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The Natural Cytotoxicity Receptor NKp46 Is Dispensable for IL-22-Mediated Innate Intestinal Immune Defense against Citrobacter rodentium

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Natural cytotoxicity receptors (including NKp30, NKp44, and NKp46 in humans and NKp46 in mice) are type I transmembrane proteins that signal NK cell activation via ITAM-containing adapter proteins in response to stress- and pathogen-induced ligands. Although murine NKp46 expression (encoded by Ncr1) was thought to be predominantly restricted to NK cells, the identification of distinct intestinal NKp46+ cell subsets that express the transcription factor Rorc and produce IL-22 suggests a broader function for NKp46 that could involve intestinal homeostasis and immune defense. Using mice carrying a GFP-modified Ncr1 allele, we found normal numbers of gut CD3+‘GFP+’ cells with a similar cell surface phenotype and subset distribution in the absence of Ncr1. Splenic and intestinal CD3+NKp46+ cell subsets showed distinct patterns of cytokine secretion (IFN-γ, IL-22) following activation via NK1.1, NKp46, IL-12 plus IL-18, or IL-23. However, IL-22 production was sharply restricted to intestinal CD3+‘GFP+’ cells with the CD127+‘NK1.1−’ phenotype and could be induced in an Ncr1-independent fashion. Because NKp46 ligands can trigger immune activation in the context of infectious pathogens, we assessed the response of wild-type and Ncr1-deficient Rag2−/− mice to the enteric pathogen Citrobacter rodentium. No differences in the survival or clinical score were observed in C. rodentium-infected Rag2−/− mice lacking Ncr1, indicating that NKp46 plays a redundant role in the differentiation of intestinal IL-22+ cells that mediate innate defense against this pathogen. Our results provide further evidence for functional heterogeneity in intestinal NKp46+ cells that contrast with splenic NK cells. The Journal of Immunology, 2009, 183: 6579–6587.

Diverse NK cell effector functions are tightly controlled via a balance of signals delivered through a diverse set of activating and inhibitory cell surface receptors (1). Among the different activating receptors on NK cells, a family of natural cytotoxicity receptors (NCRs)† has been identified that play an important role in MHC class I/HLA-independent cytotoxicity of target cells (reviewed in Ref. 2). Three NCRs (NKp30, NKp44, and NKp46) are present in the human genome, while only NKp46 (encoded at the Ncr1 locus) is present in mice. NKp46 demonstrates predominant NK cell-specific expression in several species (3, 4), although a small TCR-γδ cell population expressing NKp46 has been described in adult mice (5) and in IL-15-expanded human T cells (6). NKp46 lacks intrinsic signaling motifs and associates with ITAM-bearing adaptor molecules (including CD3ζ and FcRγI) to couple ligand binding to the intracellular signaling machinery (3, 7). Cross-linking NKp46 on mature human or mouse NK cells with plate-bound Abs elicits cytokine release and enhances degranulation of cytotoxic effector molecules (4, 8). Similar results have been obtained using anti-NKp30 and anti-NKp44 Abs, suggesting that engagement of NCRs by their respective ligands on target cells can modulate NK cell activity (9, 10). Accordingly, blockade of NCRs, alone or in combination reduces NK cell cytotoxicity in vitro against susceptible targets (reviewed in Ref. 2).

Several NCR ligands have been described, including influenza virus hemagglutinin (11) and Sendai virus hemagglutinin/neuraminidase (12), that bind NKp44 on human NK cells and NKp46 in both mice and humans. More recently, NCR ligands were found to be expressed by murine lymphoma and myeloma cell lines (13) and in human primary nevi and melanomas (14, 15). Moreover, a NKp44-Fc fusion protein binds to the surface of mycobacterium (Mycobacterium bovis, Mycobacterium tuberculosis)-infected cells and anti-NKp46 antisera partially inhibit lysis of M. tuberculosis-infected monocytes by NK cells, suggesting the existence of NCR ligands on bacteria or bacterially infected cells (16). Although the nature of certain NCR ligands remains obscure, studies using NKp46-deficient mice demonstrated increased susceptibility to lethal influenza infection (17) and aberrant tumor immunosurveillance (13), suggesting an important role for triggering NKp46 during innate immune defense.

Recently, several groups described NKp46 expression on a novel innate cell subset in the murine intestine (18–20). Using mice carrying a GFP reporter inserted into the Ncr1 locus, we were able to identify CD3+‘GFP+’ cells from the intestinal lamina propria and intraepithelial compartments that differentially expressed

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1 Abbreviations used in this paper: NCR, natural cytotoxicity receptor; LTi, lymphoid tissue inducer; LPL, lamina propria lymphocyte; WT, wild type.

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CD127 and NK1.1 (18). All intestinal NKp46+ subsets developed independently of the thymus and were found in recombina-ntactivating gene 2 (Rag2)-deficient mice. A minor subset of gut NKp46+ cells resembled mature splenic NK cells (NK1.1+CD127+Rorc+) with expression of typical NK cell markers (including NKG2D, DX5, CD122, CD11b, and Ly49 family members). Surprisingly, a predominant CD3+NKp46+ subset (CD127+NK1.1+) failed to express the above-cited NK markers and, furthermore, lacked typical effector functions (perforin, IFN-γ) associated with mature NK cells (18).

Analyses of the gene expression profiles revealed the expression of the transcription factor Rorc in subsets of propria CD3+NKp46+ cells (18, 20, 31). Rorc has been previously shown to be essential for thymocyte survival, development of lymphoid tissue inducer (LTi) cells and for the differentiation of polarized T cells that express IL-17 family cytokines (21). Surprisingly, intestinal NKp46+ cells failed to express constitutive or inducible IL-17A, although gut CD3+NKp46+ cells expressing CD127 harbored IL-22 transcripts. Moreover, the homeostasis of these NKp46+ IL-22+ cells required the presence of intestinal microflora (18, 19) and an intact Rorc gene (18, 20). These results identify novel NKp46+ cell subsets in the gut that appear hard-wired for rapid IL-22 production in response to microbial signatures.

IL-22 expression by gut CD3+NKp46+ cells appears to play a role in immune defense against intestinal pathogens. Initial immune control of the Gram-negative bacteria Citrobacter rodentium infection is T and B cell independent and critically depends on IL-22 (22). IL-22 is proposed to “cross-talk” with intestinal epithelial cells and promote innate defense through the elaboration of antimicrobial peptides (22, 23). Although earlier studies suggested that innate IL-22 production under these conditions was derived from CD11c+ dendritic cells (22), we showed that mice lacking intestinal CD3+ NKp46+ cells were unable to produce IL-22 and rapidly succumbed to C. rodentium (18). These studies suggest that IL-22-producing NKp46+ cells play an important role in intestinal homeostasis and in the early innate response that provides protection against pathogens.

Cytokine production by NK cells is controlled by cell surface ligands that trigger NK cell receptors as well as by soluble factors elaborated in the local microenvironment (24). Little is known about the mechanisms that control IL-22 production from CD3+ NKp46+ cells in the gut. Signaling through NKp46 could impact on the development, differentiation, anatomical localization, homeostasis, and/or activation of intestinal IL-22+ cells. In this study, using Ncr1GFP/+ reporter mice, we examine the development and differentiation of intestinal IL-22+ cell subsets in the absence of Ncr1 and assess the capacity of Ncr1-deficient Rag2−/− mice to resist early infection by C. rodentium.

Materials and Methods

Mice

C57BL/6 mice were purchased from Janvier. Ncr1GFP/+ mice on the C57BL/6 background (17) were bred to homozygosity to create Ncr1-deficient mice (Ncr1GFP/GFP) and with Rag2-deficient mice on the C57BL/6 background to generate Rag2−/−Ncr1GFP/+ and Rag2−/−Ncr1GFP/GFP mice. Rag2−/−Il2rb−/− and Rag2−/−Il2rg−/− mice on the C57BL/6 background have been previously described (18). Il22−/− mice on the C57BL/6 background have been previously reported (25). All mice were housed under specific pathogen-free conditions at the Laboratory Animal Facilities of the Institut Pasteur. Mice were used for experiments at 6–12 wk of age and experiments were conducted following the guidelines provided by the Animal Care and Use Committee of the Institut Pasteur and in accordance with French law.

Cell isolation and flow cytometric analysis

Total splenocytes were prepared by gentle dissociation using mesh filters followed by erythrocyte lysis using NH4Cl. Intestinal Peyers’s patches were removed and intestinal epithelial cells eliminated by incubation in 0.5 mM EDTA. Subsequently, lamina propria lymphocytes (LPL) were isolated using 0.5 mol collagenase (Sigma, St. Louis, MO) and were further purified using discontinuous Percoll gradients (40 and 75%). To analyze cell phenotype, FITC- or PE-conjugated NKp46 mAb (29A1.4; eBioscience), anti-CD3 (eBioscience), anti-NK1.1 (BD Pharmingen), anti-CD127 (BD Pharmingen), anti-CD94 (Serotec), anti-2B4 (BD Pharmingen), anti-Ly49A/D (BD Pharmingen), anti-CD11b (eBioscience), and anti-NK2D2 (BD Pharmingen) were used as previously described (18). Purified polyclonal goat Abs against mouse NKp46 were available from R&D Systems. All washings and reagent dilutions were made with PBS containing 2% FCS. A Canto II flow cytometer interfaced to the FACSDiva software (BD Biosciences) was used to acquire data that was analyzed using FlowJo software (Tree Star).

In vitro cytokine stimulation and intracellular staining

Cross-linking Abs (NK1.1 clone PK135, BD Pharmingen; 2B4, BD Pharmingen; NKp46 clone 29A1.4, a gift from E. Vivier, CIML, Marseille, France) were used to coat 96-well tissue culture plates for 4 h at 37°C. After three washes with PBS, total splenocyte or LPL preparations diluted in RPMI 1640 with 10% FCS were added (106 cells/well) for 4 h in the presence of GolgStop (BD Pharmingen). In some cases, cells were left unstimulated or stimulated with cytokines (IL-12 at 5 ng/ml plus IL-18 at 100 ng/ml; IL-23 at 40 ng/ml) for 6 h. After surface staining and incubation with LIVE/DEAD Fixable reagent (BD Pharmingen), cells were fixed in 4% paraformaldehyde and processed for intracellular cytokine detection. Cells were stained with PE-conjugated anti-IFN-γ (BD Pharmingen) and Alexa Fluor 647-labeled anti-IL-22 mAb (MH22B2.2; Ref. 26) in permeabilization buffer (0.1% saponin in HBSS) before extensive washing in PBS and analysis. For RORγt detection, an anti-mouse/human RORγt-PE (clone AFKJ5-F9; eBioscience) was used with fixation and permeabilization (eBioscience).

Immunohistology

Immunohistology was performed as previously described (18, 27). Tissues (lymph node and small intestine) were fixed in 4% paraformaldehyde overnight at 4°C, equilibrated with 30% sucrose in PBS, before extensive di-alysis against PBS. Rabbit anti-GBP mAb (In vitrogen) was used for GBP detection followed by an anti-rabbit Alexa Fluor 488 mAb (In vitrogen). Sections were stained with PE-conjugated anti-B220 mAb (BD Pharmingen). To detect CD3 expression, hamster anti-CD3 mAb (BD Pharmingen) and anti-hamster Alexa Fluor 647 mAb were used. Control staining with fluorochrome-conjugated anti-rabbit or anti-hamster Secondary Abs alone gave only nonspecific background staining (data not shown).

C. rodentium infection

Groups of five Ncr1GFP/GFP and Ncr1GFP/+ mice (male, 8 wk of age) were gavaged with 2 × 10^8 CFU C. rodentium (CDC1843-73; ATCC 51116). Mice were weighed and checked for diarrhea/bloody stool on a daily basis. Survival was monitored for the first 40 days after infection.

Statistical analysis

The statistical significance of differences between groups was determined by the unpaired Student t test. Values of p < 0.05 were considered significant.

Results

Normal development, distribution, and homeostasis of intestinal CD3+FP− cell subsets in homozygous Ncr1GFP/GFP reporter mice lacking NKp46 expression

Mice bearing a targeted insertion of the Ncr1 locus show specific GBP expression in CD3−NK cells (NK1.1+, DX5−) in the bone marrow, lymph nodes, spleen, and peripheral blood (17). To demonstrate that GBP faithfully recapitulates NKp46 expression, we further assessed cell surface NKp46 protein expression in CD3+FP− cells from Ncr1GFP/+ and Ncr1GFP/GFP mice. The anti-NKp46 mAb (clone 29A1.4; eBioscience) uniformly stained splenic CD3+FP− cells from Ncr1GFP/+ mice but not from Ncr1-deficient Ncr1GFP/GFP mice (Fig. 1A). In contrast, purified polyclonal goat Abs raised against mouse NKp46
showed poor specificity, with unexpected staining on splenic and intestinal GFP+/H11002 cells from both Ncr1GFP+/H11001 and Ncr1GFP/GFP mice and no selective staining of GFP+ cells in Ncr1GFP+/H11001 mice compared with Ncr1-deficient Ncr1GFP/GFP mice (supplemental Fig. 14 and data not shown). NK cell markers, including NK1.1, DX5, NKG2D, CD122, and CD244 (2B4), were normally expressed on splenic CD3+ GFP+/H11001 cells in the absence of Ncr1 (Fig. 1A and data not shown). Similar results were obtained when analyzing CD3+ GFP+ cells isolated from the small intestinal lamina propria and intraepithelial compartments (Fig. 1A, supplemental Fig. 2, and data not shown).

We found that the transcription factor Rorc (encoding RORγt) was expressed in the CD127+ NK1.1+ subset of intestinal lamina propria CD3+ GFP+ cells in a Ncr1-independent manner (Fig. 1B). Immunohistological analysis confirmed a normal distribution of GFP+ cells in
In the absence of Nkp46 (Fig. 2 and Table I). These results suggest that Nkp46 expression is not essential for the development and homeostasis of diverse Nkp46+ cell subsets in mice.

Cytokine production from diverse splenic and intestinal CD3+ GFP+ cell subsets in Ncr1GFP+/+ and Ncr1GFP/GFP mice following triggering via activating receptors or cytokines

Previous studies had demonstrated that the triggering of cell surface NK1.1 or Nkp46 could elicit cytokine production (IFN-γ) from splenic NK cells (8, 28). Although plate-bound anti-NK1.1 or Nkp46 Abs could stimulate IFN-γ production from splenic NK1.1+ cells, cross-linking NK1.1, 2B4, or Nkp46 on intestinal CD3+ GFP+ cells did not generate appreciable IFN-γ production (Fig. 3 and supplemental Fig. 3), even though a subset of gut Nkp46+ cells expressed IFN-γ transcripts as previously demonstrated (18) using “Yeti” reporter mice (29). Intestinal CD3+ GFP+ cells were not blocked in their capacity to produce IFN-γ, as stimulation of intestinal LPL with a combination of IL-12 plus IL-18 clearly induced IFN-γ secretion (Fig. 3).

We next assessed whether cytokines or cell surface receptor cross-linking could elicit IL-22 production from intestinal CD3− Nkp46+ cell subsets. We used a recently described anti-IL-22 Ab (MH22B2.2; Ref. 26) that specifically detects mouse IL-22 (supplemental Fig 4). Neither NK1.1 nor Nkp46 cross-linking was capable of inducing IL-22 production from splenic or intestinal CD3− Nkp46+ cells (Fig. 3 and supplemental Fig. 3). Interestingly, IL-12 plus IL-18 stimulation also resulted in IL-22 production from intestinal but not splenic CD3+ GFP+ cells, while IL-23 stimulation induced IL-22 from CD3− GFP+ cells and only in the gut (Fig. 3).

Our previous transcriptional analysis suggested that although IL-22 expression was detectable in the CD127− NK1.1+ subset, ~10-fold higher levels of IL-22 transcripts were present in intestinal Nkp46+ cells that were CD127+ but completely lacked NK1.1 expression (18). Despite the presence of IL-22 transcripts by CD3− Nkp46+ that coexpress CD127, constitutive IL-22 protein expression was not obvious in intestinal CD3− GFP+CD127+ cell subsets in Ncr1GFP+/+ or Ncr1GFP/GFP mice (Fig. 4).

To define which intestinal CD3− GFP+ subsets could produce IFN-γ and/or IL-22 in Ncr1GFP+/+ and Ncr1GFP/GFP mice, we analyzed intracellular cytokine staining patterns in intestinal CD3− GFP+ subsets that differentially express CD127 and NK1.1 following cytokine stimulation. IL-23 can induce IL-22 production in Th17-polarized T cells (30). IL-23 also induces IL-22 production from small intestinal LPL of wild-type (WT) (31) and Rag2-deficient mice (data not shown), consistent with a non-T cell source in the gut. Following stimulation with IL-23, we detected IL-22 production in intestinal CD3− GFP+ cells that was negatively correlated with NK1.1 expression and sharply restricted to the NK1.1+ fraction of CD3− GFP+ cells (Fig. 4A). This result contrasts with previous reports indicating

Table I. Homeostasis of splenic and intestinal CD3+ GFP+ cell subsets in Ncr1GFP+/+ and Ncr1GFP/GFP mice

<table>
<thead>
<tr>
<th></th>
<th>Ncr1GFP+/+</th>
<th>Ncr1GFP/GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Nkp46+</td>
<td>1,174,883 ± 616,293</td>
<td>1,004,992 ± 120,287</td>
</tr>
<tr>
<td>Small LPL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Nkp46+</td>
<td>126,645 ± 48,932</td>
<td>156,277 ± 67,374</td>
</tr>
<tr>
<td>CD127− NK1.1+</td>
<td>78,882 ± 93,371</td>
<td>93,371 ± 42,210</td>
</tr>
<tr>
<td>CD127+ NK1.1+</td>
<td>22,991 ± 7,871</td>
<td>36,302 ± 23,190</td>
</tr>
<tr>
<td>CD127− NK1.1+</td>
<td>21,832 ± 5,219</td>
<td>21,875 ± 12,689</td>
</tr>
</tbody>
</table>

* Absolute numbers of splenic and intestinal CD3− GFP+ cells (total and subsets defined by differential expression of CD127 and NK1.1) in Ncr1GFP+/+ and Ncr1GFP/GFP mice (n = 6 of each genotype) were assessed. No statistically significant differences were observed in the absence of Ncr1.
that IL-22 protein could be detected in intestinal NKp46+ cells expressing intermediate levels of NK1.1, but not in NKp46+NK1.1- cells following stimulation with phorbol ester and calcium ionophore (20) or IL-23 (19). IL-23 failed to induce IFN-γ from any gut CD3+ GFP+ cell subset, and IL-22 production from intestinal CD3+ GFP+ cells was independent of NKp46 expression and equally detected in Ncr1GFP+/ and Ncr1GFP/GFP mice (Fig. 4B).

Following IL-12 plus IL-18 in mice, a different pattern of cytokine production was observed among intestinal CD3+ GFP+ subsets (Fig. 4). As expected, NK1.1- cells could produce IFN-γ under these conditions (similar to splenic NK1.1+ cells), which was Ncr1 independent (Fig. 4B). Interestingly, the CD127+NK1.1- subset of intestinal CD3+ GFP+ cells could produce both IFN-γ and IL-22 under these conditions with cells generally producing only IL-22 or IFN-γ, although a small subset of cells clearly produced both cytokines (Fig. 4A). Stimulation with either IL-12 or IL-18 alone was capable of inducing IL-22 production, while IFN-γ production appeared to require both IL-12 and IL-18 (supplemental Fig. 5). Similar results were obtained in intestinal cells from mice lacking Ncr1 (Fig. 4B). These results indicate that different cytokine signals can elicit distinct cytokine production patterns from intestinal CD3+ GFP+ subsets.

A role for NK1.1+ cells but not the NKp46 receptor in the innate immune response to C. rodentium infection in Rag2-deficient mice

Although IL-22 production from in vitro-activated NKp46+ cells appeared independent of Ncr1 expression, a more rigorous test of NKp46- cell function would involve the capacity of these cells to produce IL-22 in vivo following infection with the Gram-negative bacterium C. rodentium (18, 31). Consistent with IL-22 production from innate cells, we found that CD3+ NKp46+ NK1.1- cells (but not CD3+ cells) were IL-22+ during the first week after C. rodentium infection (Fig. 5A and data not shown). To assess a role for NKp46 in the innate intestinal defense, we generated T and B cell-deficient mice that were competent or incompetent for NKp46 triggering (Rag2-/-Ncr1GFP/GFP and Rag2-/-Ncr1GFP/GFP mice) and orally infected them with C. rodentium. Rag2-/-Il2rg-/- mice were used as a positive control because these mice lack all NKp46+ cells, produce no IL-22, and succumb rapidly after C. rodentium infection (18). We found no difference in the capacity of Rag2-/- mice with or without NKp46 to resist early C. rodentium infection (Fig. 5, B and C). Both Rag2-/-Ncr1GFP/GFP and Rag2-/-Ncr1GFP/GFP mice showed similar clinical scores and maintained body weight up to 40 days after infection, whereas all infected Rag2-/-Il2rg-/- mice died within 8 days. Thus, innate immune responses to C. rodentium are intact in the absence of NKp46.

We previously reported that the early immune response to C. rodentium infection was intact in Rag2-/-Il2rb-/- mice that harbor normal numbers of CD3+ NKp46+ cells that express CD127 but are devoid of intestinal NK1.1+ cells (18). This protection correlated with intact IL-22 production in Rag2-/-Il2rb-/- mice (18) that was likely produced by the CD127+NK1.1+ subset of intestinal CD3+ NKp46+ cells as demonstrated above (Fig. 4A). In this study, we extend these initial observations by analyzing C. rodentium infection at later time points in Rag2-/-Il2rb-/- mice. Interestingly, we found that Rag2-/-Il2rb-/- mice (but not Rag2-/- mice) succumbed to infection by day 35 (Fig. 5, B and C). As Rag2-/-Il2rb-/- mice lack NK1.1+ cells (18) that can produce IFN-γ (Fig. 4A), these results underline the important role for intestinal NK1.1+ cells in the defense against C. rodentium. These results are also in accordance with the previous report from the Colonna laboratory using NK1.1 depletion in C. rodentium-infected Rag2-/- mice (31). Nevertheless, the kinetics of susceptibility to C. rodentium in the
absence of NK1.1− cells is clearly distinct and delayed (≈30 days) in comparison to infected Rag2−/− Il2rg−/− mice (Fig. 5, B and C) or to infected Rag2−/− mice when endogenous IL-22 is neutralized (22). Under these conditions, mice rapidly succumb to infection (≈7 days). These results point to distinct roles for CD127NK1.1− and NK1.1+ subsets of CD3−NKp46+ cells in the innate intestinal defense against C. rodentium.

Discussion

NCRs comprise a set of transmembrane signaling receptors in human NK cells (NKp30, NKp44, NKp46) and in mice (NKp46 encoded at the Ncr1 locus) that interact with specific ligands and modulate NK cell effector functions (reviewed in Ref. 32). Although NCR expression was initially characterized as NK specific (4, 7) and later proposed as part of a “universal” definition of NK cells (33), we now appreciate that NKp46 can be expressed by some T cell subsets (34, 35) and by intestinal cells that have only a marginal resemblance to classical NK cells (18–20, 31). As such, the functional heterogeneity within CD3−NKp46+ cells of a given tissue may vary considerably. In some organs (e.g., spleen), CD3−NKp46+ cells are quite homogeneous and comprise cytolytic, IFN-γ-producing NK cells. In contrast, in the gut, a subset of CD3−NKp46+ cells include classical NK cells, but the majority are characterized by the absence of most NK cell markers and effector functions and instead express the transcription factor Rorc and produce IL-22 (18–20, 31). Given that the homeostasis of this latter population is dependent on microbial flora (18, 19) and is implicated in IL-22 production in response to the intestinal pathogen C. rodentium (18, 22, 31), we assessed whether NKp46 expression was required for the development, differentiation, and function of intestinal innate lymphocytes that produce IL-22 and protect against C. rodentium.

By analyzing mice harboring a GFP-modified Rag2−/− allele (17) in the heterozygous (WT) or homozygous (knockout) state, we were able to assess whether the absence of surface NKp46 expression influenced the development and function of intestinal CD3−GFP+ cells. We found that the subset distribution of CD3−GFP+ cells expressing CD127 and/or NK1.1 as well as their overall homeostasis (cell numbers and tissue distribution)
We found that RORγt expression by this subset may indicate that intestinal CD3−NKp46+ cells exhibit substantial phenotypic and functional diversity (18–20). Using CD127 and NK1.1, we have characterized three gut CD3−NKp46+ cell subsets. The two NK1.1+ subsets (CD127+, CD127−) appear related to classical NK cells by many criteria, including expression of NK markers (NK2D, CD122, DX5, Ly49 family members), perforin and IFN-γ effectiveness, but expresses transcripts for Rorc and IL-22 (18). Using IL-22-specific Abs, we demonstrated that IL-22 protein is barely detectable in unstimulated gut CD3−NKp46+ cells, but is rapidly produced in activated cells. Thus, IL-22 protein expression may be controlled posttranscriptionally with rapid translation in cells containing a preformed pool of IL-22 transcripts in a fashion analogous to IFN-γ (37). This regulation would be consistent with the role for IL-22-producing cells during initial innate phases of the immune response.

The signals that control cytokine production from intestinal CD3−NKp46+ cells are poorly understood and could include receptors that recognize cell-associated as well as soluble ligands. In this report, we assessed the contribution of cell surface receptors (NK1.1, NKp46, 2B4) and soluble cytokines (IL-12, IL-18, IL-23) to IL-22 and IFN-γ production by intestinal CD3−NKp46+ cell subsets. In contrast to splenic NK cells, intestinal NKp46+ cells failed to produce cytokines after NK1.1+, 2B4, or NKp46 cross-linking, but were competent for cytokine secretion after stimulation with soluble factors. We found that several intestinal CD3−NKp46+ cell subsets (NK1.1+ cells but also CD127+NK1.1− cells) can produce IFN-γ following stimulation. Moreover, IL-12 plus IL-18-induced production of IFN-γ by the CD127−NK1.1− subset was accompanied by IL-22 production, with a small population of IL-22+IFN-γ double producers. The explanation for the existence of distinct cytokine-producing cells within the CD127−NK1.1− subset is not yet known, but several hypotheses can be forwarded. One possibility is that further heterogeneity exists in the CD127−NK1.1− subset with subsets that are selectively tuned toward either IL-22 or IFN-γ production. An alternative explanation is that cytokine profiles are dynamically regulated within CD127−NK1.1− cells (e.g., starting out as major IL-22 producers that evolve toward IFN-γ producers as cells become chronically stimulated). Collectively, these results suggest that intestinal CD3−NKp46+ cells may have the potential to secrete different cytokine profiles depending on the environmental context. As such, intestinal CD3−NKp46+ cell subsets could have varied roles throughout the course of an evolving immune response.

IL-22-expressing CD3−NKp46+ cells are present in the small and large intestine under steady-state conditions (18–20) and cultured colonic lamina propria cells from C. rodentium-infected mice produce IL-22 that is correlated with the presence of CD3−NKp46−NK1.1− cells (18, 22). Although other laboratories detected IL-22 protein in a subset of intestinal NK1.1+ cells (19, 20), in our hands, IL-22 production following cytokine stimulation was sharply restricted to the CD127−NK1.1− subset of CD3-NKp46+ cells. IL-22 production by intestinal NK1.1− cells would be consistent with the normal early innate immune response (up to 30 days) after C. rodentium infection in mice that genetically lack NK1.1+ cells (18) or are treated with depleting anti-NK1.1 Abs (31). Nevertheless, NK1.1− cells clearly play an important role in the ultimate defense against this pathogen as illustrated by the reduced survival of C. rodentium-infected Rag2−/−Il2rb−/− mice compared with Rag2−/− mice. Our results are consistent with the previous report showing that anti-NK1.1 treatment reduced overall survival of C. rodentium-infected WT mice (31). Because the NK1.1− subset of intestinal CD3−NKp46+ cells is a poor IL-22 producer, but can produce IFN-γ following stimulation, it remains possible that the major role for intestinal NK1.1− cells in the course of C. rodentium infection would be at a latter stage,
complementing the IL-22-mediated restriction of bacterial spread into the host. Further studies will be required to test this hypothesis.

Lastly, by comparing *C. rodentium* infection in T and B cell-deficient mice that were competent or incompetent for NKp46 triggering (Rag2*−/−Ncr1*−/−FP/Fp and Rag2*−/−Ncr1*−/−FP/Fp mice), we could provide clear evidence that NKp46 was not required for innate immune defense against this pathogen. Several explanations may account for this result. First, NKp46 ligands may not be induced during the course of *C. rodentium* infection. Little is known about the nature of NKp46 ligands outside of the identified viral hemagglutinins (11). Studies using NKp46-Fc fusion proteins may help to further investigate whether *C. rodentium* (or other bacterial infections) induce NKp46 ligands. Second, IL-22 production from intestinal CD3**+** NKp46**+** cells may be elicited by triggering of other cell surface receptors or via soluble factors. Stimulation with IL-12, IL-18, or IL-23 resulted in IL-22 production in vitro, and IL-12, IL-18 and IL-23 production by monocytes or dendritic cells has been documented following triggering of pathogen-associated molecular pattern receptors during infection (38). The critical role of IL-12 or IL-23 in vivo for immunity to *C. rodentium* was reported by MacDonald and colleagues (39), who found increased mortality and compromised *C. rodentium* control in mice lacking the IL-12p40 subunit of IL-12 and IL-23. Release of IL-12 and/or IL-23 during infection, other IL-22-producing cells may also be engaged during this process and compensate in the absence of NKp46. Further analysis of the critical signals that control IL-22 production in vivo during steady state and after infections challenge will help to unravel the biological roles for distinct CD3**+** NKp46**+** cell subsets in intestinal immune protection and homeostasis.

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**Disclosures**

The authors have no financial conflict of interest.

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