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Microbial Flora Drives Interleukin 22 Production in Intestinal NKp46$^+$ Cells that Provide Innate Mucosal Immune Defense

Naoko Satoh-Takayama,[1,2] Christian A.J. Vosshenrich,[1,2] Sarah Lesjean-Pottier,[1,2] Shinichiro Sawa,[3] Matthias Lochner,[3] Frederique Rattis,[4] Jean-Jacques Mention,[1,2] Kader Thiam,[4] Nadine Cerf-Bensussan,[5] Ofer Mandelboim,[6] Gerard Eberl,[3] and James P. Di Santo[1,2,*]

[1]Cytokines and Lymphoid Development Unit, Institut Pasteur, Paris F-75724, France
[2]Inserm U668, Paris F-75724, France
[3]Lymphoid Tissue Development Group, Institut Pasteur, Paris F-75724, France
[4]genOway, Lyon 69362, France
[5]Inserm U793, Faculté de Médecin Necker, Paris F-75724, France
[6]The Lautenberg Center for General and Tumor Immunology, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel
*Correspondence: disanto@pasteur.fr
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SUMMARY

Natural killer (NK) cells are innate lymphocytes with spontaneous antitumor activity, and they produce interferon-γ (IFN-γ) that primes immune responses. Whereas T helper cell subsets differentiate from naive T cells via specific transcription factors, evidence for NK cell diversification is limited. In this report, we characterized intestinal lymphocytes expressing the NK cell natural cytotoxicity receptor NKp46. Gut NKp46$^+$ cells were distinguished from classical NK cells by limited IFN-γ production and absence of perforin, whereas several subsets expressed the nuclear hormone receptor retinoic acid receptor-related orphan receptor t (RORγt) and interleukin-22 (IL-22). Intestinal NKp46$^+$IL-22$^+$ cells were generated via a local process that was conditioned by commensal bacteria and required RORγt. Mice lacking IL-22-producing NKp46$^+$ cells showed heightened susceptibility to the pathogen *Citrobacter rodentium*, consistent with a role for intestinal NKp46$^+$ cells in immune protection. RORγt-driven diversification of intestinal NKp46$^+$ cells thereby specifies an innate cellular defense mechanism that operates at mucosal surfaces.

INTRODUCTION

Natural killer (NK) cells develop from hematopoietic stem cells under the influence of soluble factors and stromal cell interactions provided in the bone marrow (BM) and thymic microenvironments (Di Santo, 2006). The process of NK cell maturation involves the sequential acquisition of cell-surface receptors that provide either activating or inhibitory signals (Lanier, 2005; Raulet et al., 2001). NK cells are "educated" toward host major histocompatibility complex (MHC) class I molecules as their concomitantly expressed inhibitory receptors (of the NKG2 and Ly49 families) specific for MHC class I molecules are tested (Gasser and Raulet, 2006). NK cells that pass this developmental checkpoint are considered "competent" and acquire diverse functional capacities, including granule-mediated cytotoxicity and prompt chemokine and cytokine production (predominantly of interferon-γ [IFN-γ] and tumor necrosis factor-α [TNF-α]) (Yokoyama and Kim, 2006). BM and thymus-derived NK cells seed the peripheral lymphoid organs and primarily localize in the spleen, lymph node (LN), liver, and lung and in the uterus during gestation (Huntington et al., 2007). NK cells perform critical functions during innate and adaptive immune responses, by virtue of their ability to eliminate virus-infected, transformed, or stressed cells and to recruit and amplify inflammatory responses (Trinchieri, 1995).

NK cells have been long considered as a homogeneous population of cytotoxic and cytokine-producing cells. As such, NK cells may be predicted to perform stereotyped roles during the immune response, with little flexibility in their biological functions. In contrast, several different T helper (Th) cell subsets (Th1, Th2, and Th17) differentiate from naive T cells after antigen activation in a process that is guided by transcription factors and results in the generation of distinct effector T cells with specialized functions (Ho and Glimcher, 2002; Weaver et al., 2007). In contrast, evidence for a similar level of functional diversification in the NK cell lineage is limited. Early studies identified phenotypically distinct human NK cell subsets (Lanier et al., 1986) that were subsequently shown to have different tissue localizations and functional properties (Fehniger et al., 2003; Ferlazzo et al., 2004). Recent observations in the mouse have confirmed the existence of distinct NK cell subsets (Hayakawa et al., 2006; Veinotte et al., 2008; Vosshenrich et al., 2006). NK cell subsets may be generated as a consequence of programming via transcription factors or may represent different stages of an ongoing process of NK cell differentiation, in which maturing NK cells exhibit unique functional capacities. The mechanisms that control acquisition of NK cell effector functions are not fully understood, but they may include signals derived from the environment that could be altered during infection. One example is the recent report describing interleukin-10 (IL-10) production by activated NK cells in the context of chronic Leishmania infection (Maroof et al., 2008). Collectively, these results challenge the general notion that NK cells function in a homogeneous fashion as proinflammatory killer cells.

In the intestinal tract, a coordinated system of hematopoietic and nonhematopoietic cell types synergize to provide immune defense against potential ingested pathogens (reviewed in Artis, 2008). A single cell layer of epithelial cells separates the gut lumen harboring the commensal flora and food-born pathogenic antigens from the body. The protective function of epithelial cells includes not only a primary physical barrier, but also an immunoregulatory role via the secretion of antimicrobial peptides, cytokines, and chemokines that recruit hematopoietic cells. Additional sensors include intestinal dendritic cells (DCs) that extend transepithelial dendrites into the intestinal lumen and sample its contents for signs of infection. Specialized M cells provide a "pump" that feeds Peyers' patch DCs with gut antigens to initiate adaptive immune responses that are tuned toward immunoglobin A secretion. Whereas NK cells have been documented in the intestinal mucosa (Leon et al., 2003; Tagliabue et al., 1982), the developmental pathways that generate gut NK cells and the biological roles for intestinal NK cells in mucosal immunity are not understood. NK cells, by virtue of their rapid cytokine response, might play an important role in intestinal immunity by interfacing with intestinal DCs to regulate the orchestration of immune responses. Alternatively, NK cells may eliminate stressed or infected target cells and contribute to epithelial cell homeostasis.

In this study, we characterized distinct subsets of intestinal lymphocytes that expressed the natural cytotoxicity receptor NKp46. We used this molecule as a starting point because NKp46 has been shown to be highly and specifically expressed in NK cells (Biassoni et al., 1999; Sivori et al., 1997). Surprisingly, a substantial subset of NKp46$^+$ cells in the intestine lacked perforin and did not transcribe IFN-γ, and they thus bore little functional resemblance to classical NK cells. In contrast, these cells expressed the nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma t (RORγt) and IL-22 in response to local microenvironmental signals and were involved in immune defense against the pathogen *Citrobacter rodentium*. Our results provide evidence for an intestinal "niche" that conditions the differentiation of diverse NKp46$^+$ cell subsets that appear to play a role in mucosal immunity.

RESULTS

Identification of Phenotypically Diverse NK Cell Subsets in the Murine Intestinal Tract

NK cells from several species have been shown to specifically express the natural cytotoxicity receptor NKp46 (encoded by the *Ncr1* locus) (Biassoni et al., 1999; Gazit et al., 2006; Sivori et al., 1997; Walzer et al., 2007). In order to facilitate identification of NKp46$^+$ cells, we studied *Ncr1*$^{GFP/+}$ mice on the C57BL/6 background that harbor a green fluorescent protein (GFP) reporter driven by the *Ncr1* promoter (Gazit et al., 2006). As expected, CD3$^-$NKp46$^+$ cells were present in BM, spleen, thymus, liver, and lymph nodes (Figure 1A and data not shown). CD3$^-$NKp46$^+$ cells were also clearly identified as a subset of lamina propria lymphocytes (LPLs) and intraepithelial lymphocytes (IELs) in the small and large intestines of *Ncr1*$^{GFP/+}$ mice (Figure 1A). These cells showed typical lymphocyte morphology with scant cytoplasmic differentiation and an overall appearance similar to splenic NK cells (data not shown). Intestinal CD3$^-$NKp46$^+$ cells were dispersed throughout the lamina propria, were more rarely found in the intraepithelial position, and were essentially absent from the region of the intestinal crypts (Figure 1B and data not shown).

Because CD127 (IL-7Rα) expression has been observed on thymic NK1.1$^+$ cells and a subset of lymph node NK cells (Veinotte et al., 2008; Vosshenrich et al., 2006), we used CD127 and the prototypic NK cell marker NK1.1 (recognizing the C-lectin NKR-P1C) to further characterize the gut CD3$^-$NKp46$^+$ LPL and IEL populations (Figure 1C). Splenic CD3$^-$NKp46$^+$ cells were predominantly NK1.1$^+$CD127$^-$ and uniformly expressed CD122 (IL-2Rβ), CD11b, CD49b (DX5), the activating receptors NKG2D and CD16, and a "repertoire" of MHC-specific CD94 and Ly49 receptors (Figure 1C and data not shown). We noticed that intestinal CD3$^-$NKp46$^+$ cells were far more heterogeneous, and we could identify three subsets differentially expressing CD127 and NK1.1 (Figure 1C). The first subset was CD127$^+$ NK1.1$^-$ and expressed some NK cell markers (including CD224 [2B4] and low amounts of CD122 and NKG2D), but it was negative for CD11b, CD49b, CD94, and Ly49A and Ly49D (Figure 1C). Two NK1.1$^+$ cell subsets (CD127$^+$ and CD127$^-$) appear more like splenic NK cells with expression of NKG2D, CD94, and CD224 but relatively lower densities of CD49b, CD11b, CD27, and Ly49 family members (Figure 1C and data not shown). These different CD3$^-$NKp46$^+$ cell subsets were also numerous in the small intestine IEL compartment and large intestine lamina propria (Figure S1 available online). Together, these results reveal substantial phenotypic heterogeneity in gut CD3$^-$NKp46$^+$ cells.

Intestinal NKp46$^+$ Cells Fail to Express Typical NK Cell Effector Functions

NK cells employ several mechanisms to eliminate stressed or transformed target cells, including granule-mediated cytotoxicity and activation of Fas-associated protein with death domain (FADD)-linked death receptors (Smyth et al., 2002). We therefore assessed whether intestinal NKp46$^+$ cells showed these typical NK cell cytotoxicity pathways. Optimal granule-mediated cytotoxicity requires the coordinated activity of the pore-forming protein perforin and a family of granzyme serine proteases (Kagi et al., 1994; Shresta et al., 1995; Smyth et al., 2005). To assess the expression of perforin by gut NK cells, we used mice that harbor a yellow fluorescent protein (YFP) reporter gene driven by the perforin promoter (*Pfp*$^{YFP/+}$ mice). As expected, splenic NK cells from *Pfp*$^{YFP/+}$ mice uniformly expressed YFP (Figure 2A). In contrast, most intestinal CD3$^-$NKp46$^+$ cells from these mice lacked perforin expression, although a small subset of NK1.1$^+$CD127$^-$ cells showed weak YFP expression (Figure 2A). NK cells can induce apoptosis in target cells through activation of FADD-linked death receptors such as TRAIL-R and Fas. We found that intestinal CD3$^-$NKp46$^+$ cells were TRAIL$^-$ (Figure 2B), and only a minor population expressed FasL (Figure 2C). Thus, most intestinal CD3$^-$NKp46$^+$ cells lack the critical effector molecules required for cell-mediated cytotoxicity.

NK cells have the capacity to rapidly secrete a variety of cytokines after activation. In humans and mice, NK cell subsets have been described that show enhanced cytokine production, in particular production of proinflammatory Th1 cell cytokines IFN-γ and TNF-α (Huntington et al., 2007). In order to determine the

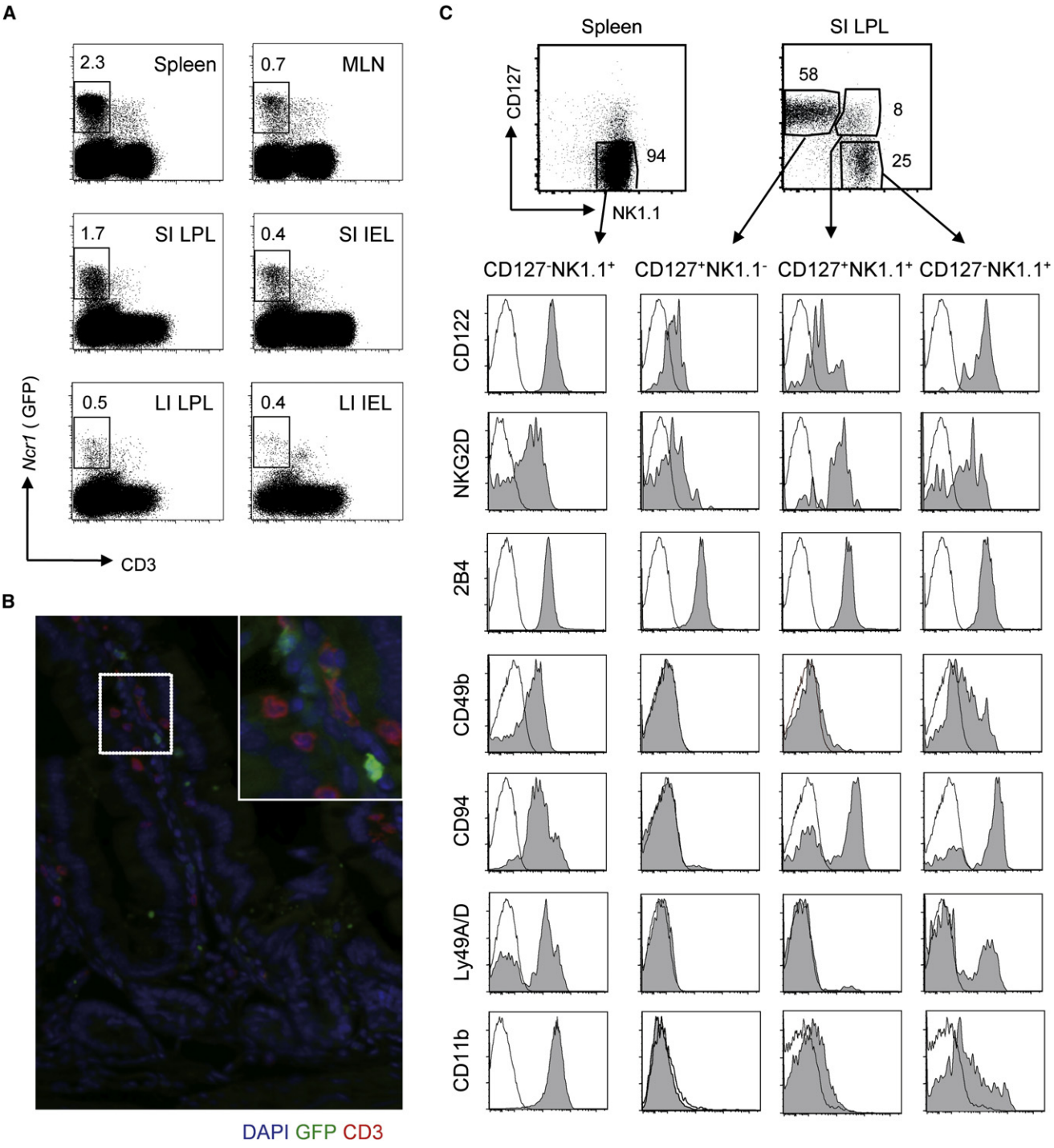


Figure 1. Intestinal NKp46+ Cells Display Unique Phenotypic Properties

(A) Frequencies of $CD3^-NKp46^+$ cells (boxed) in the indicated organs in $Ncr1^{GFP/+}$ C57BL/6 mice.

(B) Immunohistology of small intestine from $Ncr1^{GFP/+}$ mice stained with anti-GFP (green), CD3 (red), and DAPI (blue). Magnification was 20×.

(C) NK1.1 versus CD127 expression on gated $CD3^-NKp46^+$ splenocytes and lamina propria lymphocytes from the small intestine (SI LPLs). The frequencies of the gated populations are indicated. Histograms show NK cell markers (shaded area) and isotype control staining (black line). One representative experiment of six is shown.

capacity of intestinal NK cells to produce IFN-γ, we used mice that harbor a YFP reporter in the 3′-untranslated region of the *Ifng* locus ($Ifng^{YFP/+}$ mice) that can identify cells having the potential for rapid IFN-γ production because of a remodeled IFN-γ locus (Stetson et al., 2003). We confirmed that splenic NK cells in $Ifng^{YFP/+}$ mice demonstrate high YFP expression (Figure 2D),

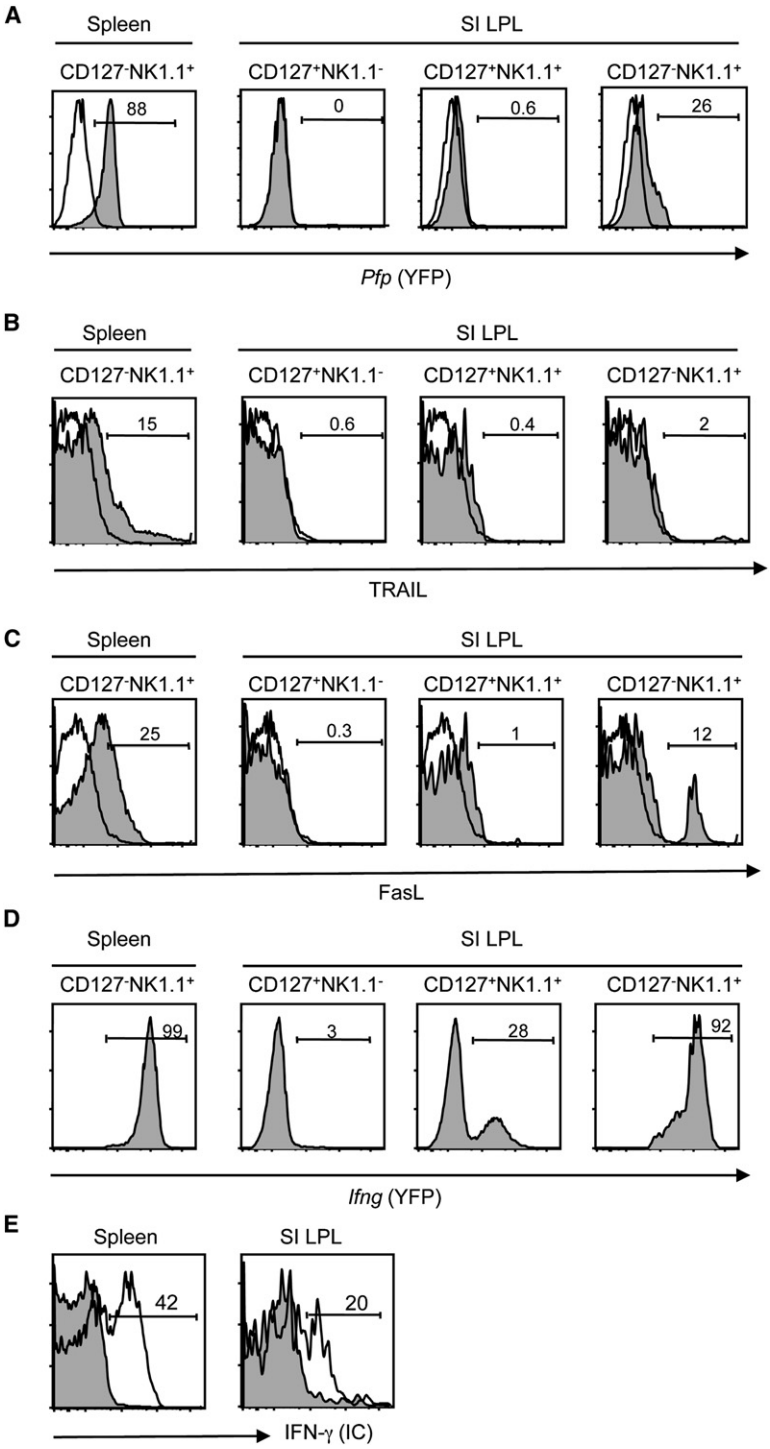


Figure 2. Intestinal NKp46+ Cells Lack Typical NK Cell Effector Functions

(A) GFP expression amounts (shaded area) in spleen NK cells and intestinal CD3−NKp46+ cell subsets from the SI LPLs of *Pfp*$^{YFP/+}$ reporter mice: CD127+NK1.1− (left), CD127+NK1.1+ (middle), and CD127−NK1.1+ (right). Nontransgenic cells are shown as control (black line).

(B and C) TRAIL expression (B) and FasL expression (C) on CD3−NKp46+ cells from spleen and SI LPLs. Shaded histograms show specific staining (isotype control indicated by a solid line). The percentages of positive cells are indicated.

(D) YFP expression amounts (shaded area) in spleen NK cells and intestinal CD3−NKp46+ cell subsets from the SI LPLs of *Ifng*$^{YFP/+}$ mice: CD127+NK1.1− (left), CD127+NK1.1+ (middle), and CD127−NK1.1+ (right).

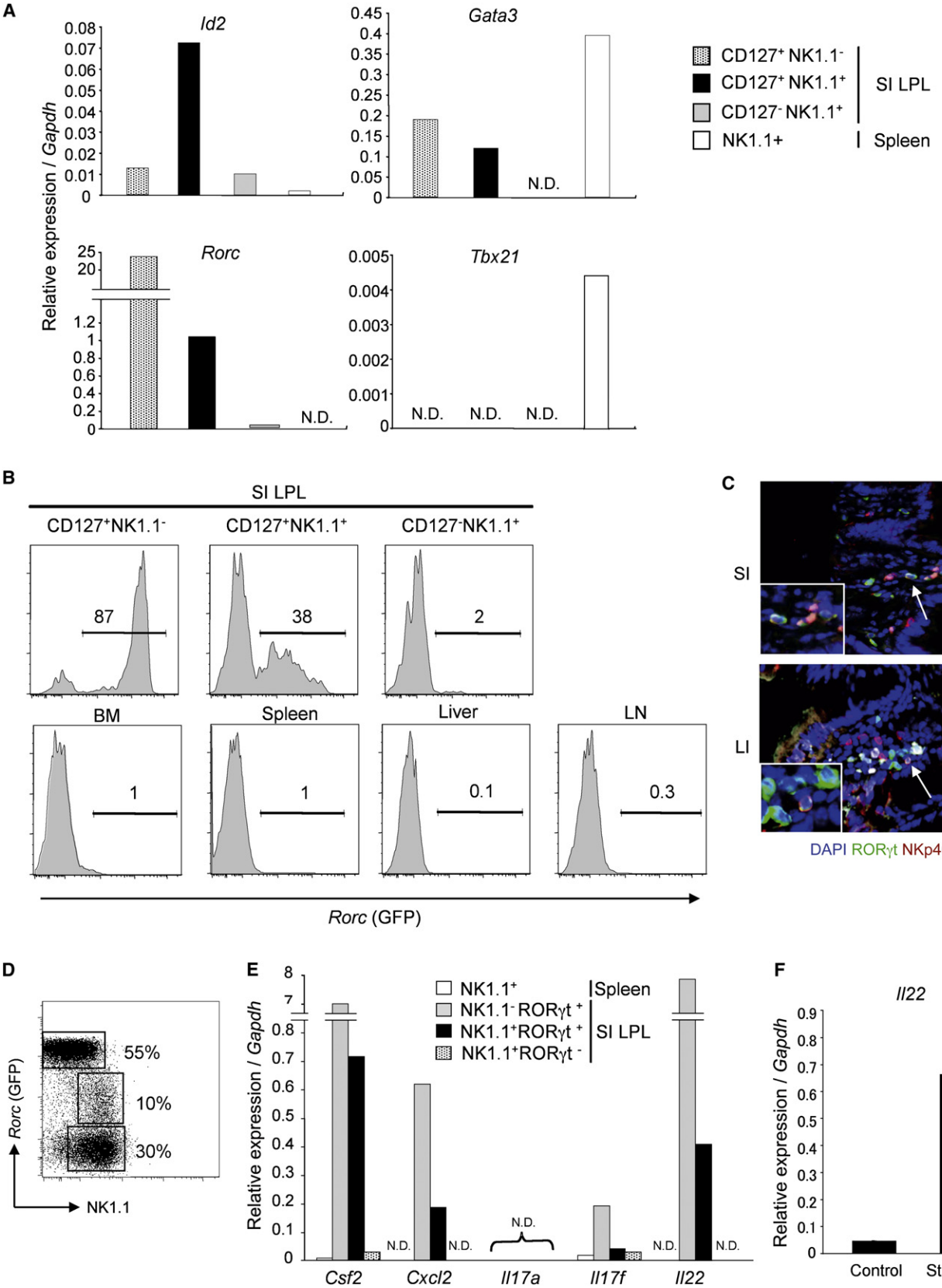
(E) Intracellular detection of IFN-γ by gated CD3−NKp46+ cells from spleen (left) and SI LPLs (right). Histogram overlays show percentages of positive cells in nonstimulated cultures (shaded) or after 4 hr stimulation (solid line). One representative experiment of three is shown.

consistent with their IFN-γ-producing potential (Figure 2E). In contrast, intestinal CD3−NKp46+ cell subsets showed distinct patterns of YFP expression, with most CD127+ cells appearing incompetent for IFN-γ production, whereas the CD127− subset had YFP expression similar to that of splenic NK cells. Collectively, these results indicated that most intestinal NKp46+ cells were functionally distinct from splenic NK cells.

Intestinal NKp46+ Cells Express the Transcription Factor RORγt and Produce IL-22

To gain more insight into the potential relationship of intestinal NKp46+ cells to known NK cell subsets and to help identify their potential effector functions, we compared their transcription factor (TF) profiles. Several TFs are critical for NK cell development in the BM and thymus (including Id2, GATA-3, PU.1, and Ikaros), whereas others modulate their differentiation into cytokine-producing killer cells (T-bet, GATA-3, IRF-2, MEF, and CEBP-γ) (Di Santo, 2006). CD3−NKp46+ cells, irrespective of their origin, expressed transcripts for *Id2* but were negative for *Foxp3* (Figure 3A and data not shown). *Tbx21* (T-bet) expression was only observed in splenic NK cells, whereas *Gata3* expression was similar in NK cells from the spleen and gut (Figure 3A). Strikingly, a subset of intestinal CD3−NKp46+ cells expressed the nuclear hormone receptor RORγt encoded by *Rorc*. We confirmed RORγt expression in intestinal NKp46+ cells by using recently described bacterial artificial chromosome (BAC) transgenic mice harboring a GFP reporter under the control of the *Rorc* promoter (Lochner et al., 2008). In *Rorc*-GFP mice, GFP expression was found in intestinal NKp46+ cells expressing CD127 (both in the LPL and IEL), whereas NK1.1+ NK cells lacking CD127 (in the intestine or elsewhere) were essentially GFP− (Figure 3B). Interestingly, almost all CD127+NK1.1− cells were GFP+ compared with CD127+NK1.1+ cells that only harbored a fraction (approximately 40%) of RORγt+ cells (Figure 3B). Immunohistology confirmed that CD3−NKp46+RORγt+ cells were located primarily as scattered cells in the intestinal LP and were rare in the cryptopatches at the base of the villi (Figure 3C).

We next assessed whether differential RORγt expression by intestinal NKp46+ cells had any impact on their functional


A
Id2
Gata3
Rorc
Tbx21
Relative expression / Gapdh
N.D.
CD127+ NK1.1-
CD127+ NK1.1+
CD127- NK1.1+
NK1.1+
SI LPL
Spleen
B
SI LPL
CD127+NK1.1-
CD127+NK1.1+
CD127-NK1.1+
87
38
2
BM
Spleen
Liver
LN
1
1
0.1
0.3
Rorc (GFP)
C
SI
LI
DAPI RORγt NKp46 CD3
D
55%
10%
30%
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NK1.1
E
NK1.1+
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NK1.1+RORγt +
NK1.1+RORγt -
Spleen
SI LPL
Relative expression / Gapdh
Csf2
Cxcl2
Il17a
Il17f
Il22
F
Il22
Relative expression / Gapdh
Control
Stimulated

properties. By comparing GFP expression with NK1.1 expression in *Rorc*-GFP mice, we could clearly identify NKp46$^+$ cell subsets that expressed distinct amounts of RORγt (Figure 3D). NKp46$^+$ cells expressing the highest amount of RORγt were essentially NK1.1$^-$ (and uniformly expressed CD127; Figure 3B). As such, this NKp46$^+$CD127$^+$NK1.1$^-$ cell subset may be related to the previously described RORγt$^+$ lymphoid tissue inducer (LTi) cells known to orchestrate development of programmed secondary and induced tertiary intestinal lymphoid aggregates (Eberl and Littman, 2004; Mebius, 2003). LTi cells express CD4 and reside in cryptopatches, which are structures found between intestinal crypts (Eberl, 2007; Ivanov et al., 2006a). Because NKp46$^+$CD127$^+$NK1.1$^-$ cells are CD4$^-$ (data not shown) and are not observed near the intestinal crypts (Figures 1B and 3C), they appear to differ from LTi cells that also express high amounts of RORγt (Eberl et al., 2004). The developmental or functional relationship of the NKp46$^+$RORγt^{hi} cells that we have identified to the previously described LTi cells is unclear. Nevertheless, NKp46 expression by RORγt^{hi} cells could caution against the use of this marker in a "universal definition" of NK cells (Walzer et al., 2007).

NKp46$^+$ cells expressing intermediate amounts of RORγt were essentially NK1.1$^+$ (Figure 3D) and coexpressed CD127 (Figure 3B), whereas NKp46$^+$ cells that were RORγt$^-$ were essentially NK1.1$^+$ (Figure 3D). Thus, two different NKp46$^+$ populations of cells expressing different amounts of RORγt$^+$ are present in the gut.

RORγt is an essential orchestrator of the Th17 cell pathway (Ivanov et al., 2007). We therefore compared steady-state expression profiles of IL-17-family cytokines and other cytokines in splenic NK cells with intestinal CD3$^-$NKp46$^+$ cells that expressed different amounts of RORγt (Figure 3E). Curiously, intestinal NKp46$^+$RORγt$^+$ cells failed to express the signature cytokine IL-17A, although they expressed transcripts for the cytokines IL-17F and IL-22 (Figure 3E). Unlike splenic NK cells, intestinal NKp46$^+$CD127$^+$ subset cells also constitutively expressed transcripts for *Csf2* (encoding granulocyte-macrophage colony-stimulating factor [GM-CSF]) and the chemokine Cxcl2 with amounts that positively correlated with RORγt expression (Figure 3E). *Il22* expression could also be induced in intestinal LPL after activation in vitro (Figure 3F). Collectively, these results identify unique functional attributes of intestinal NKp46$^+$ cell subsets that express RORγt and transcribe genes encoding GM-CSF, Cxcl2, and IL-22.

Developmental Pathways that Generate Intestinal NKp46$^+$ Cell Subsets

We next sought to identify the signals that control the development and functional diversification of intestinal NKp46$^+$ subsets. Because intestinal NKp46$^+$ cells share some phenotypic similarities (CD127$^+$, GATA-3$^+$, and CD11b^{lo}) with thymus-derived NK cells (Vosshenrich et al., 2006), we determined whether they were related. We found normal numbers and distribution of intestinal CD3$^-$NKp46$^+$ cells in nude mice lacking the transcription factor *Foxn1* (Figures 4A and 4B), suggesting that gut NKp46$^+$ cell subsets are thymus independent. Intestinal CD3$^-$NKp46$^+$ cells also normally developed in the absence of antigen-receptor recombinase *Rag2* (see below).

IL-15 plays an essential role in the development, survival, and differentiation of NK1.1$^+$ cells (Vosshenrich et al., 2005). Analysis of *Il15*$^{-/-}$ mice confirmed a similar cytokine dependency of intestinal NKp46$^+$NK1.1$^+$ cells (Figures 4A and 4B). Interestingly, the absolute numbers of gut NKp46$^+$CD127$^+$NK1.1$^-$ cells were unaffected by the absence of IL-15, suggesting that the homeostasis of NKp46$^+$ cells that differentially express NK1.1 may be controlled independently.

Because RORγt plays an important role in the differentiation of Th17 lineage (Ivanov et al., 2006b), we assessed whether RORγt was critical for the development and differentiation of gut NKp46$^+$ cell subsets by using *Rorc*$^{-/-}$ mice (Eberl et al., 2004). Strikingly, percentages and absolute numbers of intestinal NKp46$^+$CD127$^+$NK1.1$^-$ cells were strongly decreased in the absence of RORγt (Figures 4B and 4C). In contrast, homeostasis of intestinal NKp46$^+$NK1.1$^+$ cells appeared unaffected in *Rorc*$^{-/-}$ mice (Figures 4B and 4C).

The intestine is in constant contact with commensal microorganisms, which impacts on the development of gut lymphoid structures and local intestinal immune responses (Artis, 2008; Bouskra et al., 2008). We next assessed whether microbial stimulation was involved in the homeostasis of gut NKp46$^+$ cell subsets. In germ-free mice, we found that absolute numbers of intestinal NKp46$^+$CD127$^+$NK1.1$^-$ cells were strongly decreased, whereas NKp46$^+$NK1.1$^+$ cells were unaffected (Figures 4B and 4C). The similar effects of RORγt deficiency and elimination of commensal flora on NKp46$^+$CD127$^+$NK1.1$^-$ cells suggests that these processes are linked. Collectively, these results suggest that homeostasis of intestinal NKp46$^+$ cells that differentially express NK1.1 is controlled by unique mechanisms, with NK1.1$^-$ cells being predominantly maintained through signals derived from interactions with commensal flora, whereas NK1.1$^+$ cells are maintained through availability of IL-15.

Signals that Control Il22 Transcription in Intestinal NKp46$^+$ Cell Subsets

Homeostasis of NKp46$^+$NK1.1$^+$ cells was unaffected by absence of RORγt or elimination of commensal flora (Figure 4B and 4C), and we wondered whether *Il22* transcription in NKp46$^+$NK1.1$^+$ cells (essentially the CD127$^+$ subset; Figure 3E) was also

Figure 3. Intestinal NKp46$^+$ Cell Subsets Express *Rorc* and *Il22*

(A) TF expression of sorted splenic and intestinal CD3$^-$NKp46$^+$ cells. The indicated TFs were analyzed by qPCR, and the expression is normalized to *Gapdh*. N.D., not detected.

(B) GFP expression amounts (shaded area) in CD127$^+$NK1.1$^-$ (left), CD127$^+$NK1.1$^+$ (middle), and CD127$^-$NK1.1$^+$ (right) subsets of intestinal CD3$^-$NKp46$^+$ cells and BM, spleen, liver, and LN CD3$^-$NKp46$^+$ cells from *Rorc*-GFP mice.

(C) Tissue section of small and large intestines from *Rorc*-GFP stained with anti-GFP (green), NKp46 (red), and CD3 (white). Nuclei were stained with DAPI (blue). Arrowheads point to CD3$^-$NKp46$^+$RORγt$^+$ cells. Magnification was 40×.

(D) RORγt versus NK1.1 expression in NKp46$^+$CD3$^-$ cells from SI LPLs of *Rag2*$^{-/-}$ × *Rorc*-GFP mice.

(E) Detection of indicated cytokine transcripts by qPCR in NK1.1$^-$RORγt$^+$ cells (gray bars), NK1.1$^+$RORγt$^+$ cells (black bars), and NK1.1$^+$RORγt$^-$ cells (stipled bars) in NKp46$^+$ cells from the SI LPLs of *Rag2*$^{-/-}$ × *Rorc*-GFP mice compared with splenic NK1.1$^+$ cells (open bars).

(F) Steady-state and PMA plus ionomycin-induced *Il22* expression from the SI LPLs of untreated *Rag2*$^{-/-}$ mice. One representative experiment of two is shown.

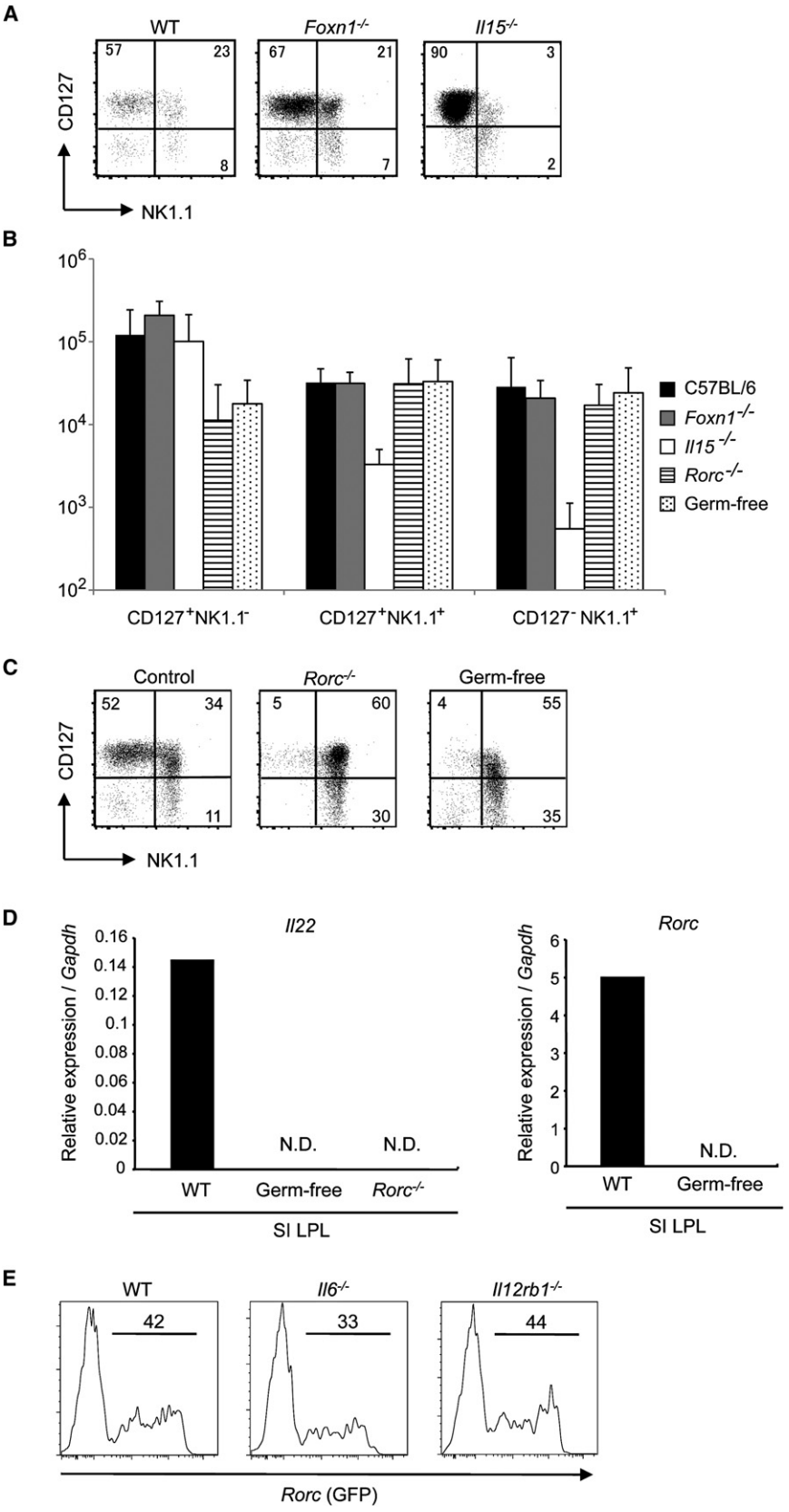

Figure 4. Intestinal IL-22 Production in NKp46+ Cell Is Driven by Commensal Flora and Requires RORγt

(A) CD127 versus NK1.1 profiles of gated CD3−NKp46+ cell subsets from SI LPLs from WT, *Foxn1*$^{-/-}$, and *Il15*$^{-/-}$ mice. The frequencies of the subsets are indicated.

(B) Absolute numbers of CD3−NKp46+ cell subsets from SI LPLs from indicated mice. Results are means ± standard deviation (SD) from n = 4–6 mice of each genotype.

(C) CD127 versus NK1.1 profiles of gated CD3−NKp46+ cell subsets from SI LPLs from WT, *Rorc*$^{-/-}$, and germ-free mice (GF).

(D) *Il22* and *Rorc* expression in isolated CD3−NKp46+NK1.1+CD127+ cells from SI LPLs of WT, *Rorc*$^{-/-}$, and germ-free mice. N.D., not detected.

(E) RORγt (GFP) expression on intestinal NKp46+NK1.1+CD127+ cells from SI LPLs of WT, IL-6-deficient, and IL-12Rβ1-deficient *Rorc*-GFP mice. The frequencies of GFP-expressing cells are indicated.

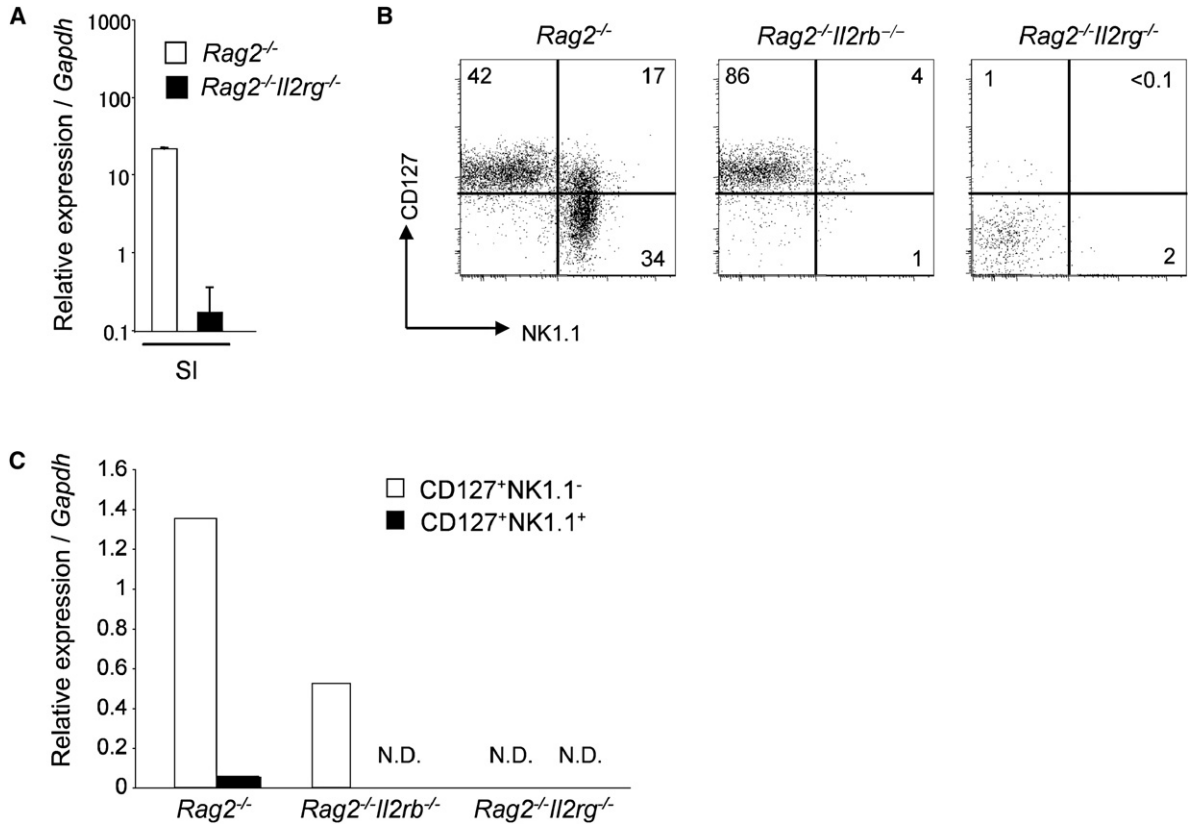

Figure 5. Mouse Models to Identify Roles for Intestinal NKp46$^+$ Cell Subsets

(A) Steady-state *Il22* expression from total SI LPLs of untreated *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice.

(B) CD127 versus NK1.1 profiles on gated NKp46$^+$ cells of SI LPLs from untreated *Rag2*$^{-/-}$ (left), *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ (center), and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (right).

(C) Steady-state *Il22* transcripts from sorted NK1.1$^+$CD127$^-$ and NK1.1$^+$CD127$^+$ subsets of intestinal NKp46$^+$ cells from SI LPLs of untreated *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Il2rb*$^{-/-}$, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. N.D., not detected.

independent of these signals. Strikingly, *Il22* expression was undetectable in NKp46$^+$CD127$^+$NK1.1$^+$ cells from germ-free and *Rorc*$^{-/-}$ mice (Figure 4D). Moreover, NKp46$^+$CD127$^+$NK1.1$^+$ cells from germ-free mice failed to express RORγt (Figure 4D), potentially explaining the lack of IL-22 transcription.

Induction of RORγt and subsequent expression of Th17 cell-like cytokines in differentiating T cells is conditioned by several soluble factors, including IL-6, IL-23, and TGF-β (Weaver et al., 2007). We found that IL-6 and IL-23 were not required for the differentiation in vivo of intestinal NK1.1$^+$ cells that constitutively express RORγt and IL-22 (Figure 4E and data not shown). Moreover, these factors could not induce RORγt expression in splenic NK cells in vitro (data not shown), although they may amplify *Il22* expression under certain conditions. Together, these results identify substantial phenotypic and functional diversity within the intestinal NKp46$^+$ cell compartment, with an important role for bacterial flora in driving development of IL-22$^+$ cells via RORγt.

Relative Roles of Distinct IL-22$^+$ NKp46$^+$ Subsets during *C. rodentium* Infection

Infection by *C. rodentium* causes acute colitis in mice (Schauer and Falkow, 1993). IL-22 is essential in the early host defense against *C. rodentium* and is required for epithelial cell secretion of several classes of antimicrobial factors (Wolk et al., 2004; Wolk et al., 2006; Zheng et al., 2008). Previous studies using *C. rodentium*-induced colitis demonstrated that mucosal IL-22 was retained in mice lacking B cells and T cells (*Rag2*$^{-/-}$ mice). DCs were proposed as IL-22 producers on the basis of CD11c and IL-22 coexpression by immunohistology, whereas NK cells were excluded on the basis of the absence of CD49b and IL-22 costaining (Zheng et al., 2008). Our identification of IL-22$^+$ NKp46$^+$ cells in the intestine (Figure 3E) that were mostly CD49b$^-$ (Figure 1C) yet expressed CD11c (Figure S2) suggested that these cells might represent the previously identified IL-22 producers (Zheng et al., 2008).

We found that intestinal *Il22* transcripts were strongly reduced in the LPLs of mice deficient in both Rag2 and the common γ chain (*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice; Figure 5A), confirming the previous observations on the role of γ_c-dependent signals in IL-22 production (Aujla et al., 2008). We suspected that absence of *Il22* expression in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice might not be due solely to the absence of intestinal NK1.1$^+$ cells, given that NKp46$^+$CD127$^+$NK1.1$^-$ cells that constitutively transcribe *Il22* could develop in the absence of IL-15 (Figure 4A). In order to test this hypothesis, we studied mice deficient in both Rag2 and the IL-2Rβ chain that is required for IL-2 and IL-15 signaling (*Rag2*$^{-/-}$*Il2rb*$^{-/-}$ mice). We predicted that these mice would lack NK1.1$^+$ cells in the gut yet retain NKp46$^+$CD127$^+$NK1.1$^-$ cells

that could produce IL-22. *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ mice would provide a means of potentially defining the relative roles for IL-22 production from distinct NKp46$^+$ subsets in the context of an intestinal bacterial infection by *C. rodentium*.

We first compared development of intestinal NKp46$^+$ cell subsets in LPLs isolated from *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Il2rb*$^{-/-}$, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. We found that NKp46$^+$NK1.1$^+$ cells were essentially absent from both *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, whereas NKp46$^+$CD127$^+$NK1.1$^-$ cells were present in *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ mice, but not *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (Figure 5B). Thus, *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ mice selectively lacked intestinal NKp46$^+$ cells that express NK1.1. Compared to that in *Rag2*$^{-/-}$ mice, expression of intestinal *Il22* was reduced in *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ mice and further reduced in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice lacking all NKp46$^+$ cells (Figure 5C). Concerning DC development in these mice, we found no differences in the distribution of different DC subsets in mice with or without *Il2rg* (Figure S2). DCs fail to express *Rorc* (Figure S3A), which is required for *Il22* transcription in lymphocytes (Ivanov et al., 2006b). Moreover, we found that isolated intestinal macrophages and DCs did not express *Il22* (Figure S3B). These results suggest that NKp46$^+$ cells represent an important source of IL-22 in the intestine.

We next infected *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Il2rb*$^{-/-}$, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice with *C. rodentium*, which requires intestinal IL-22 for its control (Zheng et al., 2008). *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were extremely susceptible to *C. rodentium*, with all mice showing dramatic weight loss and succumbing to infection by day 7 (Figures 6A and 6B). In contrast, both *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ and *Rag2*$^{-/-}$ mice resisted early infection, suggesting that in the absence of NKp46$^+$NK1.1$^+$ cells, intestinal NKp46$^+$CD127$^+$NK1.1$^-$ cells may provide sufficient IL-22 to protect against *C. rodentium*. Although percentages and absolute numbers of colonic NKp46$^+$ cell subsets were not substantially altered in response to *C. rodentium* (Figures 6C and 6D), we found that *Il22* expression was upregulated in NKp46$^+$ cells after infection (Figure 6E). IL-22 protein was present in supernatants from cultured colonic LPLs from *C. rodentium*-infected (day 4) *Rag2*$^{-/-}$ mice (86 pg/ml) and *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ mice (99 pg/ml), but not from similarly infected *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Collectively, these results suggest that NKp46$^+$CD127$^+$RORγt$^+$ cells are an important source of IL-22 and may act as a mucosal cellular effector in the protection against *C. rodentium*.

DISCUSSION

The presence of NK cells in lymphoid as well as nonlymphoid tissues raises questions as to their specific functional roles at these different sites. Although intestinal NK cells have been identified in both humans and rodents (Eiras et al., 2000; Leon et al., 2003; Tagliabue et al., 1982), previous studies have emphasized the capacity of these cells to perform "classical" NK cell functions (cytotoxicity and IFN-γ production) (Eiras et al., 2000; Leon et al., 2003; Tagliabue et al., 1982). In this report, we have re-examined intestinal NK cells in mice. As the starting point, we analyzed intestinal cells that express the natural cytotoxicity receptor NKp46 because this molecule has been shown to be highly and specifically expressed in NK cells as opposed to other cells within the hematopoietic system (Biassoni et al., 1999; Gazit et al., 2006; Sivori et al., 1997; Walzer et al., 2007). We uncovered an unexpected diversity of NKp46$^+$ cells, and we identified and characterized NKp46$^+$ subsets distinguished by their differential expression of CD127 and NK1.1 in the small and large intestine lamina propria and intraepithelial compartments. Whereas one subset (NK1.1$^+$CD127$^-$) appeared to represent "typical" NK cells, NKp46$^+$CD127$^+$ subsets in the gut were unusual, with an absence of cytotoxic effectors (perforin, granzymes, FasL, and TRAIL), low or absent IFN-γ production, expression of the transcription factor RORγt, and expression of IL-22. Because RORγt$^+$ IL-22$^+$ NKp46$^+$ cells were not identified outside the gut and were conditioned by commensal flora, we propose that the intestine provides a specific "niche" favoring the development and differentiation of NKp46$^+$ cell subsets that have particular functional properties. These observations raise questions about the developmental origins of intestinal NKp46$^+$ cells, the signals and molecular mechanisms that generate their functional diversity, and their biological roles in mucosal immunity.

We investigated the relationship between different intestinal NKp46$^+$ cell subsets by using a series of mouse mutants that have altered NK cell development. IL-15 is an essential growth and differentiation factor for innate lymphocytes and plays a critical role in the homeostasis of NK1.1$^+$ cells (Cooper et al., 2002; Ranson et al., 2003; Vosshenrich et al., 2005). IL-15 ablation reduced absolute numbers of intestinal NK1.1$^+$ cells by more than 90%. Interestingly, the NKp46$^+$ cells that are CD127$^+$NK1.1$^-$ were unaffected by the absence of IL-15, a result that could be explained if NKp46$^+$CD127$^+$NK1.1$^-$ cells were IL-15-independent precursors to NK1.1$^+$ gut cells. A similar scenario exists during NK cell development, in which NK cell precursors normally develop in the absence of IL-15, but their subsequent development requires this cytokine (Vosshenrich et al., 2005). It is interesting to note that NKp46$^+$CD127$^+$NK1.1$^-$ cells express high amounts of RORγt, similar to CD4$^+$CD3$^-$ fetal LTi cells that have NK cell-precursor activity (Mebius et al., 1997). As such, different gut NKp46$^+$ subsets could represent stages in a linear differentiation program driven by IL-15.

Alternatively, intestinal NKp46$^+$ cell subsets may be developmentally unrelated. Gut CD127$^+$NK1.1- cells and NK1.1$^+$ cells could represent distinct hematopoietic lineages despite their common expression of several NK cell markers (NKp46, NKG2D, and 2B4). A relationship of gut NKp46$^+$CD127$^+$NK1.1$^-$ cells to RORγt$^+$ LTi cells that is independent of any NK cell-precursor activity is possible. Still, gut NKp46$^+$CD127$^+$NK1.1$^-$ cells do not appear simply as a subset of LTi cells that express NKp46 because these NKp46$^+$ gut cells are not clustered in cryptopatches and they lack markers associated with LTi, including CD4 and CD117. Future studies will be necessary to define the lineage relationships between these diverse NKp46$^+$ cell subsets in the intestine.

Intestinal NKp46$^+$ cells are distinguished by their expression of the transcription factor RORγt and IL-22. Previous studies have shown that RORγt is essential for the generation of differentiated T cells that express IL-17A (Th17 cells) that play a role in promoting inflammatory immune responses at mucosal surfaces (Ivanov et al., 2006b). Th17 cells produce an unusual set of cytokines, including IL-17A, IL-17F, IL-22, and varying degrees of IFN-γ and IL-10 (Chen and O'Shea, 2008; Stockinger and Veldhoen, 2007; Weaver et al., 2007). Th17 cell differentiation is controlled via

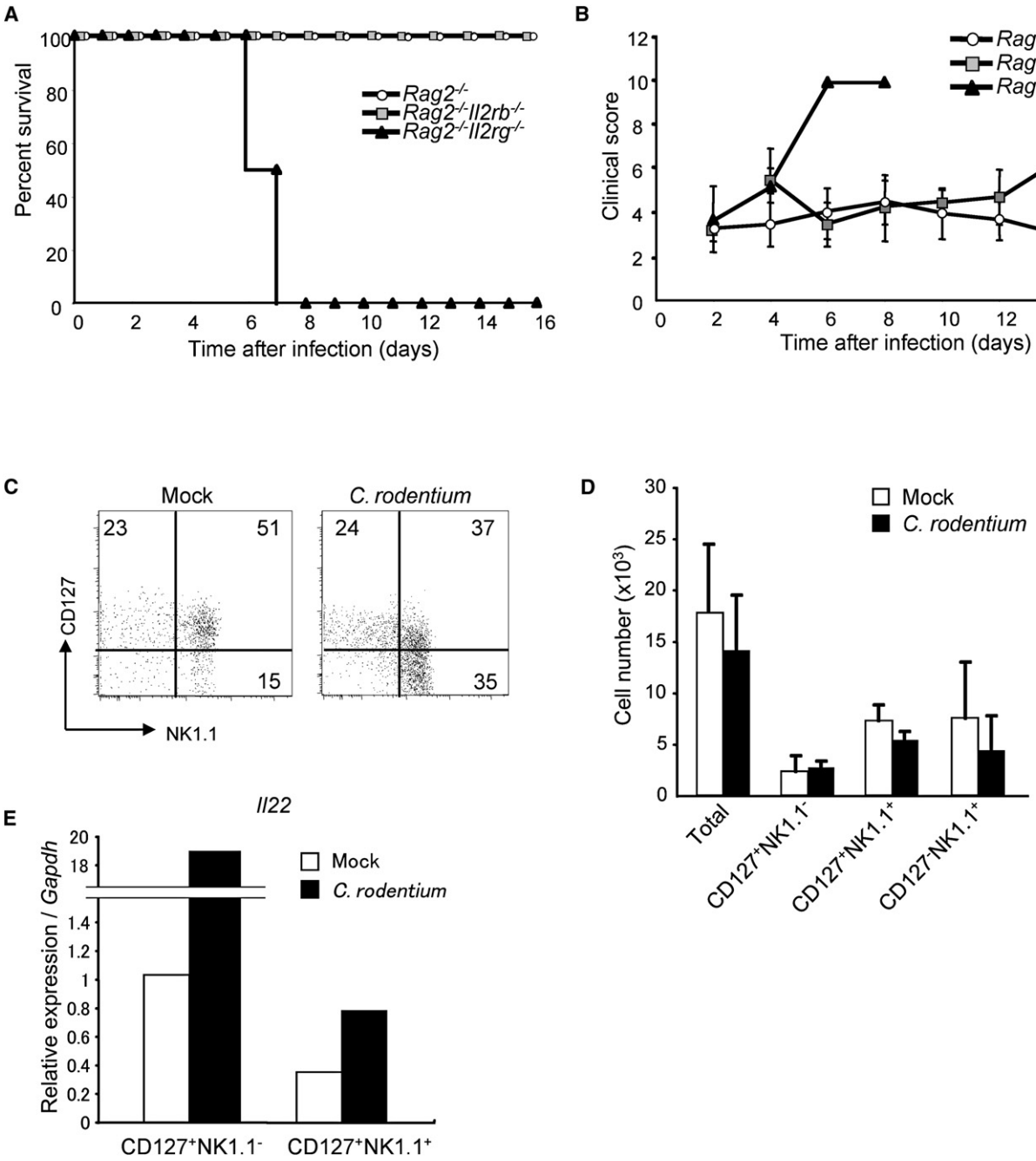

Figure 6. *Il22* Expression by Intestinal NKp46^{+} Cells Is Involved in Mucosal Immunity

(A and B) Survival (A) and clinical score (B) of *Rag2*$^{-/-}$ (circles), *Rag2*$^{-/-}$*Il2rb*$^{-/-}$ (squares), and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (triangles) after *C. rodentium* infection. Clinical score was calculated as described in Experimental Procedures.

(C and D) Frequencies (C) and absolute numbers (D) of NKp46^{+} cell subsets from colonic LPLs from mock and *C. rodentium*-infected *Rag2*$^{-/-}$ mice. Results are means ± SD from n = 4 mice (control or infected).

(E) IL-22 expression from colonic NKp46^{+} subsets of *C. rodentium*-infected *Rag2*$^{-/-}$ mice.

the soluble factors IL-6, IL-21, IL-23, and TGF-β and activates STAT3 to induce RORγt expression (Chen and O'Shea, 2008; Stockinger and Veldhoen, 2007; Weaver et al., 2007). It was therefore important to know whether gut NKp46^{+} cells differentiated in a manner analogous to Th17 cells. Unlike RORγt^{+} Th17 cells, we found that RORγt^{+} NKp46^{+} cells did not express IL-17A (and only were only weakly IL-17F^{+}), whereas they strongly expressed IL-22. Moreover, IL-6 and IL-23 were not required for development of intestinal RORγt^{+}NKp46^{+} cells, unlike that of Th17 cells (Chen and O'Shea, 2008; Stockinger and Veldhoen, 2007; Weaver et al., 2007). Still, IL-23 could boost IL-22 production from gut NKp46^{+} cells (N.S.-T. and J.P.D., unpublished data).

Thus, the mechanisms that control Th17-family cytokine expression in NKp46$^+$RORγt$^+$ cells appear to differ from those operating in Th17 cells.

We provide evidence that the functional properties of intestinal NKp46$^+$ cells are conditioned by commensal flora. In germ-free mice, NKp46$^+$RORγt$^+$IL-22$^+$ cells are strongly reduced, suggesting that an environmental niche, operative in the gut, generates these unique effector cells. Commensal organisms impact on intestinal immunity through numerous complex mechanisms (Artis, 2008). Bacterial flora is sensed by pattern-recognition receptors (TLR, NLR, and NALP) on both epithelial cells and hematopoietic cells and relay signals that result in elaboration of factors that "tune" gut lymphoid structures (Bouskra et al., 2008). Preliminary experiments show that intestinal NKp46$^+$ cell subsets are unaltered in *Myd88*$^{-/-}$ mice (N.S.-T. and J.P.D., unpublished data) suggesting that multiple sensors may be involved. Recent data has demonstrated that bacterially produced ATP can directly augment Th17 differentiation (Atarashi et al., 2008), whereas commensal flora may inhibit this pathway via IL-25 (Zaph et al., 2008). How signals derived from bacterial flora control RORγt and IL-22 in gut NKp46$^+$ cells remains to be defined, but both direct and indirect mechanisms could be operative.

We used the *C. rodentium*-infection model to assess a role for gut NKp46$^+$ cells in mucosal immune defense. Previous studies showed that IL-17A was dispensable during the early phase of *C. rodentium* infection, whereas IL-22 from a T cell- and B cell-independent cellular source was essential for survival (Zheng et al., 2008). IL-22 appeared critical for the maintenance of epithelial cell integrity and for the secretion of antimicrobial peptides (β-defensins, RegIII and S100 family members, and lipocalin2) that counteract bacterial invasion. We found that *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice showed reduced steady-state IL-22 in the intestine that correlated with heightened susceptibility to *C. rodentium*, similar to *Il22*$^{-/-}$ mice (Zheng et al., 2008). In contrast, *Rag2*$^{-/-}$ *Il2rb*$^{-/-}$ mice lacking only the NK1.1$^+$ subset of gut NKp46$^+$ cells were able to resist *C. rodentium*, suggesting that production of IL-22 by intestinal NKp46$^+$CD127$^+$NK1.1$^-$ cells may play an important role in bacterial control. Strong constitutive *Il22* expression by gut NKp46$^+$ cells distinguishes these cells from intestinal macrophages and DCs that do not express *Il22*. Although we do not rule out the possibility that the γ_c mutation may eliminate other cell types capable of IL-22 production in response to *C. rodentium*, the heightened IL-22 expression by intestinal NKp46$^+$CD127$^+$NK1.1$^-$ cells suggests that these cells are pivotal.

Expression of IL-22 (but not IL-17A) by gut NKp46$^+$RORγt$^+$ cells indicates biological roles that differ from previously described mucosal Th17 cells. Although IL-17A is dispensible during early *C. rodentium* infection (Zheng et al., 2008), it is necessary for bacterial clearance (Mangan et al., 2006). IL-17A expression is reduced in *C. rodentium*-infected *Rag2*$^{-/-}$ mice (Zheng et al., 2008), and a chronic state of infection is established that finally leads to death (Vallance et al., 2002). Nevertheless, despite bacterial persistence at later stages of infection, colon tissue of *C. rodentium*-infected *Rag2*$^{-/-}$ mice showed few signs of tissue destruction (Mangan et al., 2006; Vallance et al., 2002). This contrasts with *C. rodentium* infection in *Il22*$^{-/-}$ mice, in which compromised barrier function allowed systemic bacterial spread (Zheng et al., 2008). These results point to a noninnate cellular source of IL-17A required for elimination of *C. rodentium*, with Th17 cells as the likely candidate. IL-17A-producing Th17 cells are present in the intestine of *C. rodentium*-infected mice and are required for *C. rodentium* clearance (Mangan et al., 2006). Our results reinforce the functional dichotomy of IL-17A and IL-22 action in the gut: IL-22 is required early to maintain epithelial cell integrity and prevent bacteria spread, whereas proinflammatory IL-17A is required later for complete bacterial clearance. We suggest that intestinal NKp46$^+$ cells could provide the rapid, innate source of IL-22, whereas adaptive Th17 cells would furnish IL-17A.

The total surface of intestinal mucosa is estimated to be >100 m^2 in humans and harbors >80% of all immune cells (Artis, 2008). Given that the intestine harbors an estimated 10^{14} commensal bacteria, it is logical that several elaborate mechanisms have evolved to sustain the mucosal epithelium and to generate immune responses upon intestinal epithelial stress or breech. A crosstalk between NKp46$^+$RORγt$^+$IL-22$^+$ cells and the intestinal epithelia provides a system whereby these NKp46$^+$ cells may improve epithelial barrier function and in which perturbations in intestinal epithelial continuity can be decoded and translated to an appropriate innate immune response. The reactivity afforded by a dedicated and specific NKp46$^+$ cell-based system may provide a rapid means of protecting mucosal surfaces under diverse conditions.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from Janvier. Athymic C57BL/6 nude mice deficient in *Foxn1* were provided by the Département de Cryopréservation, Distribution, Typage et Archivage Animal (CTDA), and germ-free C57BL/6 mice were obtained from the Institut National de la Recherche Agronomique (INRA). Mice bearing fluorescent reporters driven by *Rorc* and deficient in *Il6* or *Il12rb1* (Lochner et al., 2008) or driven by *Ifng* (Stetson et al., 2003), as well as mice deficient in *Il15*, *Rag2*, *Rag2* and *Il2rb*, *Rag2* and *Il2rg* (Vosshenrich et al., 2005; Vosshenrich et al., 2007), or *Rorc* (Eberl et al., 2004), have been described. *Pfp*$^{YFP/+}$ mice were generated by inserting an IRES-eYFP cassette into the murine *Pfp* 3′ untranslated region (exon 3) in C57BL/6 embryonic stem cells (K.T. et al., unpublished data). All mice were housed in specific pathogen-free animal facilities at the Institut Pasteur and were analyzed between 6 and 12 weeks of age in accordance with regulations of the French authorities.

Cell Isolation and Flow Cytometry

IELs were isolated with 0.5 mM EDTA and gentle agitation. After removal of any residual epithelium, LPLs were isolated with collagenase digestion as described (Lochner et al., 2008). All antibodies were purchased from BD PharMingen or eBiosciences. Immunofluorescence analysis (BD FACS Canto II) was done as described (Vosshenrich et al., 2007).

RNA Isolation and Gene Expression Analysis

RNA was isolated from purified lymphocyte preparations with the RNAeasy Micro Kit (QIAGEN) and converted to cDNA with Superscript III Reverse Transcriptase (Invitrogen). In some cases, total RNA was amplified with the Message Booster Kit (Epicenter Biotechnologies). Quantitative PCR was performed with gene-specific primers and Th17 RT^2 Profiler PCR Array (SuperArray Bioscience Corporation).

Infection with *Citrobacter rodentium*

Mice were fasted for 8 hr and gavaged with 2 × 10^9 CFU of the *C. rodentium* strain CDC 1843-73 (ATCC 51116; American Type Culture Collection) before resuming normal feeding ad libitum. Mice were weighed daily and monitored for diarrhea and bloody stool. A clinical score was calculated as follows: no

diarrhea (+1), soft stool (+2), liquid stool (+3), no blood (+1), bloody stool (+2), weight gain (+1), weight stable (+2), weight loss between 1%–3% (+3), weight loss > 3% (+4).

Tissue Preparation and Histological Analysis

Small and large intestines were fixed in 4% paraformaldehyde overnight and washed in PBS before equilibration with sucrose and embedding in OCT. Immunostaining was performed as described (Lochner et al., 2008).

SUPPLEMENTAL DATA

Supplemental Data include three figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00504-9.

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