt^{+} cells but do not have the potential to develop into myeloid cells, B cells, or T cells in vivo. These data firmly place LTi-like cells within the lymphocyte lineage and demonstrate that the differentiation potential of LTi cells in vivo is more limited than previously appreciated. Although the marker profile of NKR^{-}RORγt^{+} (LTi-like) cells was quite homogenous and the development of the entire population depended on RORγt, subsets could be discriminated on the basis of CD4 expression and different levels of CD127 and c-kit. However, their shared developmental program and data from CD4-fate mapping and in vivo transfer studies support the view that these subsets represent differentiation states of LTi-like cells. Future work will need to address the developmental relationships of these subsets within the NKR^{-}RORγt^{+} population of innate lymphoid cells.

Do IL-22^{+} NKR-LTi cells constitute a stable cell fate? We identified three stages of LTi cell differentiation that proceeded along a decreasing gradient of RORγt expression. In the first transition,

(D) Dot plots depict expression of IFN-γ and IL-22 by NKR^{+}RORγt^{-} cells. Numbers indicate the percentage of cells in each quadrant.
(E–I) 5 × 10^{4} highly purified cells from the intestine of RORγt-fate map mice (H-2^{b}) were transferred into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (H-2^{d}).
(E) Five days after transfer, mice were injected with anti-CD40. Seven days after CD40 injection, donor cells were analyzed for IFN-γ production. Numbers indicate percentage of IFN-γ^{+} cells.
(F–I) Two weeks after transfer, mice were injected with control Ig or anti-CD40 and analyzed 7 days later.
(F) Histological analysis of colon sections (H&E stain). Arrowheads point to clusters of infiltrating leukocytes that can be found only in mice with colitis. Scale bars represent 100 μm.
(G) Clinical colitis score (±SEM; n = 3).
(H) Absolute numbers (±SEM; n = 3) of leukocyte clusters (>120 μm) per colon.
(I) Weight as a percentage of the initial weight at day 0. Data represent the mean weight and are pooled from three independent experiments (n = 5 mice per group). Error bars were omitted for clarity.
n.s.: statistically not significant; *p < 0.05, **p < 0.01, ***p < 0.001.

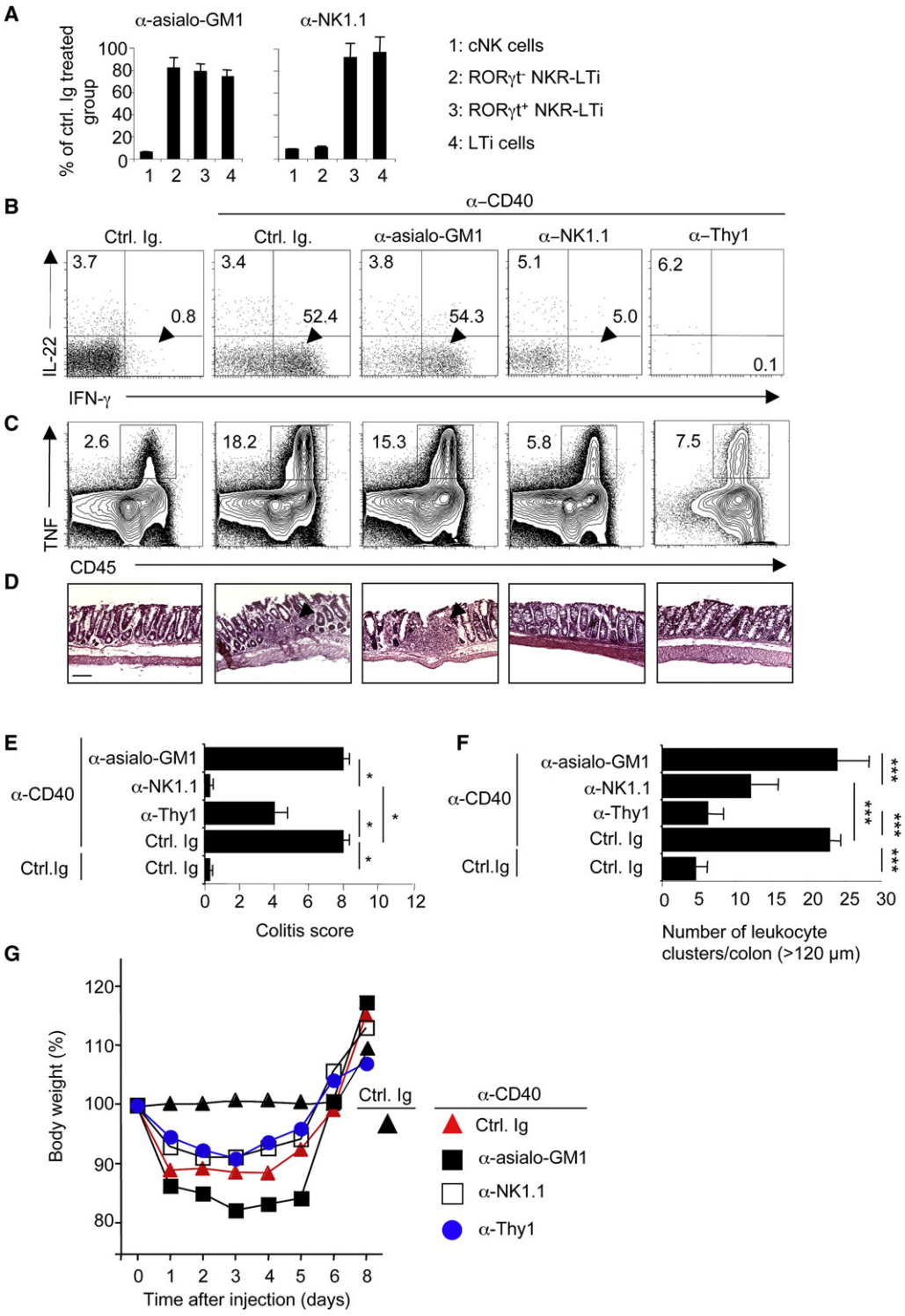

Figure 7. Depletion of RORγt⁻ NKR-LTi Cells Ameliorates Colitis

(A) Groups of RORγt-fate map were treated twice with the indicated antibodies. Two days after the last injection, intestinal lamina propria cells were stained with NKp46, RORγt, CD3, and CD19. After exclusion of all $CD3^+$ and $CD19^+$ cells, the indicated populations were quantified. The data represent the percentage (±SD; n = 5) of remaining cells compared to control Ig-treated groups.

(B–G) Groups of *Rag2*$^{-/-}$ mice were treated with the indicated antibodies and injected with control Ig or anti-CD40. Seven days later, lamina propria lymphocytes from colon were stained for CD45, NKp46, RORγt, IL-22, IFN-γ, or TNF.

(B) The dot plots represent the analysis of $CD45^+NKp46^+ROR\gamma t^-$ cells that contain both cNK cells and $ROR\gamma t^-$ NKR-LTi cells. Numbers represent percentage of cells in the respective quadrants.

LTi-like cells (stage 1) acquired NKRs while maintaining RORγt expression (stage 2). It is remarkable that a fraction of LTi-like cells in the small intestine did not acquire NKRs even 6 months after transfer. Perhaps, a subpopulation of NKR$^-$RORγt$^+$ LTi-like cells has self-renewing potential. While NKR expression remained stable, NKR-LTi cells progressively lost RORγt and this transition to the third stage may require proliferation. The fraction of RORγt$^-$ NKR-LTi cells varied from organ to organ, suggesting that molecular cues from the microenvironment may either stabilize RORγt expression or promote its loss. Although the exact nature of these cues is poorly defined, we now demonstrate that signals from the commensal microbiota stabilize RORγt expression in NKR-LTi cells. This provides a mechanistic explanation for our previous observation that the numbers of RORγt$^+$ NKR-LTi cells were decreased whereas those of NKR$^+$RORγt$^-$ cells were increased in germ-free mice (Sanos et al., 2009; Satoh-Takayama et al., 2008). The finding that the fraction of RORγt$^-$ NKR-LTi cells was less represented in the colon than in the small intestine indicates that stabilization of RORγt is not simply proportional to the number of bacteria. Perhaps, specific microbiota present in the small intestine but not in the colon promote RORγt expression. Furthermore, we have uncovered an important role for IL-7, but not for the related cytokine TSLP, in maintaining the pool of RORγt$^+$ NKR-LTi cells. IL-7 amounts were significantly decreased in germ-free mice, providing a framework for the action of IL-7 in the maintainence of RORγt$^+$ NKR-LTi cells.

The level of RORγt expression determined the phenotype and function of NKR-LTi cells. Whereas RORγt$^+$ LTi-like cells and RORγt$^+$ NKR-LTi cells were potent producers of IL-22 and expressed sLTα$_1$β$_2$, RORγt$^-$ NKR-LTi cells instead expressed IFN-γ and upregulated expression of perforin and granzyme B. Graded expression of RORγt served as a rheostat for the responsiveness of NKR-LTi cells to the related cytokines IL-12 and IL-23. These results are reminiscent of a previous report regarding the plasticity of Th17 cells that can downregulate RORγt expression and become Th1-like IFN-γ-producing cells that promote autoimmune colitis (Lee et al., 2009).

Development and/or survival of intestinal and peripheral LTi-like cells strictly required IL-7R signaling (Luther et al., 2003; Meier et al., 2007; Satoh-Takayama et al., 2010; Schmutz et al., 2009). Although LTi-like cells in PLN and spleen were absent in *Il7*$^{-/-}$ mice (Schmutz et al., 2009), intestinal LTi-like cells were present. Our data demonstrate that TSLP and IL-7 play redundant roles for the differentiation of intestinal LTi-like cells. This extends our previous data showing that overexpression of TSLP in *Il7*$^{-/-}$ mice can rescue the differentiation of PLN and splenic LTi cells (Chappaz and Finke, 2010). Because LTi cells and NKR-LTi cells but not cNK cells strictly required IL-7R signaling for their development, we investigated the development of cNK cells and IL-22-producing NKR$^+$ cells in patients lacking IL-7R expression. Patients with *IL7R* deficiency had a normal cNK cell compartment but selectively lacked IL-22-producing NKR$^+$ cells. These data demonstrate that human IL-22-producing NKR$^+$ cells share a common developmental program with LTi-like cells and may not be associated with the NK cell lineage.

Collectively, our data also enable us to more accurately name these cells. Previous attempts (NK-22, NCR22) took into account only their production of IL-22 and the expression of NKRs (Cella et al., 2009; Satoh-Takayama et al., 2010). However, IL-22-producing NKR-LTi cells are directly derived from LTi-like progenitors and are just one discrete stage in a wider ranging differentiation program. Thus, the designation NKR-LTi cells more accurately reflects their lineage relationships and the designation NK-22 or NCR22 may be appropriate only for the subpopulation of IL-22-producing RORγt$^+$ NKR-LTi cells.

Previously, RORγt$^-$ NKR-LTi cells have gone unnoticed because they were contained within the population of "NK cells" (i.e., NKp46$^+$CD3$^-$) that in fact is a composite of two developmentally and functionally distinct lymphocyte lineages: cNK cells and LTi-derived NKR$^+$ cells. We attempted to assign function to RORγt$^-$ NKR-LTi cells. In mice lacking all lymphocytes of the adaptive immune system (*Rag2*$^{-/-}$), autoimmune colitis can be triggered by the application of CD40 antibodies, suggesting that components of the innate immune system are sufficient for the induction of colitis (Uhlig et al., 2006). Colitis development required production of IFN-γ or TNF but the colitogenic cell type was unknown (Uhlig et al., 2006). A recent report demonstrated that CD40 colitis could not be triggered in mice genetically lacking RORγt (Buonocore et al., 2010). These mice lack all LTi-derived cells but also do not have lymph nodes or intestinal lymphoid clusters (Sun et al., 2000). It was not explored whether colitis was diminished because of the absence of LTi-derived cells or because of the lack of anatomical sites for leukocyte interactions (Diefenbach and Vonarbourg, 2010). By using gain- and loss-of-function experiments, we demonstrate that RORγt$^-$ NKR-LTi cells are the colitogenic, IFN-γ-producing innate lymphocyte subset. Thus, RORγt$^-$ NKR-LTi cells are required and sufficient for the development of innate colitis. Why are RORγt$^-$ NKR-LTi cells such potent inducers of colitis? An important pathological feature of colitis, as well as of other chronic inflammatory processes, are de novo generated proinflammatory leukocyte clusters (Aloisi and Pujol-Borrell, 2006; Izcue et al., 2009). RORγt$^-$ NKR-LTi cells may still have lymphoid tissue-inducing potential as they express sLTα$_1$β$_2$. Perhaps the composite qualities of proinflammatory cytokine expression and induction of ectopic lymphoid tissues make these cells uniquely positioned to induce and maintain inflammation.

(C) The contour plots represent the analysis of all CD45$^+$ cells. Numbers next to the areas indicate percentage of TNF$^+$ cells within gates.
(D) H&E staining of sections from the distal colon. Arrowheads point to inflammatory foci. Scale bar represents 100 μm.
(E) Clinical colitis score (±SEM, n = 3).
(F) Mean numbers (±SEM, n = 3) of leukocyte clusters (>120 μm) per colon.
(G) Weight as a percentage of the initial weight at day 0. Data represent the mean weight and is pooled from three independent experiments (n = 5 mice per group). Error bars were omitted for clarity.
n.s.: statistically not significant; *p < 0.05, **p < 0.01, ***p < 0.001.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice and gene-targeted mice were purchased from Janvier, Charles River Laboratories, Taconic Farms, or were provided by other laboratories. A complete list of mouse strains used is available in the Supplemental Experimental Procedures. All mice were used at 8–16 weeks of age. Antibiotic treatment was performed as described (Rakoff-Nahoum et al., 2004). Experiments were approved by and were in accordance with local animal care and use committees.

Cell Isolation and Flow Cytometry

The isolation of lymphocytes from lamina propria and other organs as well as staining of cell surface markers with fluorophore-conjugated antibodies were performed as previously described (Sanos et al., 2009; Sanos and Diefenbach, 2010). The strategy for the analysis of innate lymphoid cells is depicted in Figures S1E and S1F. Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated after Pancoll density gradient centrifugation (Biotech). Human tonsillar tissue was obtained from patients undergoing tonsillectomy and blood samples were from healthy volunteers. Informed written consent was obtained prior to sample acquisition from all donors and all investigations have been conducted according to the principles expressed in the Declaration of Helsinki. A list of antibodies used throughout the study is available in the Supplemental Experimental Procedures.

In Vivo Differentiation

For the in vivo transfer experiments, lymphocytes were isolated from the indicated organs and mice (H-2^b), for LNs and spleen $CD3^+$ and $CD19^+$ cells were removed with magnetic beads (Miltenyi) and cells were sorted twice on a MoFlo sorter (Beckman Coulter). Cells used were >98% purity in the post-sort analysis (Figures S1B–S1D) and were injected intravenously into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (H-2^d). Donor-derived lymphocyte populations were analyzed at the indicated time points. In some experiments, mice were injected every 3 days with a blocking IL-7Rα (0.5 mg) antibody (clone A7R34).

In Vitro Differentiation

For in vitro differentiation, 1000–5000 double sorted cells (purity > 98%) were seeded into microtiter plates that were coated with irradiated OP-9 cells (30 Gy) or left uncoated in medium supplemented with IL-15 (50 ng/ml), IL-7, Flt3L, and/or SCF (20 ng/ml, each; all Peprotech). Cells were analyzed after the indicated time points. In some experiments cells were labeled prior to the culture with a cell proliferation dye according to the manufacturer's instructions (eBioscience).

In Vitro Stimulation and Intracellular Cytokine Staining

The indicated lymphocyte populations were stimulated overnight with 50 ng/ml IL-12 or 10 ng/ml IL-23 (Peprotech). Brefeldin A (Sigma) was added for the last 4–6 hr of the stimulation period. Cytokine expression was analyzed by intracellular staining.

Experimental Colitis

CD40 colitis was induced as described (Uhlig et al., 2006). For transfer colitis, 5×10^4 cells of the indicated cell populations were transferred into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice and 2 weeks later, colitis was induced. Mice were sacrificed 7 days after CD40 injection and colitis was evaluated according to clinical and histological parameters (see Supplemental Experimental Procedures). In some experiments, NKR^+ cells were depleted by injections of 250 μg anti-Thy1 (clones 30H12 and M5/49.4.1), 250 μg anti-NK1.1 (clone PK136), or 100 μg anti-asialo GM1 at days −1, +1, and +4.

Real-Time PCR

Real-time PCR was performed as previously described (Sanos et al., 2009). The amount of mRNA was normalized to that of the "housekeeping" gene *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase). Primer sequences are reported in the Supplemental Experimental Procedures.

Statistical Analyses

ANOVA test was used to determine significance of the clinical colitis scores and of leukocyte clusters. Student's t test was used for all other data sets. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.immuni.2010.10.017.

ACKNOWLEDGMENTS

We thank G. Häcker for support and for critically reading the manuscript; the members of the A.D. laboratory for valuable discussions; A. Fischer, H. Pircher, and K. Connor for comments on the manuscript; J. Brandel for help with the figures; and N. Göppert and K. Oberle for technical assistance. We are grateful to D. Littman and F. Santori for discussion and providing crucial reagents, to G. Niedermann and C. Johner for experimental support, and to M. Follo, K. Geiger, and J. Wersing for cell sorting. The work was supported by the Deutsche Forschungsgemeinschaft (Di764/3, GRK1104, SGBM, CRC620 to A.D., A.M., T.H., E.A.K., M.F.), the BMBF (CCI, A.D.), the Max-Planck-Society (IMPRS-MCB), the Swiss National Science Foundation grant PP00A-116894/1 (D.F.), the National Institutes of Health (grants R37AI033068, CA069381, and AI088445 to C.F.W.) and the Cluster of Excellence Inflammation at Interfaces (Borstel-Kiel-Lübeck-Plön; EXC306 to C.H.).

Received: May 6, 2010
Revised: August 16, 2010
Accepted: September 28, 2010
Published online: November 18, 2010

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