

Lethal influenza infection in the absence of the natural killer cell receptor gene *Ncr1*

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The elimination of viruses and tumors by natural killer cells is mediated by specific natural killer cell receptors. To study the *in vivo* function of a principal activating natural killer cell receptor, NCR1 (NKp46 in humans), we replaced the gene encoding this receptor (*Ncr1*) with a green fluorescent protein reporter cassette. There was enhanced spread of certain tumors in 129/Sv but not C57BL/6 *Ncr1^{gfp/gfp}* mice, and influenza virus infection was lethal in both 129/Sv and C57BL/6 *Ncr1^{gfp/gfp}* mice. We noted accumulation of natural killer cells at the site of influenza infection by tracking the green fluorescent protein. Our results demonstrate a critical function for *Ncr1* in the *in vivo* eradication of influenza virus.

The successful eradication of an invading pathogen is dependent on the coordinated action of the innate and adaptive immune systems. Natural killer (NK) cells are able to quickly kill a wide range of pathogens such as viruses, bacteria and intracellular parasites^{1–3}, as well as tumors⁴. The activity of NK cells is tightly controlled by both inhibitory and activating receptors⁵. Among these, NCR1 (also called Ly94 and Mar-1, or NKp46 in humans) is the only receptor reported so far to be expressed specifically on NK cells in all mammals tested, including humans⁶, monkeys⁷, mice⁸, rats⁹ and bovines¹⁰. However, the precise *in vivo* function of NCR1, or any other NK cell-activating receptor, in the killing of tumors and viruses has remained unclear.

NKp46 is a transmembrane type I glycoprotein containing two immunoglobulin domains and a positively charged arginine residue in the transmembrane domain, which associates with the T cell receptor- ζ signaling molecule^{6,11}. The discovery of activating NK cell receptors slightly modified the ‘missing-self’ hypothesis, which originally stated that NK cells kill unless inhibited by expression of self major histocompatibility complex (MHC) class I proteins¹². It is now known that a delicate balance between self MHC-mediated inhibition and stress-induced activation^{5,12} controls the cytotoxic activity of NK cells. Several *in vitro* studies have demonstrated that NKp46 is important in the recognition and destruction of various tumors^{13–17} and virus-infected cells^{18,19}. Low concentrations of NKp46 have been noted in several clinical situations, including acute myeloid leukemia²⁰, human immunodeficiency virus 1 infection²¹ and bare lymphocyte syndrome, type I (ref. 22). The presence of NK cells in tumors has been reported in renal cell carcinoma²³, and NK cells have been

identified *in situ* by staining with an NKp46-specific monoclonal antibody²³. A cellular ligand for NKp46 or NCR1 has not yet been identified, but evidence suggests that heparan-sulfate proteoglycans are involved in NKp46 ligand recognition²⁴. However, the *in vivo* function of NKp46 or NCR1 in the killing of tumor cells has not been investigated directly.

The importance of NK cells in virus defense *in vivo* has been demonstrated most precisely in an adolescent human who lacked NK cells and was therefore susceptible to virus infections²⁵. In mice, depletion experiments have demonstrated increased mortality in the absence of NK cells after infection with influenza²⁶. Finally, the critical involvement of NK cells has been established in various virus infections and in immune deficiencies^{27,28}. The recognition of influenza virus-infected cells is mediated through direct interaction of NKp46, NKp44 and the hemagglutinin proteins of various strains of influenza in a sialic acid-dependent way^{18,19,29}. The *in vivo* function of NKp46 in viral resistance has not yet been investigated.

Here we have generated mice in which a green fluorescent protein (GFP) cassette was ‘knocked into’ the *Ncr1* locus, thereby rendering *Ncr1* nonfunctional. There was substantial expression of the reporter gene only on NK cells. NCR1 is the only mouse receptor known to be expressed specifically on NK cells. Therefore, visualization of GFP allowed accurate identification of NK cells *in vivo* in unmanipulated mice. Influenza virus infection was lethal in *Ncr1^{gfp/gfp}* 129/Sv and B57BL/6 mice, demonstrating that *Ncr1* (NCR1 in humans) is essential for influenza eradication *in vivo*. We monitored the *in vivo* accumulation of the NK cells at the site of infection by tracking GFP expression. In addition, there was reduced clearance of the

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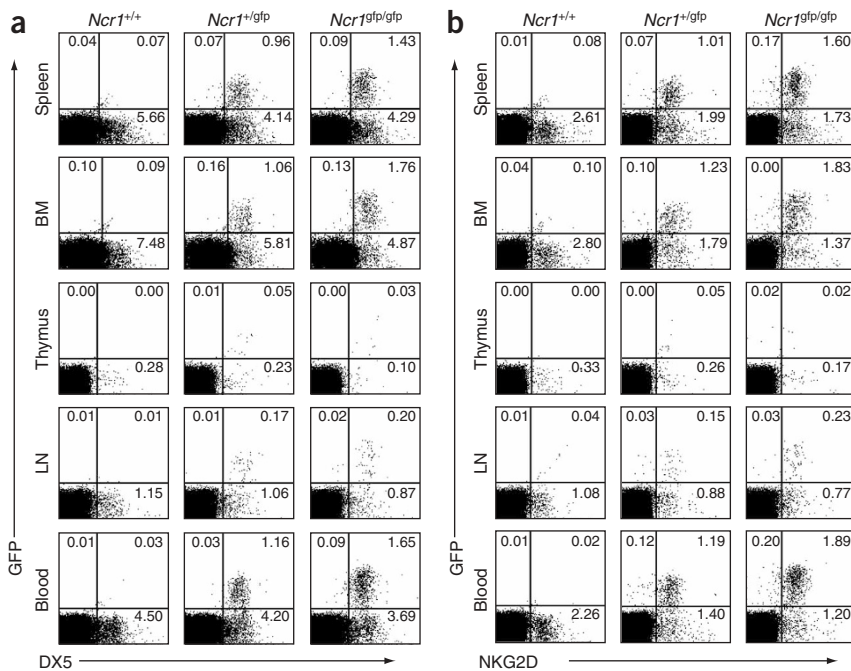


Figure 1 *Ncr1*^{gfp} expression in NK cells. (a,b) Flow cytometry of GFP expression in NK cells from various tissues (left margins). Numbers in quadrants indicate percentages of gated cells in each. BM, bone marrow; LN, lymph node. Representative of three independent experiments.

MHC class I-deficient RMAS tumor cells *in vivo* in *Ncr1*^{gfp/gfp} mice on a 129/Sv background but not on a C57BL/6 background, demonstrating that NCR1 contributes to tumor eradication.

RESULTS

Generation of *Ncr1*^{gfp/gfp} mice

We initially maintained the *Ncr1*^{gfp/gfp} mice on the 129/Sv strain because gene targeting was done in R1 embryonic stem cells (Supplementary Figure 1 online), which are of the 129/Sv background. In addition, the DAP12 adaptor protein, which transduces signals from Ly49-activating receptors, is impaired in 129/Sv mice³⁰. As NCR1 signals via the T cell receptor- ζ chain, its activity would not be impaired in 129/Sv mice. Therefore, the 129/Sv background would allow a distinct assessment of the *in vivo* function of NCR1. Offspring were born at a mendelian ratio of 105:208:95 (wild-type/heterozygous/homozygous) and developed normally. We genotyped mice by Southern blot and PCR (Supplementary Figure 1). As a monoclonal antibody recognizing NCR1 was not available, we used RT-PCR to verify the lack of *Ncr1* transcripts (Supplementary Figure 1). We later deleted the phosphoglycerate kinase-neomycin-resistance (PGK-*neo*^r) cassette by crossing *Ncr1*^{gfp/gfp} mice with transgenic mice expressing Cre recombinase in the germline and verified that the PGK-*neo*^r cassette was excised (Supplementary Figure 1). There-

fore, *Ncr1*^{gfp/gfp} mice lacked *Ncr1* transcripts and instead transcribed the gene encoding GFP under control of the endogenous *Ncr1* regulatory elements.

GFP expression only in NK cells

NK cells are distinguished from other cell types by their expression of CD56 (in human) or DX5 (in mice)³¹ and their lack of CD3 expression. Although those markers are also expressed by some T cell subsets, NCR1 expression is specific and restricted to NK cells⁸. As we had introduced a GFP reporter into *Ncr1*, we first tested whether green cells were detectable in *Ncr1*^{gfp/gfp} mice. A population of GFP⁺ cells was distinctly visible in *Ncr1*^{+/gfp} and *Ncr1*^{gfp/gfp} mice but not in *Ncr1*^{+/+} littermates (Fig. 1). To verify that all GFP⁺ cells were NK cells, we stained the cells for DX5 and showed that all GFP⁺ cells also expressed DX5 (Fig. 1a). In agreement with published studies³¹, not all DX5⁺ cells expressed GFP. Most of the DX5⁺GFP⁻ cells also expressed CD3, indicating that these were T cells (data not shown). Similar analysis of expression of the NK cell-activating receptor NKG2D showed that almost all GFP⁺ cells also expressed NKG2D, whereas not all NKG2D⁺ cells expressed GFP (Fig. 1b). Most of the NKG2D⁺GFP⁻ cells also expressed CD3 (data not shown). Analysis of cells from various organs demonstrated GFP expression in bone marrow, blood and spleen cells (Fig. 1). There was a small

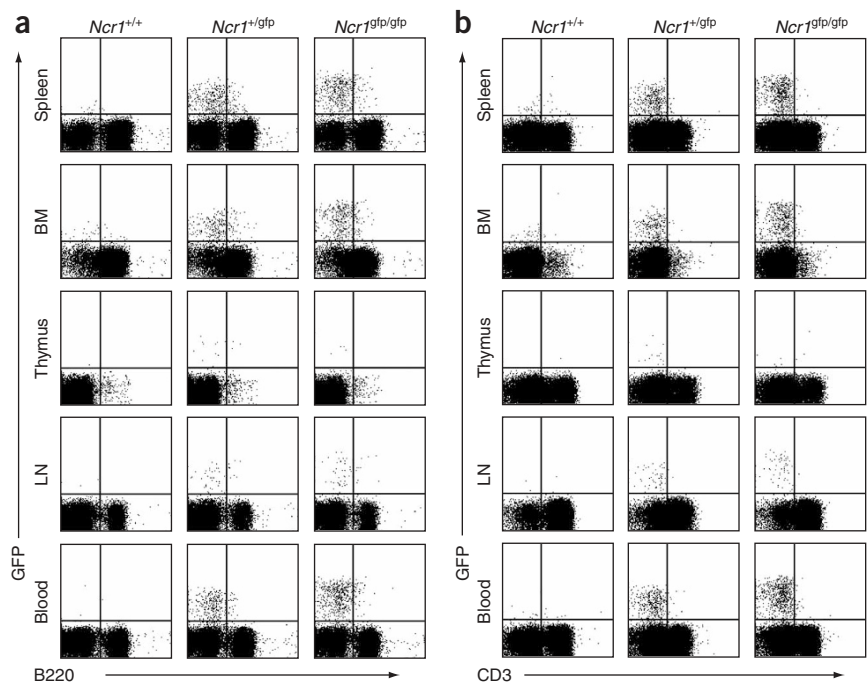


Figure 2 NK cell-specific *Ncr1*^{gfp} expression. Flow cytometry demonstrates a lack of GFP expression in B cells (a) or T cells (b) from various tissues (left margins). Representative of three independent experiments.

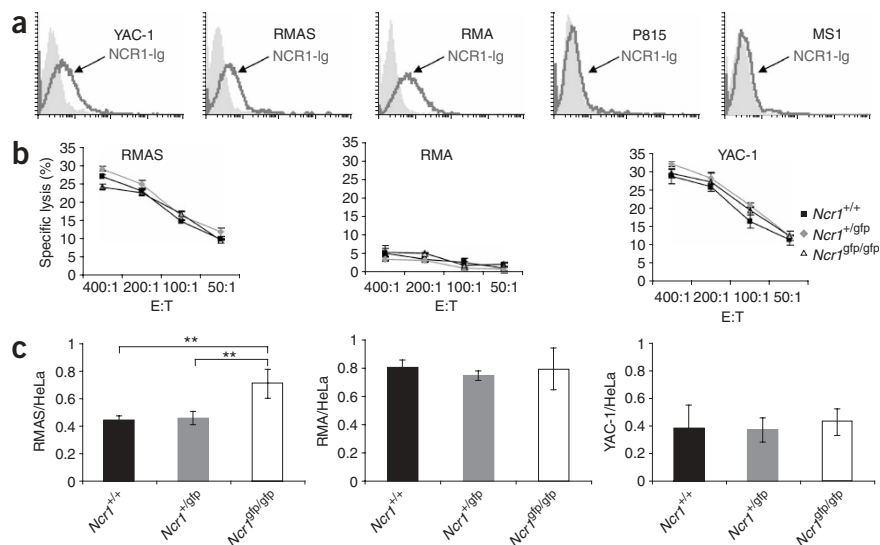


Figure 3 Reduced clearance of tumor cells *in vivo*. (a) NCR1-Ig recognizes NK cell targets, as demonstrated by its binding to various mouse cell lines (above histograms). Gray shading, background secondary antibody staining; dark lines, specific staining. Representative of three independent experiments. (b) Natural cytotoxicity of poly(I)·poly(C)-activated splenocytes from mice of various genotypes (key) against various target cell lines (above graphs). E:T, effector/target ratio. Data represent percent specific lysis \pm s.d. and are representative of three independent experiments. (c) Reduced *in vivo* killing of MHC class I-deficient RMAS cells in *Ncr1^{gfp/gfp}* mice. Labeled target cells and control HeLa cells in the lung were quantified by flow cytometry 5 h after injection into the tail vein. Representative of three independent experiments.

percentage of NK cells in the lymph nodes, whereas almost no green cells were detected in the thymus (Fig. 1).

We next analyzed whether GFP expression could be detected in B cells or T cells by staining with antibody to B220 (anti-B220) or anti-CD3, respectively. Weak B220 staining was apparent in a small fraction of the GFP⁺ population (Fig. 2a) and no CD3⁺ T cells expressed GFP (Fig. 2b). These results are in agreement with published reports demonstrating that some NK cells express B220 (ref. 32). They also indicate that the GFP reporter faithfully 'marked' only NK cells *in vivo*.

Enhanced tumor spread in *Ncr1^{gfp/gfp}* mice

The importance of human NKp46 in the *in vitro* lysis of many tumors is well established^{14,5,18,19,29}. To test whether NCR1 recognizes classical mouse NK cell-sensitive targets, we fused the extracellular portion of the NCR1 receptor with the Fc portion of human immunoglobulin G1

(IgG1) to generate the NCR1-Ig fusion protein as described before¹⁸. 'Classical' NK cell-sensitive targets such as the RMA, RMAS and YAC-1 cell lines were recognized by NCR1-Ig, whereas other cell lines, including P815 or MS1, were not (Fig. 3a). Next we investigated whether the killing of targets recognized by NCR1-Ig would be affected in *Ncr1^{gfp/gfp}* mice. As little or no killing of RMA, RMAS or YAC-1 cells was induced by freshly isolated splenocytes (data not shown), we activated NK cells *in vivo* by injecting poly(I)·poly(C) and, 18 h later, tested killing activity *in vitro*. There was no difference in the killing activity of NK cells from *Ncr1^{gfp/gfp}*, *Ncr1^{+/gfp}* and *Ncr1^{+/+}* mice (Fig. 3b). In general, there was poor killing when RMA cells were used (Fig. 3b), probably because RMA cells express H-2^b MHC class I molecules, which are recognized by inhibitory NK cell receptors expressed in 129/Sv mice. We obtained similar results when we used other tumor lines such as MS1 or P815, when we activated splenocytes *in vitro* with interleukin 2 and when we purified NK cells from splenocytes using magnetic beads (data not shown). Thus, it seems that *in vitro* killing of tumor cells is not affected by a lack of *Ncr1*, despite their recognition by NCR1-Ig.

However, although NK cells had to be activated to achieve meaningful results *in vitro*, NK cells *in vivo* are part of the innate immune response, ready to kill without prior stimulation. We therefore examined the NK cell killing function *in vivo* using a published lung tumor clearance assay³³, with minor modifications because of the presence of GFP in our mice. We used fluorescence-labeled (Vybrant DiD) human HeLa cells, which are not killed by mouse NK cells (data not shown), as a reference internal control. We labeled target cells with Vybrant Dil and injected these cells together with equal numbers of labeled HeLa cells into the tail veins of mice. We explanted lungs 5 h later and quantified labeled cells by flow cytometry. The *in vivo* killing of RMAS cells was impaired in *Ncr1^{gfp/gfp}* mice compared with that in *Ncr1^{+/gfp}* or *Ncr1^{+/+}* mice (Fig. 3c). The RMAS/HeLa ratio was approximately 0.4 in *Ncr1^{+/+}* and *Ncr1^{+/gfp}* mice but was 0.65 in *Ncr1^{gfp/gfp}* mice ($P = 0.005$). RMAS cells are derived from RMA cells

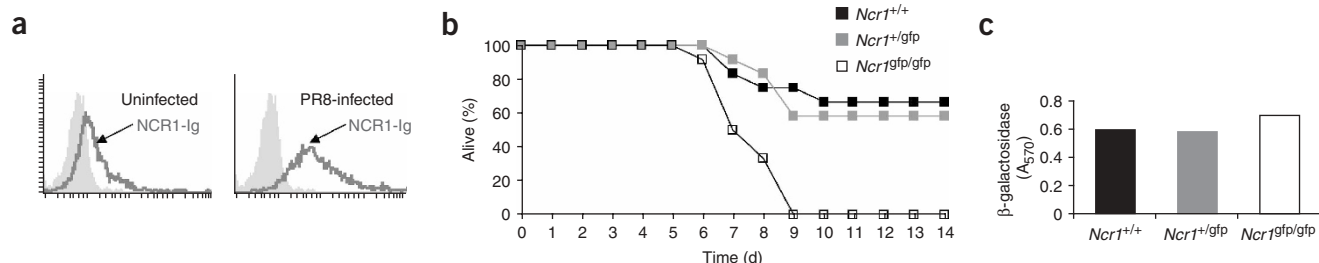


Figure 4 Lethal influenza infection in 129/Sv *Ncr1^{gfp/gfp}* mice. (a) Enhanced binding of NCR1-Ig to infected cells. RMAS cells were infected with influenza PR8 virus and were stained with NCR1-Ig. Gray shading, secondary antibody staining; dark lines, specific staining. Representative of five independent experiments. (b) Decreased survival of *Ncr1^{gfp/gfp}* mice after influenza infection. Viability was monitored daily after infection. Representative of three independent experiments. (c) Normal control of vaccinia infection in *Ncr1^{gfp/gfp}* mice. β -galactosidase activity was quantified as absorbance at 570 nm (A_{570}) in the ovaries of mice infected with vaccinia virus encoding β -galactosidase. Representative of two independent experiments.

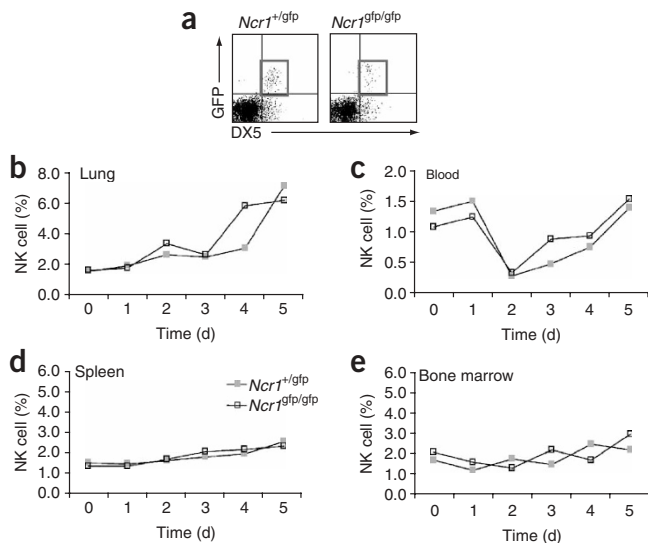


Figure 5 NK cells accumulate at the site of influenza infection. (a) Flow cytometry of NK cells in the lungs of influenza-infected mice; boxed areas indicate DX5⁺GFP⁺ cells. Representative of ten independent experiments. (b–e) Quantification of NK cells in the lung (b), blood (c), spleen (d) and bone marrow (e) of mice of various genotypes (key) at various times after intranasal influenza infection (horizontal axes). All mice were infected on the same day. Representative of three independent experiments.

and express little to no surface MHC class I molecules^{12,33}. These two cell lines were used to establish the ‘missing-self’ hypothesis and to demonstrate that NK cells efficiently kill tumors that have lost MHC class I expression¹². The *in vivo* RMA/HeLa ratio was approximately 0.8 in all strains (Fig. 3c), confirming that RMA cells are killed less efficiently than RMA5 cells by NK cells. Thus, in agreement with the ‘missing-self’ hypothesis, the killing of tumor cells by NK cells was influenced by dominant negative signals emanating from MHC class I–specific inhibitory receptors. However, in the absence of those inhibitory receptors, killing depended on the presence of activating receptors. The next issue was whether the killing of other cells would also be impaired *in vivo* in *Ncr1^{gfp/gfp}* mice. YAC-1 cells were killed with an efficiency similar to that of RMA5 cells in all strains (Fig. 3c). These experiments suggest that NCR1 is directly involved in the killing of tumors such as RMA5 *in vivo* and that there is no direct correlation between the *in vitro* and *in vivo* killing of tumor cells (probably because after activation, additional NK cell receptors are involved in killing, similar to the NKp44 situation in humans³⁴). When MHC class I molecules are present, killing is inhibited and NCR1 is no longer involved in residual cytotoxicity, and the killing of YAC-1 cells is probably mediated by other activating receptors such as NKG2D.

Lethal influenza infection in *Ncr1^{gfp/gfp}* mice

Studies have indicated that viral hemagglutinin proteins interact with human NKp44 and NKp46 but not with NKp30 and that this interaction is dependent on receptor sialylation^{18,29}. To determine whether those interactions are relevant *in vivo*, we first tested whether NCR1-Ig recognizes influenza-infected cells. Infection of RMA5 by influenza A/PR8 (H1N1) virus enhanced NCR1-Ig binding (Fig. 4a), suggesting that similar to human NKp46, mouse NCR1 specifically recognizes influenza-infected cells. To test whether those interactions are important *in vivo*, we inoculated mice intranasally with influenza A/PR8. This assay directly addresses the function of NCR1 in a very complex *in vivo* physiological situation. In this assay, mice are

subjected to life-threatening virus infection, and the NK cells in these mice express an array of activating and inhibitory receptors (except for NCR1 in *Ncr1^{gfp/gfp}* mice). Most *Ncr1^{+/+}* and *Ncr1^{+/gfp}* mice (66.6% and 58%, respectively) survived infection (Fig. 4b). In contrast, all of their *Ncr1^{gfp/gfp}* littermates died (Fig. 4b). All mice were infected, as demonstrated by body weight reduction and hemagglutination-inhibiting antibodies to influenza (data not shown).

To test whether the virus susceptibility of *Ncr1^{gfp/gfp}* mice is specific for influenza, we infected mice with a vaccinia virus expressing β-galactosidase. Vaccinia-infected cells showed no increase in the binding of NCR1-Ig (data not shown). We assessed β-galactosidase expression in the ovaries 3 d after infection. There was similar infection of *Ncr1^{gfp/gfp}*, *Ncr1^{+/gfp}* and *Ncr1^{+/+}* littermates (Fig. 4c), suggesting that NCR1 is required specifically for controlling influenza infection.

Because we detected GFP⁺ NK cells in the lung even before influenza infection (Fig. 5a), we monitored the *in vivo* accumulation of NK cells in influenza-infected mice. The number of NK cells in the lungs increased fourfold during illness (Fig. 5b). We stopped the experiment on day 5 because mice were already severely ill. NK cell accumulation in the lung was almost identical in influenza-susceptible *Ncr1^{gfp/gfp}* and influenza-resistant *Ncr1^{+/gfp}* littermates, indicating that NK cell trafficking *in vivo* is unaffected by the absence of NCR1 and that the death of *Ncr1^{gfp/gfp}* mice was not due to defects in trafficking of NK cells to the lung.

We tracked NK cells in various organs during influenza infection and noted a salient reduction in the percentage of NK cells in the blood 2 d after infection (Fig. 5c). That reduction correlated with the increased percentage of NK cells in the lung (Fig. 5b), suggesting that early in the course of influenza infection, NK cells are recruited to the lung directly from the blood. Later, the percentage of NK cells in the blood increased back to the preinfection percentage, whereas the percentage of NK cells in the lung continued to increase (Fig. 5b,c). There were modest increases in NK cell percentages in the bone marrow and spleen throughout infection (Fig. 5d,e). NK cells are generated in the bone marrow¹. It therefore seems possible that NK cell proliferation is increased 3 d after infection and NK cells emigrate from the bone marrow. These results suggest that at early time points after infection, NK cells rapidly migrate to the site of infection directly from the blood. Later, at the peak of infection, when NK cells are most needed to fight the invading virus, NK cells are found in large numbers in the infected organ. However, that accumulation does not leave other organs unprotected.

Strain specificity of the function of NCR1

PGK-*neo^r* cassettes can affect the function of other proximal genes. Therefore it was possible that the effects reported here could have been attributed to the presence of the PGK-*neo^r* cassette. In addition, as stated above, the Ly49-activating receptors that signal via DAP12 are not functional in 129/Sv mice³⁰. Therefore, the functional consequences of *Ncr1* deficiency noted here might differ in mice expressing a ‘normal’ repertoire of activating NK cell receptors. To address the former issue, we excised the PGK-*neo^r* gene by breeding *Ncr1^{gfp/gfp}* mice with 129/Sv (Prm-Cre) or C57BL/6 (Zp3-Cre) mice expressing Cre in the germline. To address the latter issue, we backcrossed *Ncr1^{gfp/gfp}* mice to the C57BL/6 background (which expresses functional DAP12) for ten generations (Fig. 6).

In agreement with our results reported above (Fig. 1), the only GFP⁺ cells found in the 129/Sv mice after PGK-*neo^r* excision or in the C57BL/6 mice were NK cells (Supplementary Figs. 2–5 online). The intensity of GFP expression was greater after excision of the PGK-*neo^r*

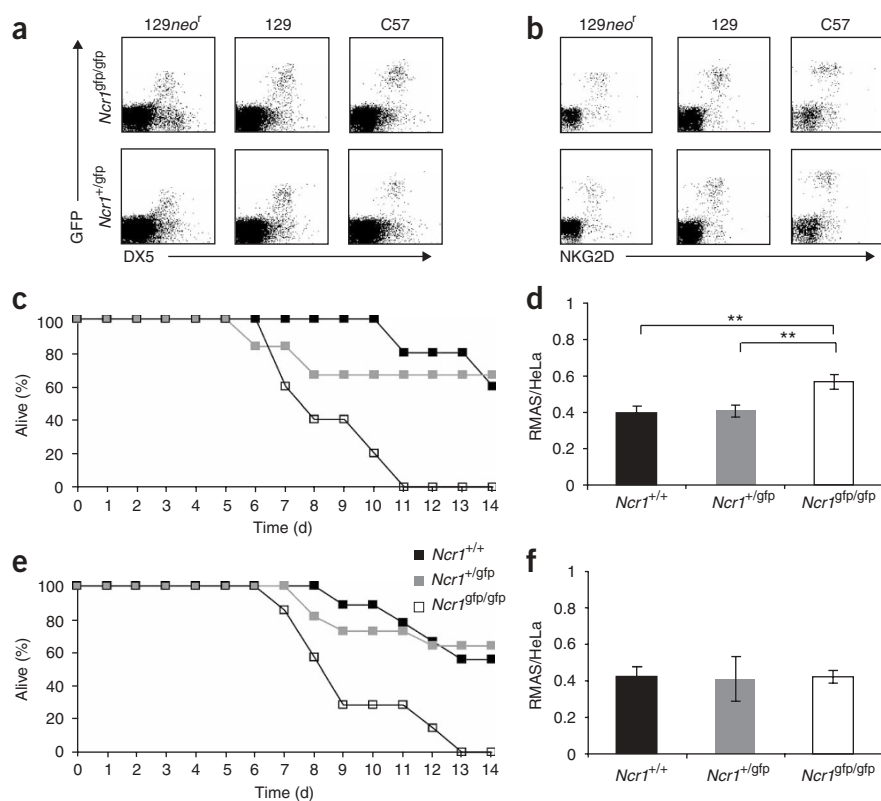


Figure 6 Strain-specific NCR1 functions. **(a,b)** GFP expression in NK cells of *Ncr1*^{gfp/gfp} and *Ncr1*^{+/gfp} mice before and after excision of the PGK-*neo*^f cassette: 129*neo*^f, 129/Sv before excision; 129, 129/Sv after excision; C57, C57BL/6 after excision and ten generations of backcrossing. Representative of three independent experiments. **(c)** *Ncr1*^{gfp/gfp} 129/Sv mice remain susceptible to influenza infection after excision of the PGK-*neo*^f cassette. Viability was monitored daily after infection. Representative of two independent experiments. **(d)** Reduced RMAS clearance is maintained in *Ncr1*^{gfp/gfp} 129/Sv mice after PGK-*neo*^f excision. Labeled RMAS target cells and control HeLa cells in the lung were quantified by flow cytometry 5 h after injection into the tail vein. Representative of two independent experiments. **(e)** Influenza is lethal in *Ncr1*^{gfp/gfp} C57BL/6 mice. Viability was monitored daily after infection. Representative of three independent experiments. **(f)** Normal RMAS clearance in *Ncr1*^{gfp/gfp} C57BL/6 mice, assessed as described in **d**. Representative of two independent experiments.

However, in the presence of MHC class I molecules (on RMA cells) or perhaps other activating ligands and/or receptors (on YAC-1 cells and in C57BL/6 NK cells), or when NK cells were activated, the specific function of NCR1 in tumor clearance was diminished, suggesting that NCR1 participates in the delicate balance of activating and inhibitor signals regulating NK cell cytotoxicity. Indeed, it has been shown that the killing of YAC-1 but not RMAS cells is mediated via the NKG2D receptor³⁷.

Studies have shown *in vitro* that various viral hemagglutinin proteins interact with the sialic acid residues on human NKp44 and NKp46 (ref. 19). We have demonstrated here that despite the expression of MHC class I proteins and other activating receptors on NK cells and despite the fact that NK cells accumulate *in vivo* at the site of infection, the activity of NCR1 against influenza was crucial and rescued mice of two different genetic backgrounds from influenza-induced death. Tumor spread is limited to the lifespan of each person, but influenza infection is a threat to an entire population. Therefore, NKp46 (NCR1 in mice) might have evolved first to fight influenza infection and then to fight tumor spread in specific situations (such as in the absence of MHC class I-mediated inhibition).

We first did experiments before excision of the PGK-*neo*^f cassette. However, there was no difference functionally in the *Ncr1*^{gfp/gfp} 129/Sv mice before or after PGK-*neo*^f excision; all died from influenza and RMAS clearance was impaired in both. However, there was more GFP expression after PGK-*neo*^f excision. Thus, as commonly appreciated, we suggest that careful attention should always be given to the presence of the PGK-*neo*^f gene.

Here we have directly investigated the function of a single gene *in vivo* and have devised a way to track cells expressing this gene. As the detection of NK cells has always been complicated by the expression of 'classical' NK cell markers on other cell types, *Ncr1*^{gfp/gfp} mice provide a unique ability to isolate and track NK cells *in vivo* without experimental manipulation or complicated staining protocols. The GFP reporter 'marks' only NK cells in both 129/Sv and C57BL/6 mice.

The differentiation of NK cells might have been impaired in *Ncr1*^{gfp/gfp} mice, as activating signals are essential for the maturation

cassette (Fig. 6a,b; median fluorescence intensity of 110 or 180 for *Ncr1*^{gfp/gfp} mice of the 129/Sv background before or after PGK-*neo*^f excision, respectively). There was even higher GFP intensity in the C57BL/6 mice after PGK-*neo*^f excision and ten generations of backcrossing, and all GFP⁺ cells expressed DX5 (Fig. 6a,b; median fluorescence intensity of 420 for *Ncr1*^{gfp/gfp} mice of this background).

Regardless of whether the PGK-*neo*^f cassette was deleted or whether *Ncr1* deletion was assessed on a 129/Sv or a C57BL/6 background, *Ncr1*^{gfp/gfp} mice died from influenza infection, whereas approximately 60% of *Ncr1*^{+/+} and *Ncr1*^{+/gfp} littermates survived the infection (Fig. 6c,e). Thus, even in the presence of a full spectrum of NK cell-activating receptors, NCR1 is critical for the survival of influenza-infected mice. Also regardless of whether the PGK-*neo*^f cassette was deleted, *in vivo* clearance of RMAS tumor cells was impaired in *Ncr1*^{gfp/gfp} 129/Sv mice compared with that in *Ncr1*^{+/+} and *Ncr1*^{+/gfp} littermates ($P < 0.01$; Fig. 6d). In contrast, clearance of RMAS cells was similar in *Ncr1*^{gfp/gfp}, *Ncr1*^{+/gfp} and *Ncr1*^{+/+} C57BL/6 littermates (Fig. 6f). Thus, the killing of tumor cells by NK cells *in vivo* is efficient in some strains such as the C57BL/6, even in the absence of NCR1, as other NK cell-activating receptors probably compensate for the lack of NCR1 in those strains.

DISCUSSION

The ability to directly and quickly destroy hazardous cells without the need for prior stimulation positions NK cells at the front line of innate immune defense. Two mechanisms regulate NK cell cytotoxicity. Inhibition is achieved mostly by inhibitory NK cell receptors that interact with MHC class I proteins^{12,35}, and activation is achieved by activating NK cell receptors that recognize stress-induced proteins³⁶, viral proteins¹⁸ or unknown tumor ligands²⁹. Here we have demonstrated that the NCR1 activating receptor was directly involved in the *in vivo* killing of RMAS tumor cells that lack MHC class I molecules.

of the closely related T cell lineage³⁸ and NKp46 is known to be expressed in humans from an early stage of cell maturation³⁹. The identification of normal NK cell populations in both 129/Sv and C57BL/6 *Ncr1*^{gfp/gfp} mice indicated that multiple activating pathways are redundant or that NCR1 signaling is not essential during NK cell development. The former option is supported by the fact that NK cells develop normally even in the absence of the tyrosine kinases Zap70 and Syk⁴⁰. The latter option is supported by the observation that *in vitro* maturation of human NK cells from progenitors has been achieved without any reported stimulation of activating receptors³⁹. Results obtained with the *Ncr1*^{gfp/gfp} mice indicate that despite its critical involvement in cytotoxicity, *Ncr1* is not essential for the generation of a normal NK cell population *in vivo*.

In summary, *Ncr1*^{gfp/gfp} mice demonstrate the critical involvement of NCR1 in the control of influenza and some tumors. These mice will enable direct investigation of the function of NCR1 in other virus infections and tumor models, as well as in bacterial and parasite infections and in autoimmune disorders. Furthermore, the NK cell-specific expression of GFP in these mice will allow study of NK cell trafficking *in vivo* and imaging of NK cells in various pathological conditions.

METHODS

Mice. The *Ncr1* targeting vector was generated by PCR amplification from ES-R1 embryonic stem cell line genomic DNA with the following primers: left arm forward, 5'-GGAGAGCTCTGGTCAAACCTCGAGCAAC-3', and left arm reverse, 5'-GAAGAATTCGGGTCGGTAGGTGGAAGG-3' (including restriction sites for *XhoI* and *EcoRI*, respectively); and right arm forward, 5'-AAGCGGCCGACAGATTAACAAATTGGG-3', and right arm reverse, 5'-CTGCCCGGTAGCTGGAACAGCGCAG-3' (including restriction sites for *EagI* and *SacII*, respectively). IRES-GFP and PGK-*neo*^r cassettes were subcloned in pBluescript-KS II using *EcoRI* plus *Sall* or *Sall* plus *XbaI*, respectively. The targeting construct was electroporated into R1 embryonic stem cells⁴¹ and targeted clones were selected with G418 and analyzed by Southern hybridization. The generation of founder chimeras was done by aggregation at the Transgenic & Knockout Facility of the Weizmann Institute (Rehovot, Israel). The PGK-*neo*^r cassette was deleted by breeding of targeted mice with 129/Sv (Prm-Cre) or C57BL/6 (Zp3-Cre) mice expressing Cre in the germline (stocks 003328 and 003651, respectively; The Jackson Laboratory). Targeted mice were backcrossed for ten generations onto the C57BL/6 background. All experiments were done in the specific pathogen-free unit of Hadassah Medical School (Ein-Kerem, Jerusalem) according to guidelines of the ethical committee.

Flow cytometry. Peripheral blood was obtained from the tail, single-cell suspensions of organs were obtained with a cell strainer and bone marrow was obtained from the tibia and femur. Antibodies specific for NK cells (DX5; Caltag), NKG2D (CX5; eBioscience) and B220 and CD3 (RA3-6B2 and 145-2C11, respectively; BD Bioscience) were conjugated to phycoerythrin. A monoclonal antibody specific for CD16 and CD32 (2G.4.2; Pharmingen) was used for blocking of Fc receptors before staining. The NCR1-Ig fusion protein was generated as described^{18,29}, and staining of cell lines was visualized with secondary phycoerythrin-conjugated goat anti-human (Jackson ImmunoResearch).

Cytotoxicity assays. For the *in vitro* assay, 200 µg poly(I)-poly(C) (Sigma) was injected and splenocytes were removed 18 h later. Target cells were labeled with [³⁵S]methionine and were incubated for 5 h at 37 °C with effector splenocytes at various effector/target ratios. Specific killing was calculated as described^{18,29}. For the *in vivo* assay, we used a published fluorescence labeling method³³ with a modification to avoid possible influence of the intrinsic GFP present in *Ncr1*^{gfp/gfp} mice. Cells were labeled with Vybrant cell labeling solutions (Molecular Probes). HeLa cells were labeled with Vybrant DiD (Molecular Probes), and RMA, RMA or YAC-1 cells were labeled with Vybrant Dil (Molecular Probes). Cells were mixed at a concentration of 20 × 10⁶ cells of

each population/ml in PBS, and 200 µl was injected into the tail vein (average of three mice in each experiment). Lungs were collected 5 h later, single-cell suspensions were obtained with cell strainers and fluorescence was analyzed by flow cytometry. The ratio of the tested targets to the internal control HeLa cells was calculated.

Viral infection. Influenza A PR8 virus was grown in hen eggs. Mice (6–8 weeks of age) were anesthetized and were inoculated intranasally with 50 µl of diluted virus (3 × 10³ plaque-forming units; a dilution of 1/31,622 from 4096 HAU). Each experiment included at least five mice of each genotype. Viability was determined daily. Antibodies to influenza were assessed in the serum of mice that survived infection. For analysis of NK cell trafficking during the disease, mice were infected with influenza as described above and were killed at various times after infection, and lungs were analyzed as described above. Vaccinia infections were as described⁴²; mice were infected with 2 × 10⁷ plaque-forming units and, 3 d later, β-galactosidase activity was assayed with chlorophenol red-β-D-galactopyranoside substrate, measured at a wavelength of 570 nm.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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