Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor

Emilie Narni-Mancinelli a,b,c, Julie Chais a,b,c,1, Aurore Fenisa,b,c, Yann M. Kerdiela,b,c, Nadia Yessaada,b,c, Ana Reynerdasa,b,c, Claude Gregoirea,b,c, Herve Lucheab,c, Sophie Ugolinab,c, Elena Tomaselloa,b,c, Thierry Walzerab,c,d, and Eric Viviera,b,c,d,4

a Centre d’Immunologie de Marseille-Luminy, Université de la Méditerranée UM 631, Campus de Luminy Case 906, 13288 Marseille, France; b Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche S 631, 13009 Marseille, France; c Centre National de la Recherche Scientifique, Unité Mixte de Recherche 6102, 13288 Marseille, France; and d Assistance Publique, Hôpitaux de Marseille, Hôpital de la Conception, 13385 Marseille, France

Edited* by Christophe Benoist, Harvard Medical School, Boston, MA, and approved September 19, 2011 (received for review July 26, 2011)

NKp46 is a cell surface receptor expressed on natural killer (NK) cells, on a minute subset of T cells, and on a population of innate lymphoid cells that produce IL-22 and express the transcription factor retinoid-related orphan receptor (ROR)-γt, referred to as NK cell receptor (NKR)+ROR-γt cells. Here we describe NKp46Cre knock-in mice in which the gene encoding the improved Cre (iCre) recombinase was inserted into the Nkp46 locus. This mouse was used to noninvasively trace cells expressing NKp46 in vivo. Fate mapping experiments demonstrated the stable expression of NKp46 on NK cells and allowed a reappraisal of the sequential steps of NK cell maturation. NKp46 genetic tracing also showed that gut NKR+ROR-γt and NK cells represent two distinct lineages. In addition, the genetic heterogeneity of liver NK cells was evidenced. Finally, NKp46Cre mice also represent a unique mouse model of conditional mutagenesis specifically in NKp46+ cells, paving the way for further developments in the biology of NKp46+ NK, T, and NKR+ROR-γt cells.

natural killer cell differentiation | NKp46 knock-in

Natural killer (NK) cells are effector and regulatory lymphocytes of the innate immune system that contribute to tumor surveillance, hematopoietic allograft rejection, control of microbial infections, and pregnancy (1). NK cells can be cytotoxic and secrete an array of cytokines and chemokines, such as IFN-γ and β-chemokines.

NKp46 (NCR1, CD335) is a marker of NK cells in all mammalian species tested so far, including human, nonhuman primates, mouse, rat, and cow (2–8). NKp46 is an Ig-like superfamily cell surface receptor, which is a member of a group of natural cytotoxicity receptors (NCRs) with NKp44 (NCR2, CD336) and Nkp30 (NCR3, CD337) (9). NKp46 is associated with immunoreceptor tyrosine-based activation motif-bearing polypeptides, such as CD3-ζ and FcR-γ, which transduce potent activating signals upon triggering (4, 10). NKp46 is involved in tumor cell recognition via still-unidentified ligands (11, 12) and has also been described as binding viral hemagglutinins (13, 14). Finally, it has been reported that NK cells contribute via NKp46 to type 1 diabetes through the destruction of pancreatic β-islets (15). NKp46 is found on all mature NK cells regardless of their anatomic localization in both human and mouse. The selective expression of NKp46 on NK cells has two reported exceptions: rare T-cell subsets (6, 16, 17) and a mucosal population of NKp46+ innate lymphoid cells (ILCs) that express the transcription factor retinoid-related orphan receptor (ROR)-γt and produce IL-22, a key cytokine for the activation and defense of epithelial cells (18–28).

The NKp46 amino acid sequence is highly conserved in all mammals. The striking homologies in the regulatory regions of the NKp46 gene from opossum to human prompted us to generate a transgenic mouse line, called NDE, in which the diphtheria toxin receptor (DTR) and the enhanced green fluorescent protein (eGFP) expression were driven by a 450-bp conserved promoter region (P1) upstream of NKp46 (Ncr1) start codon (6). Although the selectivity of expression of eGFP in NDE mice mirrored that of endogenous NKp46 in a fraction of transgenic mice, variegation at the transgenic locus led to unpredictable variation in the penetrance of the transgene expression in mouse littersmates. Another transgenic mouse expressing the improved Cre (iCre) recombinase under the control of the Nkp46 promoter was recently reported and crossed to eGFP reporter mice (29). However, on average only 80% of NKp46+ NK cells expressed eGFP, and no eGFP expression was detected in T cells, suggesting that the regulation of the iCre transgene expression in this model did not match with the endogenous expression of NKp46, hence hampering the use of these mice for selective gene targeting in NKp46+ cells. To circumvent these caveats of expression pattern that are classically observed in transgenic mice (30), we generated a knock-in mouse line in which the gene encoded the improved recombinase (iCre) was inserted by homologous recombination at the 3’ end of NKp46, in an attempt to drive its expression by all endogenous NKp46 regulatory elements. Here we report that iCre expression faithfully corresponds to the endogenous expression of NKp46, on bona fide NK cells, on a subset of gut ILCs, as well as on very discrete subpopulations of T cells, allowing us to trace the fate of the heterogeneous NKp46+ populations of cells.

Results

Characterization of NKp46Cre Knock-in Mice. We generated a knock-in mouse line in which iCre was inserted by homologous recombination at the 3’ end of the Nkp46 gene (Fig. L4 and Fig. S1). NKp46Cre/wt mice were obtained at Mendelian frequencies, developed normally, were fertile, and showed no significant variations in the numbers of lymphoid and myeloid subsets compared with wild-type (wt) littersmates. NK cell counts, phenotype, and in vitro effector function were not affected in NKp46Cre/wt mice, despite a down-regulation in the density of cell surface NKp46 that did not alter the percentage of NKp46+ NK cells (Fig. S2).

Rosa26eYFP reporter mice (R26r<sup>eYFP</sup>−/−) carry under the Rosa26 promoter a loxP-flanked STOP sequence that prevents the expression of the downstream eYFP gene. The STOP sequence is removed and eYFP is expressed in cells where iCre is expressed


Conflict of interest statement: E.V. is a co-founder and shareholder of Innate-Pharma.

*This Direct Submission article had a prearranged editor.

1E.N.-M. and J.C. contributed equally to this work.

2Present address: Abramson Family Cancer Research Institute, Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104.

3Present address: Université de Lyon, Institut National de la Santé et de la Recherche Médicale U851, F-69007 Lyon, France.

4To whom correspondence should be addressed. E-mail: vivier@ciml.univ-mrs.fr.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1112064108/-DCSupplemental.
Diphtheria toxin (DT) treatment in NKp46+ cells was detected in the spleen (Fig. S3). Reciprocally, splenic restricted NKT cells, dendritic cells, neutrophils, and macrophages

promoter a

Rosa26

expression of NKp46 on NK1.1+CD3

CD4

neutrophils macrophages

Nkp46

expression of YFP expression (Fig. 1). (A) Flow cytometric measurement of eYFP expression on gated NK cells (C) or indicated cell population (D) from indicated organs of NKp46iCrEiCre/wt mice and control NKp46iCre/wt mice heterozygous at both loci and re- ferred as to NKp46iCrEiCreiCre/wt and NKp46iCrER26RiCre/wt and NKp46iCrER26RiCre/wt mice were generated to ensure the irreversible expression of the eYFP reporter gene in NKp46+ cells and their progeny, irrelevant of the possible arrest in NKp46 transcription.

We monitored the expression of eYFP to analyze the distribution of iCre in NKp46iCrER26RiCre/EYFP mice. The cell surface expression of NKp46 on NK1.1+CD3+ splenic cells matched with the expression of YFP expression (Fig. 1B). NK cells isolated from bone marrow (BM), peripheral blood, spleen, parietal lymph nodes (LNs), mesenteric LNs, lungs, and thymus uniformly expressed eYFP (Fig. 1C). Besides NK cells, discrete subsets of CD4+CD8−NK1.1+ T cells also express NKp46 (6, 16, 17). Consistent with these data, minute percentages of eYFP+αβT cells and γδT cells were detected in the spleen (Fig. S3). Reciprocally, splenic NKp46+ cells, such as B cells, CD4+ and CD8+αβT cells, CD1d-restricted NKT cells, dendritic cells, neutrophils, and macrophages did not express eYFP (Fig. 1D). Thus, the expression of eYFP in NKp46iCrER26RiCre/EYFP mice corresponded to the endogenous cell surface expression of NKp46, showing the faithful expression of iCre driven by NKp46 regulatory regions.

We also generated NKp46iCrER26DTR mice by crossing NKp46iCrEiCre to Rosa26loxP-DTRiCre mice, which carry under the Rosa26 promoter a loxP-flanked STOP sequence that prevents the expression of the downstream diphtheria toxin receptor (DTR) gene (32). Diphtheria toxin (DT) treatment in NKp46iCrER26DTR mice induced a depletion of NK cells in blood, spleen, BM, and LN (Fig. 2A and Fig. S4). The depletion induced by DT treatment in NKp46iCrER26DTR mice was restricted to NK cells (Fig. 2B),
Cells were isolated from spleen, LNs, and lung. We observed the induction of NKp46 selectively on NK1.1+CD3+ across species (7). We took advantage of the use of mouse markers that are not conserved in human pressed (35). One of the caveats of this classification resides in the use of mouse markers that are not conserved in human (e.g., DX5). In contrast to these molecules, NKp46 is conserved in the expression of c-kit (the stem cell factor receptor CD117), stage IV by the CD49β1 integrin DX5, and stage V by the CD11bβ2 integrin (34). On fully mature NK cells (stage V), c-kit and CD27 expression are lost, whereas KLRG-1 and CD43 are expressed (35). One of the caveats of this classification resides in the use of mouse markers that are not conserved in human (e.g., DX5). In contrast to these molecules, NKp46 is conserved across species (7). We took advantage of the Nkp46CreR26R eYFP mice to revisit the stages of NK cell differentiation. Stage I (CD122 NK1.1+) BM NK cells were negative for both NKp46 and eYFP, whereas NKp46 and eYFP were expressed on a majority of NK1.1+ BM NK cells (Fig. 3A and B). Furthermore, after in vitro culture in IL-15 of sorted CD122 CD3 CD19 Nkp46 BM cells, we observed the induction of NKp46 selectively on NK1.1+CD3− NK cells (Fig. S5). Thus, the cell surface expression of NKp46 was acquired on BM NK cells after NK1.1. The monitoring of NK cell maturation using CD27 and CD11b markers further supported this kinetics of differentiation (36, 37), because the acquisition of NKp46 occurred at the CD27−CD11b+ stage and remained stable (Fig. 3C and Fig. S6). Similar data were obtained when NK cells were isolated from spleen, LNs, and lung.

Following the same track, we then focused on CD16 (FcyRIIIA). Indeed, the cell surface expression of CD16 defines a checkpoint in human NK cell differentiation that is associated with NK cell maturation, as evidenced by the perforin-dependent cytotoxic function of CD16+CD56dim NK cells in contrast to CD56brightCD16− human NK cells (38). In addition, recent data support a model in which CD56brightCD16− NK cells differentiate into CD16+CD56dim NK cells (39). Although mouse NK cells also express CD16, the kinetics of CD16 surface expression on mouse NK cells is still unknown, in part due to the lack of reagent able to discriminate CD16 from CD32 (FcyRIIB). We addressed this issue using a recently described anti-CD16 antibody (40). As shown in Fig. 4A, CD16 was induced at the CD27−CD11b+ stage, but after NKp46, because CD16− cells contain both eYFP− and eYFP+ NK cells in comparable amounts, whereas CD16+ cells are uniformly eYFP+. We then analyzed the kinetics of induction of CD94, NK2D, DX5, CD11b, c-Kit, and Ly49 receptors (using a pan Ly49 mAb). We found that CD94 was expressed very early in CD122−NK1.1− cells (Fig. 4B). Because the majority of NK2D−low cells expressed CD16, the data indicated that CD16 induction preceded that of NK2D (Fig. 4C). NK2D was expressed before DX5, because DX5− cells contain NK2D+ cells, whereas all DX5− cells are uniformly NK2D+ (Fig. 4D). As for c-Kit, despite its low cell surface expression in both CD11b−low and CD11b+ cells, the c-Kit− cells contained a majority of DX5+ cells, indicating that c-Kit expression occurs after DX5 induction (Fig. 4E). Finally, regarding Ly49 expression, most c-Kit− cells are Ly49+, whereas most c-Kit+ cells are Ly49− (Fig. 4F), suggesting coinduction of these molecules.

We then analyzed whether the acquisition of the cell surface expression of Nkp46 and CD16 was associated with modifications...
in NK cell function. The induction of NKp46 expression was associated with a higher ability to secrete IFN-γ upon stimulation with IL-12 and -18, although the response of NKp46−NK1.1+ NK cells and NKp46+NK1.1+ NK cells to phorbol 12-myristate 13-acetate (PMA) and ionomycin was comparable (Fig. S7A). This higher reactivity of NKp46+NK1.1+ NK cells to IL-12 and -18 stimulation was associated with the induction of cell surface IL-12 and -18 receptors (Fig. S8). In contrast, the response of CD16−NK1.1+ NK cells and CD16+NK1.1+ NK cells to IL-12 and -18 or PMA and ionomycin was similar (Fig. S7B). Unexpectedly, the IFN-γ production of CD11b−/lowNK1.1+ and CD11b+NK1.1+ NK cells was also comparable (Fig. S7C). Thus, despite the reported acquisition of NK cell functional response to various stimulations at the CD11b+ stage (34), our data define the induction of NKp46 as a key step in the acquisition of NK cell reactivity to IL-12 and -18.

Together these data converged to propose the acquisition of NKp46 expression as a useful and stable tool to mark a previously undescribed stage 2 in NK cell differentiation. These findings also lead us to propose a revised model of NK cell differentiation based on the sequential and stable acquisition of cell surface molecules as follows: CD122 (stage 1), NK1.1 (stage 2), NKp46 (stage 3), CD16 (stage 4), and CD11b (stage 5) (Table 1). Stage 6 of NK cell maturation is still defined by the disappearance of CD27 in CD11b+ mature NK cells, awaiting the identification of a cell surface molecule that would be selectively and stably induced at this stage. Indeed, although KLRG-1 and CD43 are preferentially expressed in stage 6 NK cells (35), their cell surface expression is initiated at stage 5. This model is fully compatible with the previous classification (34) but refines the early stages of NK cell development, i.e., between stage II, as defined earlier by the induction of NK1.1 and CD94, and stage III, as defined by the induction of Ly49 and c-kit.

**Gut NKp46+ Cells.** NKp46+ cells comprise not only bona fide NK cells and a minute subset of T cells, but also a population of mucosal cells. We thus took advantage of the NKp46CreR26REYFP mice to revisit the heterogeneity of NKp46+ cells in various organs by comparing for the expression of eYFP and the cell surface of NKp46. In BM, spleen, LNs, and lungs, NKp46 and eYFP were co expressed (Fig. S9A Upper). Less than 10% of splenic and BM eYFP+ lymphocytes expressed low surface density of NKp46, but they did not correspond to a population of eYFP+NKp46− cells because they appeared as a continuum with NKp46+NK1.1+ cells (Fig. S9A Lower). Confirmation of this interpretation was obtained by using a brighter revelation of the NKp46 fluorescence using an anti-NKp46 goat antiserum and Alexa 647-conjugated secondary reagent instead of an anti-NKp46 mAb (Fig. S10).

---

**Table 1. NK cell maturation**

<table>
<thead>
<tr>
<th></th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
<th>Stage 5</th>
<th>Stage 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD122</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NK1.1</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NKp46</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD16</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD11b</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD27</td>
<td>−</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DX5</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>c-Kit</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ly49</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD94</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD43</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>KLRG1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

A revised model of NK cell maturation is proposed based on the sequential expression of CD122, NK1.1, NKp46, CD16, and CD11b at NK cell surface.
A distinct picture emerged from the analysis of gut lymphocytes because two populations of cells expressing eYFP were detected in the small intestine (Fig. 5A Left). Indeed, besides gut eYFP+ cells that uniformly express NK1.1 and the same level of eYFP compared with NKp46+ BM, splenic, LN, and lung cells, a subset of gut eYFP+ cells with lower cell surface expression of NK1.1 was detected. Further analysis revealed that eYFP+NK1.1+ cells were CD3−NKp46+CD45R+CD11b+ (Fig. 5A Right) and corresponded to bona fide small intestine NK cells (28). In contrast, eYFP+ NK1.1− and eYFP− NK1.1+ cells corresponded to a continuum of NKp46+CD11b− expressing intermediate levels of CD45 (Fig. 5A Right). These cells corresponded to the NKp46−ROR−γt− subset that produces IL-22 (22, 23, 26, 28, 41), consistent with the coexpression of eYFP and ROR−γt in gut cells from isolated lymphoid follicles (Fig. 5B) and their ability to produce IL-22 at steady state and upon IL-23 stimulation (Fig. S11). In contrast, only the eYFP− NK1.1− cells produced IFN-γ upon IL-12 and -18 stimulation, consistent with their bona fide small intestine NK cell identity (Fig. S11).

Because the expression of iCre induced the irreversible expression of eYFP in NKp46CreR26R-YFP mice, the distinct levels of eYFP observed at a single cell level could not result from a distinct regulation at the NKp46 locus but, rather, indicated a distinct regulation of the Rosa26 locus in eYFP+ vs. eYFP− cells, as shown in other reporter mouse models (42). Thus, the genetic tracing in NKp46CreR26R-YFP mice indicated that the Rosa26 locus was differently regulated in bona fide NK cells and in NKp46−ROR−γt− cells, supporting by another approach that NK cell receptor (NKR)+ROR−γt+ and NK cells represent two distinct lineages (19, 24, 28).

Liver NKp46+ Cells. The analysis of liver lymphocytes in NKp46Cre−R26R-YFP mice also revealed an unexpected complexity within eYFP+ cells. As in the gut, two populations of eYFP+ and eYFP+ lymphocytes were detected. eYFP+ cells were NK1.1+ and comprised a continuum of NKp46+ and NKp46− cells (Fig. 6), whereas eYFP− cells comprised a more homogeneous NK1.1−NKp46+ cell population. Besides the cell surface expression of NKp46, eYFP+ and eYFP− cells expressed a panel of cell surface receptors present on NK cells, such as the eYFPhi NK cell subset. This sign of immaturity in a subset of bone marrow cells was reported and associated with the low surface expression of DX5 and the constitutive expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (45). A similar phenotype was observed for the eYFPhi liver NK cells (Fig. 6), indicating that they corresponded to the CD11b−/lowDX5−/TRAIL− NK cell subset previously described in the same organ. The cell surface expression of Ly49 molecules was mostly restricted to the eYFP+ liver NK cells as in the spleen (Fig. S12B), which is consistent with the bona fide NK cell phenotype of eYFP+ liver NK cells and the more immature phenotype of eYFP− liver NK cells. Genetic tracing revealed that these two liver subsets are quite divergent, because the level of eYFP expression in CD11b−/low and CD11b+ BM NK cells was similar, in contrast to the distinct eYFP levels in CD11b−/low and CD11b+ liver NK cells (Fig. S13). Thus, the levels of YFP are not merely associated with various stages of NK cell maturation, but with a more profound lineage commitment of NK cell subsets, highlighting the use of Nkp46CreR26R-YFP mice for in vivo fate-mapping experiments.

Conclusions

We report here the fate mapping of NKp46+ cells in vivo through the generation and characterization of an unprecedented model of Nkp46Cre knock-in mice. Earlier attempts to create a mouse model selectively targeting NKp46+ cells by using nontargeted transgenesis have failed due to a complex and still poorly understood regulation of the NKp46 locus (6, 29). To visualize iCre activity in Nkp46Cre mice, we crossed them with R26R-YFP reporter mice. In Nkp46CreR26R-YFP mice, the fluorescent reporter permanently labeled cells that had switched on the expression of the Nkp46 gene. Using these mice, we have shown that the expression of iCre faithfully corresponded to the endogenous expression of NKp46. The genetic tracing of NKp46+ cells in vivo allowed us to reveal the stability of NKp46 cell surface expression. In addition, the acquisition of NKp46 marked a checkpoint of NK cell maturation. Based on these data, we propose a unique model of NK cell differentiation, which also includes CD16 as a marker of NK cell maturation. One advantage of this unique model resides in the use of cell surface molecules that are conserved in both mouse and human, with the exception of mouse NK1.1. Along this line, preliminary data obtained on CD34+ hematopoietic cell progenitors from human cord blood indicate that the induction of surface CD56 precedes that of NKp46 in an in vitro NK cell differentiation assay, supporting the hypothesis that CD56 could be positioned in the human NK cell differentiation pathway as NK1.1 in the mouse.

Furthermore, the differential expression of YFP in Nkp46CreR26R-YFP mice showed that gut NKR+ROR-γt+ and NK cells represent two distinct lineages. In addition, fate mapping experiments revealed the genetic heterogeneity of the two subsets of CD11b−/lowDX5+TRAIL− and CD11b+DX5−TRAIL− liver NK cells. It is puzzling that besides bona fide eYFP+ CD3−NKp46+NK1.1+ NK cells, subsets of NKp46dim/low eYFP+ cells were present in gut and liver. Besides their common NKp46dim/low eYFP phenotype, gut and liver eYFP+ cells did not appear to be directly related, because gut eYFP+ cells were detected in the small intestine (Fig. 5A Left).

Fig. 6. Characterization of liver NKp46+eYFP+ and NKp46dim/-eYFP+ cells. Flow cytometric characterization of NK1.1+CD3+eYFPhi and NK1.1+CD3+eYFP+ liver cells from NKp46Cre−/−R26R-YFP mice for indicated markers (open histograms) or control isotype (gray histograms). Results are representative of two to four experiments. Of note, it has recently been shown that liver NK cells have been reported and associated with the low surface expression of DX5 and the constitutive expression of tu-
NK1.1$^{dim}$ and corresponded to NKR$^+$ ROR-$^+$ cells, whereas liver eYFP$^+$ cells were NK1.1$^+$ and expressed TRAIL. Together, these results define Nkp46$^{Cre}$ mice as a unique mouse model of specific targeting in Nkp46$^+$ cells, allowing the generation of unique mouse strains based on the crossing of Nkp46$^{Cre}$ mice to a variety of floxed mice to dissect the biology of Nkp46$^+$ NK, T, and gut NKR$^+$ ROR-$^+$ cells.

**Material and Methods**

**Mice and Crosses.** We have generated a Nkp46$^{Cre}$ knock-in mouse line in which the gene encoded the Cre recombinase (Cre) was inserted by homologous recombination at the 3' end of the Nkp46 gene (see SI Materials and Methods). Recombinant offspring of chimeric mice was crossed with transgenic flIps (FLP)-expressing mice (46). Nkp46$^{Cre}$ mice were crossed to R26RERTH$^+$ (31) (CDTA, Orleans, France). All mice used in these studies were Nkp46$^{Cre}$R26RERTH$^+$, Nkp46$^{Cre}$iCre, or WT control litters. All mice were bred in pathogen-free breeding facilities at Centre d'Immunologie de Marseille-Luminy and used between 6 and 10 weeks of age. Experiments were conducted in accordance with institutional guidelines for animal care and use. Nkp46$^{Cre}$ mice were crossed to Rosα26S$^{iCRE}$ mice (32) to obtain Nkp46$^{Cre;}$ Rosα26S$^{iCRE}$ mice referred as to Nkp46$^{–/–}$/Rosα26S$^{iCRE}$ mice. Six- to eight-week-old offspring mice were used for experiments. Unnickled Diphtheria Toxin from Corynebacterium diptheriae (Calbiochem, 500 ng/mouse) diluted in PBS was injected i.p.

**Cell Preparation.** Cells were prepared as described (22). For details, see SI Materials and Methods.

**Flow Cytometry.** Flow cytometric analysis was done on a FACS Canto (Becton Dickinson, San Diego, CA). For details on reagents, see SI Materials and Methods.

**Tissue Immunofluorescence.** Portions of small and large intestine were isolated from mice and treated as previously described (22). For staining details, see SI Materials and Methods.

**ACKNOWLEDGMENTS.** We thank C. Cognet for help and advice and the Centre d'Immunologie de Marseille-Luminy (CIML) mouse house, cytometry, and knockout/knock-in facilities. E.V. and S.U. are supported by European Research Council Advanced Grants, grants from Agence Nationale de la Recherche (ANR) and Ligue Nationale Contre le Cancer (Equipe Labellisée “La Ligue”), and institutional grants from Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, and Université de la Méditerranée (to CIML). E.V., E.T., and N.Y. are supported by ANR Grant N°080636AS. E.V. is a scholar from Institut Universitaire de France. E.N.-M. is a recipient of Agence Contre le Cancer. J.C. was supported by Région Provence Alpes Côte d’Azur and Innate-Pharma.

---

**Supporting Information**

**Narni-Mancinelli et al. 10.1073/pnas.1112064108**

**SI Materials and Methods**

**Cell Preparation.** Mice were anesthetized and immediately perfused with PBS before organs were collected. Spleens, thymus, and LNs were removed, triturated in RPMI 5% FCS at 4°C and then passed through a 100-μm cell strainer (BD). Bone marrow cell suspensions were obtained by flushing femurs and tibias. Red blood cells were then lysed with ACK buffer (NH₄Cl, KHCO₃, EDTA.Na₂·2H₂O, pH7.4). Livers and lungs were cut in small pieces and incubated at 37°C for 20 min in HBSS medium (Invitrogen) containing 4,000 U/ml collagenase I (Invitrogen). Samples were then enriched for lymphocytes by Percoll gradient centrifugation (Amersham-Pharmacia). Blood lymphocytes were enriched by Lympholyte Mammals centrifugation (Tebu-Cerdane). Cells from small intestine were prepared as described (1).

**Flow Cytometry.** All mAbs were from PharMingen except the anti-Ly49D and H (eBioscience) and Ly49A and IL-18Rα (Biolegend). The anti-CXCR3, anti-CXCR6, the rat monoclonal anti-mouse FcγRIIIA/CD16, the goat anti-mouse NKp46 serum and control serum were purchased from R&D Systems. Anti-CD16 mAbs were revealed with a goat anti-rat IgG coupled to Alexa-647 and anti-NKp46 polyserum was revealed with a donkey anti-goat Alexa-647 (Molecular Probes). CD1d-restricted NKT cells were identified by alphagalactosylceramide-loaded CD1d tetramer staining kindly given by L. Brossay (Brown University, Providence, RI). IL-22 was a kind gift of J.C. Renauld (Ludwig Institute for Cancer Research, Brussels, Belgium), and anti-RORγt was kindly given by D. Littman (Howard Hughes Medical Institute, New York, NY).

**NK Cell Stimulation Assay.** Anti-NK1.1 (PK136), anti-Ly49D (4E5), and anti-NKp46 mAbs (29A1.4) were bound on plastic (96-well plates) overnight in carbonate buffer pH7.4. Spleen lymphocytes were stimulated 4 h in the presence of Golgi-stop and Golgi-plug (PharMingen). Alternatively, NK cells were activated by adding 10 ng/ml recombinant IL-12 combined with 20 ng/ml IL-18 (R&D Systems) or with a mix of PMA (200 ng/ml) and ionomycin (5 μg/ml) or with 40 ng/ml of IL-23 (R&D Systems).

**In Vitro NKp Differentiation.** BM cells were incubated for 20 minutes at 4°C with a cocktail of mAb: rat anti-mouse CD4, CD8, CD3, CD19, CD11b, IA-IE, and TER-119 and incubated with anti-rat IgG-coated magnetic beads (Biomag) for 45 minutes at 4°C. Cells attached to beads were removed with a magnetic separator. Immature CD122⁺NKp46⁻CD3⁻CD19⁻ NK cells were sorted using a FACSaria Cell Sorter and analyzed or put in culture in complete medium supplemented with 25 ng/ml of IL-15 for 4 d.

**Tissue Immunofluorescence.** Mouse NKp46 was stained with polyclonal goat anti-mouse NKp46 serum, followed by Alexa Fluor 488–conjugated donkey anti-goat (Molecular Probes). RORγt was stained with Armenian hamster anti-RORγt, followed by indocarbocyanine-conjugated goat anti-Armenian hamster serum (Jackson ImmunoResearch). Nuclei were counterstained with Sytox Blue (Molecular Probes). eYFP was revealed with Alexa 488 conjugated rabbit anti-GFP (Invitrogen). After being stained, slides were dried, mounted with Prolong Gold (Invitrogen), and examined with an LSM 510 Confocal microscope (Zeiss).

Fig. S1. Generation of NKp46<sup>cre</sup> knock-in mice. (A) Schematic representation of the strategy used to generate NKp46<sup>cre</sup> mice. A 6.6 Kb fragment of the BAC clone no. RP23-106A10 (Imagenes) containing the entire exon 7 of Nkp46 and centered on the stop codon was inserted in the plasmid PACYC177 and flanked with Sall sites using Red/ET recombination (Gene Bridges). In brief, the following primers containing 85-bp homology with nkp46 were used to amplify PACYC177 replication origin and ampicillin resistance gene: 5′-aatatgattagaatattgtatgcaattctcaattaataaaaatttaaaaaaatgaagctagtccacacaagttttttcaattgctgagcgcgtagggagtattttatgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacagtcga-cgcgctagggagtattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgggtcatttgcattgtacatgctgaagccgttattgg
Fig. S2. NK cells in NKp46Cre mice. (A) Spleen cells of wt control and NKp46Cre were analyzed by flow cytometry after staining with NK1.1, Nkp46, CD3, CD27, CD11b mAbs. Data show representative FACS dot plots in one out of three experiments. The frequency of cells in each gate is indicated. (B) wt and NKp46Cre spleen cell suspensions were stimulated for 4 h in wells coated with the indicated antibodies, in the presence of soluble anti-CD107a antibody. Cells were then stained for surface NK1.1, NKp46, and CD3 and intracellular IFN-γ. Results show the expression of IFN-γ and CD107a in gated NK cells and are representative of three independent experiments.

Fig. S3. NKp46 and eYFP expression by TCRγδ+ and TCRβ+ cells. NKp46 expression revealed by the anti-NKp46 polyclonal serum is shown on gated eYFP+TCRγδ+ and TCRβ+ cells prepared from the spleen of NKp46CreR26R+eYFP mice. Results are representative of three experiments.
**Fig. S4.** Depletion of NK cells in DT-treated NKp46<sup>Cre</sup>R26<sup>R</sup>DT<sup>R</sup> mice. Groups of mice were treated i.v. with DT, and cell recovery was analyzed 2 d later. Data show NK cell proportions among live leukocytes of nontreated (−) or DT-treated mice.

**Fig. S5.** Early expression of NKp46 on immature CD122<sup>+</sup>NK1.1<sup>+</sup> NK cells. Immature BM NK cells identified as CD122<sup>+</sup> NKp46<sup>−</sup>CD3<sup>−</sup>CD19<sup>−</sup> cells were sorted and analyzed or put in culture in complete medium supplemented with 25 ng/mL IL-15 for 4 d. Data show the sorting gate (Top), and the expression of NK1.1 and NKp46 after sort (Middle) and after 4 d of culture (Bottom).
Fig. S6. NKp46 is expressed on immature NK cells. NKp46 expression is shown on CD122^+NK1.1^− line− bone marrow NK cells according to their expression of CD27 and CD11b markers as in Fig. 3C. Results are representative of at least four independent experiments.

Fig. S7. Acquisition of NK cell effector function. (A–C) NKp46^iCre^ spleen cell suspensions were stimulated with IL-12/IL-18 or PMA/ionomycin. Results show the expression of IFN-γ (open histograms) or control isotype (gray histograms) on indicated cell population. Results are representative of two to three experiments.
Fig. S8. Acquisition of IL-18Rα and -12Rβ1 during NK cell maturation. IL-18Rα and -12Rβ1 expressions are shown in NK1.1⁺NKp46⁻CD3⁻ and NK1.1⁺NKp46⁺CD3⁺ splenic NK cells.

Fig. S9. Flow cytometric measurement of NK1.1 and eYFP in spleen, BM, LNs and lungs of NKp46<sup>iCreR26R</sup>eYFP mice. Data are representative of at least three experiments.

Fig. S10. NKp46 staining matches eYFP expression. NKp46 expression is shown on total splenic lymphocytes using the anti-NKp46 mAb and polyclonal serum. Results are representative of two independent experiments.
**Fig. S11.** NKp46<sup>+</sup>eYFP<sup>hi</sup> cells produce IL-22. **NKP46<sup>iCre</sup>R26R<sup>cre+eYFP</sup>** spleen cell suspensions were stimulated for 4 h with IL-12/IL-18 or IL-23. Cells were then stained for surface NK1.1, Nkp46, CD19, and CD3 and intracellular IL-22 or IFN-γ. Results show the expression of IL-22 and IFN-γ in indicated cell populations. Results are representative of two experiments.

**Fig. S12.** Characterization of liver NKp46<sup>+</sup>eYFP<sup>+</sup> and NKp46<sup>dim/low</sup>eYFP<sup>+</sup> cells. (A) Flow cytometric characterization of NK1.1<sup>+</sup>CD3<sup>-</sup>eYFP<sup>hi</sup> and NK1.1<sup>+</sup>CD3<sup>-</sup>eYFP<sup>+</sup> liver cells from NKp46<sup>iCre</sup>R26R<sup>cre+eYFP</sup> mice for indicated makers (open histograms) or control isotype (shaded histograms). (B) Ly49 receptors analysis is shown on NK1.1<sup>+</sup>eYFP<sup>+</sup>CD3<sup>-</sup> NK cells from spleen and liver and NK1.1<sup>+</sup>eYFP<sup>+</sup>CD3<sup>-</sup> NK cells from liver of NKp46<sup>iCre</sup>R26R<sup>cre+eYFP</sup> mice.

**Fig. S13.** NK cell maturation state and eYFP expression are not correlated. eYFP expression is shown on NK1.1<sup>+</sup>lin<sup>-</sup>CD11b<sup>-</sup> immature and CD11b<sup>+</sup> mature NK cells in bone marrow and liver of NKp46<sup>iCre</sup>R26R<sup>cre+eYFP</sup> mice. Results are representative of two to four experiments.