Expression, Function, and Molecular Properties of the Killer Receptor Ncr1-Noé

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*J Immunol* published online 14 September 2015
http://www.jimmunol.org/content/early/2015/09/13/jimmunol.1501234

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**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/09/13/jimmunol.1501234.DCSupplemental

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Expression, Function, and Molecular Properties of the Killer Receptor Ncr1-Noé

Ariella Glasner, Hrvoje Simic, Karmela Miklić, Ziv Roth, Orit Berhani, Isam Khalaila, Stipan Jonjic, and Ofer Mandelboim*

NK cells kill various cells using activating receptors, such as the natural cytotoxicity receptors (NCRs). NKp46 is a major NCR and is the only NCR expressed in mice (denoted Ncr1). Using Ncr1-deficient mice (Ncr1<sup>−/−</sup>) we demonstrated that Ncr1 controls various pathologies, and that in its absence Ncr1-related functions are impaired. In 2012, another Ncr1-related mouse was generated, named Noé, in which a random mutation, W32R, in position 32, impaired the Ncr1-Noé cell surface expression. Interestingly, in the Noé mice, Ncr1-dependent deficiencies were not observed. Additionally, the Noé-NK cells were hyperactivated, probably due to increased Helios expression, and the Noé mice demonstrate increased clearance of influenza and murine CMV. In contrast, in the Ncr1<sup>−/−</sup> mice infection with influenza was lethal and we show in the present study no difference in murine CMV infection between Ncr1<sup>−/−</sup> and wild-type (WT) mice. Because the foremost difference between the Noé and Ncr1<sup>−/−</sup> mice is the presence of a mutated Ncr1-Noé protein, we studied its properties. We show that Ncr1-Noé and various other Ncr1 mutants in position 32 can be expressed on the surface, albeit slowly and unstably, and that ligand recognition and function of the various Ncr1-Noé is similar to the WT Ncr1. We further show that the glycosylation pattern of Ncr1-Noé is aberrant, that the Ncr1-Noé proteins accumulate in the endoplasmic reticulum, and that the expression of Ncr1-Noé proteins, but not WT Ncr1, leads to increased Helios expression. Thus, we suggest that the NK hyperactivated phenotype observed in the Noé mice might result from the presence of the Ncr1-Noé protein.

Natural killer cells are lymphocytes of the innate immune system, which specialize in the eradication of tumor cells and cells infected with viruses or bacteria (1–12). A balance of signals derived from activating and inhibitory receptors controls the activity of NK cells (13–18). Among the activating NK cell receptors is NKp46, a member of the natural cytotoxicity receptors (NCRs) family, which also includes NKp30 and NKp44. Interestingly, out of the three NCRs, only NKp46 has a mouse ortholog, denoted Ncr1 (19). NKp46/Ncr1 directly recognizes the hemagglutinin (HA) proteins of influenza viruses (4, 5, 20), poxviruses (8), and Newcastle disease viruses (9). Furthermore, although the identities of the cellular ligands of NKp46/Ncr1 are still unknown, it is well established that NKp46/Ncr1 is involved in a myriad of immunological activities (5, 7, 10, 21–24).

The role played by Ncr1 in various pathologies was demonstrated most compellingly by using Ncr1 knockout mice. Our group previously generated a mouse named Ncr1<sup>−/−</sup>, where exons 5–7, which contain the transmembrane and cytoplasmic domains of Ncr1, were replaced by GFP (5). The heterozygous Ncr1<sup>+/−</sup> mice are immune competent, expressing functional Ncr1 from one allele and GFP from the other, and thus all their NK cells are GFP<sup>+</sup> (5). The Ncr1<sup>−/−</sup> heterozygous mice are excellent for detecting NK cells (as well as some ILC subsets) and for studying their trafficking in vivo under various conditions (5, 7, 10, 21–24).

Using the Ncr1<sup>−/−</sup> mice, it was revealed that Ncr1 is involved in controlling influenza virus infections (5, 22, 25), tumor and metastases eradication (6, 7, 10, 24), the development of type I and type II diabetes (11, 23, 26, 27), liver fibrosis (28), and bacterial infections (3, 29). In all of these studies, the activity of the Ncr1<sup>−/−</sup> NK cells was impaired in an Ncr1-dependent manner.

In 2012, a new Ncr1-related mouse named Noé was generated (30). This mouse was created using random N-ethyl-N-nitrosourea mutagenesis. The Noé NK cells displayed a hyperactivated phenotype and were more effective against various challenges both in vitro and in vivo. The hyperactivated phenotype was reported to correlate with higher levels of the Helios transcription factor in the Noé NK cells as compared with WT NK cells. Surprisingly, although the hyperactivating phenotype of the Noé mice was general and Ncr1-independent, the Noé NK cells’ hyperactivation was attributed to one mutation in the Ncr1. This mutation resulted in the replacement of a tryptophan residue located in position 32 of the Ncr1 protein with arginine (30). Because the Ncr1-Noé protein was not detected on the cell surface, the Noé mice were considered to be Ncr1-deficient.

Recently, another mouse was generated by crossing Nkg2d knockout mice with the Ncr1<sup>−/−</sup> mice (31). In these mice, similarly to the Ncr1<sup>−/−</sup> mice, NK cell hyperactivation was not
observed and Helios was not overexpressed, either in the single or in the double knockout mice (31). The major difference between the Noé and Ncr1<sup>W32R<sup> mouse is that the Ncr1 protein was mutated in the Noé mice. Consequently, we sought to investigate the properties of the Ncr1-Noé protein to better understand the striking discrepancies observed between the Ncr1-deficient mice.

Materials and Methods

Mice and murine CMV infection

The Ncr1<sup>W32R</sup> mouse was previously described (5). Ncr1<sup>W32R</sup> was originally generated in 129/Sv mice and crossed to the C57BL/6 and BALB/c strains for >10 generations. For murine CMV (MCMV) infection, the mice were i.p. infected with the indicated doses (see Fig. 1) of MCMV and sacrificed 3 days later. Tissue samples were further processed for virus titration.

Cells

The cells used in this study were the human HEK293T cells, mouse BW thymoma cells, human YTS NK cells, mouse mammary cell AT1, mouse fibroblast B12, murine carcino-gen-induced lymphoma EL4, mouse lymphoblast-like mastocytoma P815, human melanoma 1106 Mel, human breast cancer SKBR3, human chronic myelogenous leukemia K562, and human Burkitt’s lymphoma Raji.

Ncr1 plasmids, reporter cells, Ig fusion proteins and mAbs

The Ig fusion proteins used in this study included the various Ncr1 Ig proteins, NKp44 Ig, NKp46 Ig, and KIR2DL1 Ig. Fusion proteins were generated in HEK293T cells, as previously described (20). Single point mutations in Ncr1 (W32R, W32A, and W32E) were generated as follows: single point mutations in positions Trp<sup>32</sup> were prepared using the general 5’ primer, including NolI, AAT GCGGCCGC GCC ACC ATG CTG CCA ACA CTC ACT GCC, and 3’ primer, including XhoI, AAT CTCGAG TCAC-AAGGGCCCAAGAAGTGG. The mutation primers were as follows: W32R, 5’-CTCATACAGGGCGCAACCACCC-3’, GGGTTTGGCGCTGAGTG; W32A, 5’-AA CCC ATC ATC-3’, GTTTGGCCCGTCGAAGTCGTT; W32E, 5’-AAA CCC ATC ATC GAG GCC AAA C-3’, GGT TTGGCCCGTCGAAGTCGTT. The triple N-linked mutated Ncr1 (3Nmut) was generated as previously described (5, 22).

Staining with all fusion proteins was performed with 5 µg of each (saturation concentration). FACS analyses of cell lines stained with the fusion proteins were visualized using a secondary allophycocyanin-conjugated goat anti-human mAb. For the generation of the BW reporter cells, the extracellular parts of the Ncr1 and various Ncr1 mutants described above were fused to the mouse CD3-ζ chain using the primers: Ncr1-ζ, 5’-AAT GCGGCCGC GCC ACC ATG CTG CCA ACA CTC ACT, Ncr1 EC ζ fusion, 5’-AAT CTCGAG TCAC AAGGGCCCAAGAAGTGG; Ncr1 EC ζ construct was prepared as a lenti-viral vector and transfected into the human HEK293T cells. The various Ncr1 ζ constructs were expressed in a lentiviral vector and transduced in BW cells. The BW assays were performed as previously described (20). Brefly, BW cells expressing the various Ncr1 receptors fused to the mouse ζ-chain were incubated at a ratio of 1:1 with target cells at 37°C and 5% CO₂. Following 48 h of incubation, the supernatant was collected, and IL-2 levels were measured using standard ELISA. Blocking of the interaction of the various Ncr1 proteins with viral HA was performed using the anti-HA1 mAb H2S2E23 (gift from Jonathan Yewdell, National Institutes of Health). The 12E7 mAb was used as control.

For the generation of YTS cells expressing the chimeric proteins consisting of the extracellular part of Ncr1 fused to the transmembrane and cytoplastic domains of 2B4, the general 5’ primer was used as mentioned above, and the 2B4 3’ primer, including XhoI, AATCTCGAGTCAGGAA TAAACATCAAAAGTTCT. The fusion primers were as follows: 5’-TG-GGATCACACAACACCAAGAATT TTTTGGTGATCATCGTGATTCTA, 3’-GGATCACACAACCCAGAATT TTTTGGTGATCATCGTGATTCTA, 3’-TAGAATTACAGGTATGCCAACAAAAATTTCTGCTGTTTGTGATCCCA, the various anti-mNcr1 mAbs used, including mNcr1.6, 7, 8, 9, 12, 13, and 15 were generated by us. The commercially available 29A.1 (Biotest, Karf Saba, Israel) mAb was used where indicated.

Immunoprecipitations, Western blotting, and enzymatic digestion

Immunoprecipitation of Ncr1 was performed following standard immunoprecipitation protocol, using agarose A/G beads and mNcr1.6 that we generated. For Western blotting (WB), the precipitated proteins were run on a 10% SDS-PAGE gel, transferred, and stained with various anti-mNcr1 mAbs. For the lectin WB assays, the various fusion proteins (5 µg) were run on 10% SDS-PAGE gel, transferred to a nitrocellulose membrane (Tamar Laboratory Supplies, Zion, Israel), and blotted with a biotin-conjugated α2,3-linked Maackia amurensis lectin II (MAL; Vector Laboratories, Burlingame, CA), a biotin-conjugated α2,6-linked Sambucus nigra agglutinin (SNA) lectin (Vector Laboratories), or with biotin-conjugated HA Ig. The staining was visualized using a streptavidin-HRP secondary reagent and EZ ECL substrate (Biological Industries, Kibbutz Beit-Haemek, Israel). Enzymatic digestion with endoglycosidase H (Endo H) and PNGase F (Ornat, Rehovot, Israel) were performed according to standard protocol.

CD107 degragation assays and A/Puerto Rico/8/34 H1N1 (PR8) influenza virus

For CD107a degragation assays, several YTS cells were incubated with various targets in a ratio of 1:1 in the presence of 0.1 µg allopocyccinan-conjugated CD107a mAb for 2 h at 37°C (Biotest). CD107a levels on the YTS cells were determined by flow cytometry. When influenza virus was used, 1 × 10<sup>6</sup> cells in 2 ml complete medium were treated with 20 µl, 1000 hemagglutination units of the influenza virus overnight at 37°C. 5% CO₂. Propagation of the human influenza virus A/Puerto Rico/8/34 H1N1 (PR8) was performed as previously described (32).

Preparation of the fusion proteins for sugar content analysis and HPLC

The Ncr1 Ig and Ncr1 W32R Ig N-linked glycans were released in solution with PNGase F as described by Roth et al. (33).

Normal phase HPLC was performed with the low-salt buffer system using a 4.6- by 250-µm GlycoSep N column (OGS) (Waters, Milford, MA). The solvents were used buffer B (50 mM ammonium formate [pH 4.4]) and buffer C (acetonitrile). The glycans were eluted by linear gradient with buffer B, with initial conditions of 20% buffer B at a flow rate of 0.4 ml/min. The concentration of buffer B was changed from 35 to 53% during 132 min and then from 53 to 100% during the next 3 min, with a constant flow rate. The column was washed with 100% buffer B for 5 min at a flow rate of 1 ml/min before re-equilibration in the initial solvent system. Fluorescence was measured at 420 nm with an excitation of 330 nm (with 16-nm bandwidths).

Immunofluorescence

HEK293T cells transfected with the various Ncr1 constructs were grown on glass slides and fixed in 4% paraformaldehyde. Cells were fixed and permeabilized in cold (–20°C) methanol. Cells were blocked in CAS-block Life Technologies and incubated with anti–protein disulfide isomerase (PDI; a gift from Dr. Hidde Ploegh of the Whitehead Institute of Biomedical Research, Cambridge, MA), anti-CD107a (Merck Millipore, catalog no. AB2971), and anti-Ncr1 (mAb 29A.1; Biotest, catalog no. AP2225). A confocal laser scanning microscope (Zeiss Axiovert 200 M; Carl Zeiss MicroImaging, Thornwood, NY) was used to analyze the stained slides.

Statistical analysis

ANOVA was used to identify significant group differences. To assess colocalization, the FV10-ASW version 3.00.01.15 statistical packet was used, followed by a Student t test, and subsequently comparing the average Pearson coefficient values in at least 30 cells in each group. A p value <0.05 was considered significant in all studies.

Results

Control of MCMV infection is not dependent on Ncr1

It was previously reported that NK cells in the Noé mice are hyperactivated and more resistant to in vivo challenges, such as MCMV infection (30). To investigate whether the enhanced control of MCMV would occur in the Ncr1<sup>W32R<sup> mouse and to study whether the MCMV control is strain specific, we infected three genetically distinct Ncr1<sup>W32R<sup> strains with MCMV: 129sv (Fig. 1A), BALB/c (Fig. 1B), and C57BL/6 (Fig. 1C). We selected these three strains because past work using depleting Abs against NK cells demonstrated that NK cell function was critical for controlling MCMV infection in all three strains (34–37).

To avoid NK cell activation via the receptor–ligand interaction between Ly49H and m157, C57BL/6 mice were infected with
the results observed with the Noe´ mice, where Noe´ mice were the heterozygous or WT mice. These results are in stark contrast to infection in different organs (lungs, spleen, and liver), 3 d postinfection (p.i), and virus titers in different organs were determined using standard plaque assays.

FIGURE 1. The absence of Ncr1 does not affect MCMV infection. Adult 129/SV1 (A), BALB/c (B), and C57BL/6 WT (C) mice, heterozygous (Ncr1+/gfp) mice, or mice lacking the Ncr1 receptor (Ncr1−/−/gfp) were i.p. injected with tissue culture–grown MCMV. Mice were sacrificed at 3 (BALB/c and 129/SV1) or 5 d (C57BL/6) postinfection (p.i), and virus titers in different organs were determined using standard plaque assays. The figure is representative of at least three experiments.

A

129/Sv

WT MCMV 2x10^6 PFU i.p., 3 days p.i.

<table>
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<tr>
<th>Organ</th>
<th>Viral titer</th>
<th>Log_{10} PFU/organ</th>
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<tbody>
<tr>
<td>Lungs</td>
<td>6</td>
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<tr>
<td>Spleen</td>
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<tr>
<td>Liver</td>
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B

Balb/c

WT MCMV 2x10^6 PFU i.p., 3 days p.i.

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<tr>
<th>Organ</th>
<th>Viral titer</th>
<th>Log_{10} PFU/organ</th>
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<tr>
<td>Lungs</td>
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<td>Spleen</td>
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<td>Liver</td>
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C

C57BL6

Dm157 MCMV 3x10^6 PFU i.p., 5 days p.i.

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<th>Organ</th>
<th>Viral titer</th>
<th>Log_{10} PFU/organ</th>
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<tr>
<td>Lungs</td>
<td>8</td>
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<tr>
<td>Spleen</td>
<td>7</td>
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<tr>
<td>Liver</td>
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The Ncr1−/−/gfp mice do not express Ncr1

In the Ncr1−/−/gfp mice, exons 5–7 of the Ncr1 gene were replaced by GFP, and thus there is no expression of the full-length Ncr1 protein. Contrary to the Ncr1+/+gfp mice, the Noe´ mice express the full-length Ncr1 protein, yet it carries a mutation in position W32. The Ncr1−/−/gfp mice exhibited impaired Ncr1-related activities (5, 7, 10, 21–24) and therefore it is clear that Ncr1 does not function in these mice. Nevertheless, it is plausible that a truncated version of Ncr1 may exist in the Ncr1−/−/gfp mice. To address this possibility, we generated six new anti-Ncr1 mAbs. The anti-Ncr1 mAbs were produced by immunizing Ncr1−/−/gfp mice with fusion proteins composed of the extracellular part of Ncr1 fused to human IgG1. Interestingly, we were unable to generate mAbs against Ncr1 using Ncr1-proficient mice, probably because these mice express Ncr1. Specific anti-Ncr1 mAbs were generated, as all six new mAbs (mNcr1 6–8, 12, 13, and 15), as well as the commercial mAb 29A.1, positively stained BW cells expressing Ncr1, whereas no staining was observed with the parental BW cells (Supplemental Fig. 1A). Importantly, the new anti-Ncr1 mAbs recognized the Ncr1 protein expressed on mouse NK cells derived from the Ncr1−/−/gfp mice, but not NK cells derived from the Ncr1+/−/gfp mice (Supplemental Fig. 1B).

To test whether fragments of Ncr1 are present in the Ncr1+/−/gfp mice, we initially tested which of the anti-Ncr1 mAbs efficiently precipitates Ncr1 and selected anti-Ncr1 mAb mNcr1.6. We subsequently prepared lysates of NK cells obtained from Ncr1+/−/gfp and Ncr1−/−/gfp mice and precipitated Ncr1. WB was performed with anti-Ncr1 mAbs mNcr1.6, 9, and 13 (Supplemental Fig. 1C). The Ncr1 protein could only be detected in the Ncr1−/−/gfp but not in the Ncr1+/−/gfp mice (molecular mass, ∼37 kDa; Supplemental Fig. 1C). Interestingly, it seemed that a truncated version of Ncr1 could also be recognized by some of the anti-Ncr1 mAbs (mNcr1.6 and mNcr1.13, Supplemental Fig. 1C). However, neither the full-length Ncr1 nor the truncated versions were detected in the Ncr1+/−/gfp mice (Supplemental Fig. 1C). Accordingly, we concluded that it is unlikely that truncated versions of Ncr1 exist in the Ncr1+/−/gfp knockout mice. Furthermore, even if they do exist, they do not contribute to functions associated with Ncr1, because the Ncr1-dependent activities are impaired in the Ncr1+/−/gfp (5, 7, 10, 21–24).

Ncr1-Noe´ can be expressed on the cell surface

We next turned to investigate the properties of the Ncr1-Noe´ protein. Our first assumption was that the mutation in Ncr1-Noe´ might have disrupted the binding site recognized by the anti-Ncr1 Abs used to detect its expression on the cell surface. Additionally, we were interested in determining whether the Noe´ phenotype is sequence specific. To that end, we cloned the WT Ncr1 and mutated it in position 32, converting the tryptophan residue into alanine (W32A), glutamic acid (W32E), or arginine (W32R, this is Ncr1-Noe´) (30). All constructs were cloned into lentivirus vectors that express GFP and were stably expressed in HEK293T cells. Infection of the HEK293T cells by all lentivirus vectors that express GFP were efficiently recognized by the anti-Ncr1 mAbs mNcr1 6–8, 12, 13, and 15, as well as the commercial mAb 29A.1. We next tested whether the Ncr1-Noe´ can be expressed on the cell surface. Interestingly, we were able to detect specific surface staining of Ncr1, Ncr1 W32A, Ncr1 W32E, and Ncr1-Noe´ (W32R) (Fig. 2B, Supplemental Fig. 2A). However, expression of the Ncr1 mutants, including Ncr1-Noe´, was delayed by several days when compared with the WT Ncr1. Importantly, note that although the expression of the Ncr1 mutants was delayed, the GFP expressions of all mutants disappeared from the cell surface close to 100%, as indicated by GFP expression (Fig. 2A).

Next, we used the six new mNcr1 mAbs that we generated to test whether Ncr1-Noe´ can be expressed on the cell surface. Interestingly, we were able to detect specific surface staining of Ncr1, Ncr1 W32A, Ncr1 W32E, and Ncr1-Noe´ (W32R) (Fig. 2B, Supplemental Fig. 2A). However, expression of the Ncr1 mutants, including Ncr1-Noe´, was delayed by several days when compared with the WT Ncr1. Importantly, note that although the expression of the Ncr1 mutants was delayed, the GFP expressions of all constructs were detected simultaneously. Additionally, the expression of all mutants disappeared from the cell surface close to 100% following their expression. Lastly, the expression of all Ncr1 mutants was lower that than that of the WT Ncr1 (Fig. 2B, Supplemental Fig. 2A).

Ncr1-Noe´ is activated upon tumor and influenza virus recognition using the BW reporter system

To test whether Ncr1-Noe´ mutants were functional, we initially attempted to express the WT and various Ncr1-Noe´ constructs in primary mouse NK cells using several methods, including lentiviral infection. Our numerous attempts proved to be unsuccessful. Therefore, to determine the functionality of the various Ncr1-Noe´ proteins, we applied two types of reporter assays. First, we employed a cell-based reporter system we previously used, denoted BW reporter assay (20). We cloned the extracellular part...
of the WT and mutated Ncr1 variants (W32A, W32E, and W32R) and fused them to the mouse \(\zeta\)-chain. In the BW reporter system, triggering of Ncr1 by an appropriate ligand, leads to IL-2 secretion (20), thus reporting on the functional interaction between Ncr1 and its ligand. The chimeric constructs were then stably expressed in BW cells. As seen in Fig. 3A, the GFP levels of the various transfectants were similar. The expression of all the receptors on the BW cells was verified by staining with the specific anti-Ncr1 mAbs that we generated, as well as with the commercial anti-Ncr1 Ab 29A.1 (Fig. 3B, Supplemental Fig. 2B). Notably, and in agreement with the above results (Fig. 2, Supplemental Fig. 2A), the expression level of the mutated Ncr1 proteins on the surface of the BW cells was lower than the expression of the WT Ncr1 (Fig. 3B, Supplemental Fig. 2B). In further agreement with the expression pattern of the Ncr1-Noe´ mutants, it took a longer period for the Ncr1-Noe´ variants to be expressed on the surface of the BW cells, and their expression on the cell surface was unstable.

To confirm the various receptors ability to respond to stimuli, we incubated the BW cells with plate-bound anti-Ncr1 mAb, mNcr1.15, and determined the maximum amount of IL-2 secretion (Fig. 3C).

We have previously demonstrated that Ncr1 recognizes the HA of the influenza virus; therefore, our target cells in the BW reporter assays were EL4 cells incubated in the presence or absence of the influenza virus.

As can be seen in Fig. 3C, significant elevation of IL-2 secretion was observed when all BW reporters (Ncr1, W32A, W32E, and W32R) were incubated with EL4 cells, suggesting that EL4 cells express an unknown ligand for Ncr1 (Fig. 3C). To validate that this elevated secretion is mediated by the various Ncr1 proteins, we blocked the interaction with the anti-Ncr1 mAb, mNcr1.7. IL-2 secretion was reduced to basal levels, thus confirming that the various Ncr1 proteins interact with an unknown ligand on EL4 cells (Fig. 3C, designated EL4 mNcr1.7).

In the presence of influenza, further enhancement of IL-2 secretion was observed with all BW Ncr1 reporters (Fig. 3C, designated EL4 PR8). IL-2 secretion was significantly reduced when blocked with mNcr1.7 (Fig. 3C, designated EL4 PR8 mNcr1.7), indicating that Ncr1 is directly involved in the elevation of IL-2 secretion. When the tested targets (either in the presence or absence of mAbs) were incubated with the BW cells transfected with a control empty vector, there was little or no IL-2 secretion (Fig. 3C).

To demonstrate that the various Ncr1 proteins recognize the viral HA protein, we incubated the BW transfectants with EL4 and EL4 PR8 cells with or without an anti-HA1 blocking mAb. The anti-HA1 mAb had no effect on IL-2 secretion following incubation of the various BW cells with EL4 cells (Fig. 3D). In contrast, the increased IL-2 secretion observed following incubation of the various BW cells with the EL4 PR8 cells was significantly reduced after blocking with the anti-HA1 mAb (Fig. 3D), indicating that the various Ncr1 proteins (WT and mutants) directly interact with viral HA.

Next, we wanted to further demonstrate that the various Ncr1-Noe´ proteins directly recognize HA. To that end, we produced fusion proteins composed of the extracellular parts of the WT Ncr1 and the various Ncr1-Noe´ mutants (W32A, W32E, and W32R) fused to human IgG1. All constructs were then stably expressed in HEK293T cells, and the secreted proteins were purified on a protein G column and run on SDS-PAGE gels (Fig. 3E).

Additionally, we cloned the HA protein of the PR8 virus in-frame with human IgG1, stably expressed it in HEK293T cells, and purified the secreted protein using a protein G column. The HA Ig was biotinylated and used in WB assays for recognition of the Ncr1 and Ncr1-Noe´ fusion proteins. In agreement with our above results demonstrating that all Ncr1-Noe´ proteins interact with influenza, all Ncr1 variants, including Ncr1-Noe´, indeed interacted with HA Ig (Fig. 3F). To confirm that the HA Ig staining of the various Ncr1 proteins is specific, we generated fusion proteins of human NKp46, NKp44 (that were shown to bind viral HA) (20, 22, 25, 38, 39), and KIR2DL1. We then ran these proteins on SDS-PAGE gels (Fig. 3G), blotted them, and stained them with the biotinylated HA Ig. As shown in Fig. 3H, NKp46 and NKp44 indeed directly bound the HA Ig fusion protein. As expected, KIR2DL1, an inhibitory receptor expressed on NK cells that recognizes MHC class I proteins, bound the HA Ig fusion protein to a much lesser extent (Fig. 3H).

**Ncr1-Noe´ is activated upon tumor and influenza virus recognition using the YTS reporter system**

The second system we used to show the functionality of the Ncr1 mutants was the human NK tumor line, YTS. The cytotoxicity of YTS cells is primarily executed by 2B4 (40). Therefore, we prepared chimeric constructs containing the extracellular part of Ncr1 and the various Ncr1 mutants, fused to the transmembrane and cytoplasmic domains of 2B4 (named Ncr1 2B4, Ncr1 W32A 2B4, Ncr1 W32E 2B4, and Ncr1 W32R 2B4). In this reporter system, as opposed to other cells (293T: Fig. 2, Supplemental Fig. 2A; BW: Fig. 3, Supplemental Fig. 2B), the transduction efficiency of the various Ncr1-Noe´ mutants was less efficient as compared with the empty vector or WT Ncr1 (Fig. 4A).

Because NKp46 is expressed to some extent on YTS cells, but it is not functional (40), we wanted to verify that no cross-reactivity of the mouse Abs to the human NKp46 receptor exists. We stained YTS transfectants with the blocking Ab mNcr1.7 and with the mAb mNcr1.15 (Fig. 4B). All of the YTS transfectants were specifically stained by the anti-mNcr1 mAbs, and no staining was observed on the empty vector-transfected YTS cells (Fig. 4B).
Similar results were obtained when other anti-Ncr1 mAbs were used (data not shown). In agreement with our above observations, whereas the Ncr1 2B4 was expressed quite rapidly on the cell surface, \( \sim 2 \) wk were required for the Ncr1-Noe´ mutants to be expressed on the cell surface. Furthermore, and as above, expression of all Ncr1-Noe´ mutants was unstable and disappeared after a week in culture. Finally, the expression levels of the Ncr1-Noe´ variants were, in general, lower than that of the WT Ncr1 (Fig. 4B).

To test the functionality of the Ncr1 proteins expressed on YTS cells, we performed CD107a degranulation assays. YTS cells expressing the various Ncr1 proteins were incubated with EL4 cells only, or with EL4 cells incubated with influenza virus. As can be seen in Fig. 4C, the YTS Ncr1 transfectants degranulated in response to incubation with the EL4 tumor cells (Fig. 4C, designated EL4), suggesting (as in the case of the BW reporter system, Fig. 3), once again, that EL4 cells express an unknown tumor ligand for Ncr1. This degranulation was significantly reduced when the YTS Ncr1 transfectants were blocked with the specific blocking mAb, mNcr1.7 (Fig. 4C, designated EL4 mNcr1.7). In agreement with the above results (Fig. 3), all the YTS Ncr1 transfectants degranulated to a higher extent in the presence of influenza virus (Fig. 4C, designated EL4 PR8). This enhanced degranulation was significantly reduced in the presence of the mNcr1.7 (Fig. 4C, designated EL4 PR8 mNcr1.7). Little degranulation was observed with YTS cells expressing an empty vector (Fig. 4C). Consequently, we concluded that the various Ncr1-Noe´ mutants are able to reach the cell surface, however slowly, as compared with the WT Ncr1, and that their expression is unstable and lower than that for the WT Ncr1. However, during this short period in which the Ncr1-Noe´ proteins are present on the cell surface they are functional.

Ncr1-Noe´ proteins bind tumors similarly to the WT Ncr1

Because we generated fusion proteins composed of the various Ncr1-Noe´ proteins fused to human IgG1, we were next interested to test whether they can recognize murine and human cell lines. Mouse and human primary NK cells were used as negative controls and indeed no binding was detected to these primary cells (Supplemental Fig. 3). We then tested four mouse (4T1, B12, BW, and P815) and four human (1106 mel, SKRB3, K562, and Raji) cell lines for recognition by WT and mutated Ncr1 Ig proteins. As can be seen in Fig. 5 all the mouse and human tumor lines tested were recognized similarly by all fusion proteins. To demonstrate
FIGURE 4. Ncr1-Noe is activated upon tumor and influenza virus recognition using the YTS reporter system. (A) YTS cells were transduced with the various constructs as indicated above each dot plot. The mean fluorescence intensity of the GFP expression is indicated. The figure is representative of five independent experiments. (B) FACS staining of YTS cells transfected with an empty vector or with the various Ncr1 constructs. The gray-filled histograms represent the isotype control staining, and the black line (open) histograms are stainings with the anti-Ncr1 mAbs. The figure combines representative staining obtained in five independent experiments. (C) CD107a degranulation (percentage of baseline) of the various YTS cells (as indicated, above each graph) following incubation with EL4 in the absence or presence of influenza (indicated as EL4 PR8), and in the absence or presence of the anti-mNcr1.7 blocking Ab. The experiment was conducted three times. Statistical analysis was performed on data from all experiments. Values are shown as mean ± SEM. *p < 0.05.

the specificity of the staining, we preincubated the various fusion proteins with either a control mAb (12E7, lower histograms in each staining) or with the blocking anti-Ncr1 mAb mNcr1.7. Incubation with the mAb mNcr1.7 (but not with 12E7) reduced the binding of all WT (Fig. 5A) and Ncr1-Noe (Fig. 5B–D) fusion proteins, suggesting that all tumor cell lines tested express an unknown ligand for Ncr1.

Altered glycosylation of Ncr1-Noe³

We next aimed to understand why the expression of Ncr1-Noe is problematic. Because we noticed that the electrophoretic mobility of the three mutated Ncr1 proteins (W32A, W32E, and W32R) seemed to be slightly different from the WT Ncr1 (Fig. 3E), we speculated that maybe the glycosylation of the various Ncr1-Noe proteins is altered.

To test this, we used the WT and Ncr1-Noe fusion proteins presented in Fig. 3E (for the sake of clarity, the Coomassie staining is presented here again; Fig. 6A is the same as Fig. 3E). The proteins were transferred onto a membrane and Western blotted with two lectins: MAL, which preferentially binds sialic acid residues attached in an α2,3 linkage (Fig. 6B); and SNA lectin, which preferentially binds sialic acids attached to a terminal galactose in an α2,6 linkage (41) (Fig. 6C). Both lectins recognize the WT Ncr1 Ig, indicating that the Ncr1 Ig possesses sialic acids attached both in an α2,3 and α2,6 linkage (Fig. 6B, 6C). Recognition of all mutated Ncr1 proteins by MAL was markedly reduced when compared with the WT Ncr1 (Fig. 6B, quantified in Fig. 6D). In contrast, all the fusion proteins were recognized more or less to the same extent by SNA lectin (Fig. 6C, quantified in Fig. 6E). Thus, we concluded that the mutation at position 32 of the Ncr1 caused an α2,3 glycosylation defect in the Ncr1 protein.

To further investigate the glycosylation pattern of the various Ncr1 proteins we digested the WT and mutated Ncr1 proteins with PNGase F, Endo H and PNGase F. Endo H cleaves only hybrid and high-mannose N-linked epitopes (42). PNGase F cleaves all N-glycans (43). As shown in Fig. 6F, digestion with Endo H did not cause any change in the electrophoretic mobility of the WT or mutated Ncr1 proteins, suggesting that the mutated proteins do not contain hybrid or high-mannose N-linked epitopes. Conversely, digestion with PNGase F slightly reduced the electrophoretic mobility of all WT and mutated Ncr1 Ig proteins (Fig. 6F), suggesting that all Ncr1 WT and mutated proteins contain N-linked epitopes.

To directly demonstrate that the glycosylation pattern of Ncr1-Noe is altered, we subjected the WT and Ncr1-Noe Ig fusion proteins to N-glycan release assays. The normal phase HPLC profiles of the N-glycans of the WT and Ncr1-Noe proteins are shown in Fig. 6G and 6H, respectively. For the release of N-glycans, the chromatogram of the WT Ncr1 showed three prominent peaks, which were eluted from the column after 70, 85, and 95 min (Fig. 6G). Strikingly, the N-glycans release observed for the Ncr1-Noe was completely different, as three prominent peaks were eluted from the column at ~83, 93, and 97 min (Fig. 6H).

Next, we investigated whether the aberrant glycosylation of Ncr1-Noe is associated with a particular glycosylation stemming from a specific amino acid of Ncr1. We generated fusion proteins composed of the extracellular parts of the WT Ncr1 and Ncr1 mutated in each of the predicted N-linked glycosylated residues (N139A, N216A, and N238A, and a triple mutant, N139 216 238A, named 3Nmut) and fused them to human IgG1. All constructs were stably expressed in HEK293T cells, and the secreted proteins were purified on a protein G column. Interestingly, all N-linked mutated Ncr1 fusion proteins exhibited two bands when run on an SDS-PAGE gel (Fig. 6I). The presence of two protein bands, which differ in their intensities, is a phenomenon we occasionally observed for the WT Ncr1 protein as well. The WT Ncr1 exhibits one prominent band; however, when large amounts of Ncr1 Ig are loaded onto the SDS-PAGE gels, two protein bands are frequently visualized, equivalent in size to the bands observed in the single N-linked mutations of Ncr1. Importantly, two protein bands were observed when the Ncr1 protein was precipitated from primary NK
cells (Supplemental Fig. 1C). Interestingly, the two protein bands in the WT Ncr1 can clearly be visualized in the WB with MAL and the SNA lectin (Fig. 6J, 6K), although the WT Ncr1 Ig appears as a single protein band in the Coomassie staining (Fig. 6I). We think that these two bands represent two different glycosylation products. The WT Ncr1 and the upper protein bands of all the N-linked mutants were primarily recognized by MAL (Fig. 6J). Differences in the SNA lectin recognition of the various fusion proteins were noted (Fig. 6K). In the WT Ncr1 protein the SNA lectin recognized the lower band only (Fig. 6K). In the Ncr1 N238A Ig the upper bands were primarily recognized, whereas in the Ncr1 N139A Ig, Ncr1 N216A Ig, and in Ncr1 3Nmut Ig both protein bands were recognized (recognition of the triple N-mutant was reduced, but still evident) (Fig. 6K). The fact that the MAL and SNA lectins recognized the triple N-mutant (Fig. 6J, 6K) suggests that the Ncr1 protein carries yet unidentified glycosylated residues. Importantly, none of the N-linked glycosylation mutants, including the triple mutant, displayed a lectin staining pattern similar to the Ncr1-Noé proteins (Fig. 6J and 6K quantified in Fig. 6L and 6M, compared with Fig. 6B and 6C quantified in Fig. 6D and 6E).

Thus, we concluded that the predicted N-glycosylated residues of Ncr1 are indeed glycosylated. Additionally, the glycosylation defects observed in the Ncr1-Noé protein seemingly resulted from a general change of the N-glycosylations, rather than the abolishment of specific N-glycosylations.

**FIGURE 5.** Ncr1 and Ncr1-Noé recognize murine and human tumors. (A–D) FACS staining of four mouse (four left histograms in each row) and human (four right histograms in each row) tumor cells with various Ncr1 Ig fusion proteins (as indicated under the histograms). The gray-filled histograms represent staining with the secondary mouse Ab, and the black line histograms are stainings with the specific fusion proteins. The red line histograms are stainings with the specific fusion protein following blocking with anti-Ncr1 mAb mNcr1.7 or with a control mAb 12E7 (indicated on the left of the histograms). The figure combines representative stainings obtained in five independent experiments.

**Ncr1-Noé is arrested in the endoplasmic reticulum**

Ncr1-Noé proteins exhibit a glycosylation defect and are poorly expressed on the cell surface. Therefore we wanted to examine whether the Ncr1-Noé proteins were retained in the cells. To test this, we transduced YTS cells with the full-length WT Ncr1 and Ncr1-Noé variants to determine their intracellular localization. This was done by staining for Ncr1 along with the endoplasmic reticulum (ER) marker PDI or the endosomal marker CD107a (Fig. 7A). Minimal colocalization was observed between the WT Ncr1 and either PDI or CD107a (Fig. 7A). In contrast, all Ncr1-Noé proteins, which were poorly expressed on the cell surface (Figs. 2–4, Supplemental Figs. 1, 2), were colocalized with PDI, but not with CD107a (Fig. 7A, quantification in Fig. 7B). These results illustrate that the Ncr1-Noé proteins were arrested in the ER.

To test whether this expression pattern was unique to the YTS NK line, we additionally expressed the full-length Ncr1 and Ncr1-Noé variants in HEK239T and repeated the above procedures. Similar results were obtained with this cell line (Supplemental Fig. 4A, quantified in Supplemental Fig. 4B and 4C).

**Helios transcript is elevated in Ncr1-Noé–expressing cells**

It was reported that the Helios transcript was twice as abundant in the Noé mouse NK cells when compared with WT mice (30). However, elevated levels of the Helios transcript were not observed in the Ncr1<sup>gfp/gfp</sup> mouse (Ref. 31 and data not shown). We
FIGURE 6. Mutations in position 32 of Ncr1 affect its N-linked glycosylations. (A and I) Coomassie staining of the various fusion proteins (5 μg) run on 10% SDS-PAGE gel in reducing conditions. The Coomassie staining in (A) is identical to the one presented in Fig. 3E and is shown again for the sake of clarity. (B, C, J, and K) Western blots performed on the various Ncr1 fusion proteins. Blotting was performed either with MAL (B and J) or SNA lectin (C and K). (D, E, L, and M) The ratio of the WB staining to Coomassie staining (quantified by pixel intensity) of MAL (D and L) and SNA lectin (E and M). The figures are representative of three independent experiments. Statistical analysis was performed on data from all experiments. Values are shown as mean ± SEM. *p < 0.05. (F) Coomassie staining of the various Ncr1 fusion proteins (5 μg) run on 10% SDS-PAGE gel in reducing conditions: untreated (control), treated with Endo H, or treated with PNGase F. (G and H) HPLC chromatogram of N-linked glycans released from the Ncr1 Ig (G) and Ncr1-Noe Ig (H) fusion proteins. To facilitate better visualization, contrasts in the figure depicting the Coomassie staining and the WB figures were adjusted.

speculated that the reported elevation in the Helios transcript might result from the presence of the mutated Ncr1-Noe protein.

To test whether the Ncr1-Noe proteins generate a different signal from the WT Ncr1, leading to elevated Helios expression, we initially attempted to generate bone marrow chimeras using cells from the Ncr1<sup>W32E</sup> mice, reconstituted with WT Ncr1 and Ncr1-Noe mutants. However, only ~1% of the bone marrow chimera cells were successfully transduced with WT Ncr1, and no transduction was detected with the Ncr1-Noe constructs (in agreement with the less stable phenotypes of the Ncr1-Noe proteins).

Alternately, we used two cell lines, the YTS NK cells and HEK293T cells, where we expressed the full-length WT and Ncr1-Noe proteins. We proceeded to assess the level of the Helios transcript. As seen in Fig. 8, in both the YTS (Fig. 8A) and HEK293T transfectants (Fig. 8B), Helios expression was markedly upregulated in the presence of all Ncr1-Noe proteins. Interestingly, the upregulation of Helios expression was not restricted to the YTS NK line, and increased Helios expression was also detected in the HEK293T cells, albeit a lower fold increase was observed in these cells (Fig. 8).

Discussion

NKp46/Ncr1 is one of the major NK cell–activating receptors and the only NCR to exist in mice. It has been implicated in the control of several pathogens and malignancies. The activity of Ncr1 has been extensively studied in numerous pathologies in vivo using the Ncr1<sup>W32E</sup> mice, which we have generated (5). These mice (available now at The Jackson Laboratory, JR 22739, B6.129-Ncr1<sup>tm1Oman</sup>/J) displayed impaired Ncr1 activities when compared with mice with functional Ncr1. Interestingly, and in contrast to what has been observed with the Ncr1<sup>W32E</sup> mice, the Noé mice exhibited a hyperactive, Ncr1-independent phenotype that is probably mediated by elevated Helios levels (30).

Because Ncr1-Noe was not expressed on the surface of the NK cells, it was suggested that the Noé mice were Ncr1 deficient. However, in the Ncr1<sup>W32E</sup> mice, as well as in the double-knockout mice of Ncr1 and NKg2d, hyperactivation of NK cells was not observed, and importantly Helios levels were normal (Ref. 31 and data not shown).

Thus, we wondered how such different, almost completely opposite phenotypes are observed in mice in which Ncr1 was absent. We hypothesized that the hyper–NK cell reactivity observed in the Noé mouse, and the elevated Helios levels, might be attributed to the presence of Ncr1-Noe and subsequently sought to characterize the molecular and functional properties of Ncr1-Noe.

We began our investigation by demonstrating that MCMV infection is similar in the presence or absence of Ncr1 (using three different strains of Ncr1<sup>W32E</sup>), in striking contrast to the Noé mice, which were shown to control MCMV better than the WT mice (30). The results presented in the present study were supported by additional works demonstrating that Ncr1 indeed plays no role in MCMV infection (36) and in decidual human NK recognition of human CMV (44).

Another striking difference between the Ncr1<sup>W32E</sup> and the Noé mice relates to influenza virus infection. The Ncr1<sup>W32E</sup> mice were shown to be more susceptible to influenza infection (5, 22), whereas the Noé mice were surprisingly better at controlling its propagation (30).

We generated six new mAbs against Ncr1 and observed that the full-length Ncr1 Noe protein, as well as additional Ncr1 proteins expressing other mutations in position 32, can be expressed on the cell surface of various cell lines, such as the NK tumor line YTS and HEK293T cells.
Importantly, we determined that changing the identity of the amino acid at position 32 does not matter, and that Ncr1 W32A, W32E, and W32R behave very similarly: all proteins can be expressed on the cell surface, however they appeared in delay, their expression was lower than the WT Ncr1 and significantly decreased following a short period in culture. Possibly, the Ncr1-Noe´ proteins translocate very slowly to the cell surface and once they reach the cell surface their expression is unstable.

In our reporter systems, expression of the various Ncr1-Noe´ mutants was lower than the expression of the WT Ncr1. However, despite the low expression levels of the Ncr1-Noe´ mutants, similar signals (IL-2 secretion or CD107a degranulation) were observed with all Ncr1-Noe´ mutants. It is possible that the low expression levels of the Ncr1-Noe´ mutants on the cell surface were sufficient to activate the cells and perhaps the high expression achieved with the WT Ncr1 transfectant was superfluous. Alternatively, the ER arrest of the proteins and increased Helios expression in the cells might result in a general hyperactivation, compensating for the low cell surface expression. An additional explanation could be that the differently glycosylated Ncr1-Noe´ proteins, when expressed on the cell surface, recognize their targets better than does the WT Ncr1, and thus the low surface expression is compensated for.

The aberrant expression pattern of the Ncr1-Noe´ mutants was not dependent on their transmembrane and cytoplasmic domains, as even the chimeric proteins composed of the extracellular parts of all Ncr1-Noe´ mutants fused to the transmembrane domains and the cytoplasmic tails of either mouse ζ or to human 2B4 demonstrated the same impaired expression pattern. Perhaps Narni-Mancinelli et al. (30) did not detect the cell surface expression of Ncr1-Noe´ in HEK293T cells because they transiently expressed Ncr1-Noe´ in cells, and under these short-term conditions, Ncr1-Noe´ is indeed not yet expressed on the cell surface.

We next characterized the properties of the Ncr1-Noe´ proteins to understand the mechanisms underlying the delayed surface expression. We tested for alterations in the glycosylation patterns of the various Ncr1-Noe´ proteins (W32A, W32E, and W32R), using several methods, including HPLC analyses of sugar content, enzymatic treatments, and WB with lectins. We show that the Ncr1-Noe´ N-linked glycosylations were qualitatively different from the N-linked glycosylations found on Ncr1. To further explore the behavior of the Ncr1-Noe´ protein and its variants, we performed colocalization assays. These assays revealed that Ncr1-Noe´ is arrested in the ER.

Interestingly, mutations in each of the N-glycosylation mutants of Ncr1, or even the triple N-glycosylated mutant, did not reproduce the sugar phenotype of the Ncr1-Noe´ mutants. Thus, we
propose that the Ncr1-Noe mutation does not affect the N-glycosylation of a particular residue, but rather it affects the glycosylation pattern of the entire protein. How the W32 mutation might affect the N-linked glycosylations is a very interesting question. As the tryptophan at position 32 is located far from any N-linked glycosylated residue, it is difficult to explain how this might occur. It was suggested that the W32 residue forms a pi structure with the tryptophan at position 48 (30). Because the mutation at position 32 might have disrupted this pi structure, perhaps this caused structural changes in the protein, which indirectly interfered with its ability to translocate within the cell and glycosylation pattern. It is also possible that the W32 mutation caused a global folding change in the protein (unrelated to the pi structure), which alters its maturation and glycosylation processes, rather than interferes with the glycosylation step directly. Finally, although less likely, the Ncr1-Noe mutation may directly affect its glycosylation pattern. It will be interesting to determine in the future the mechanisms by which the N-glycosylations are altered.

As the hyperactivity observed in the Noe mouse was attributed to increased Helios transcription in the Noe NK cells, we sought to determine whether the Ncr1-Noe proteins transfer different signals than the WT Ncr1. We were unable to express the Ncr1-Noe proteins in bone marrow mouse chimera cells, possibly due to their unstable properties. We therefore expressed these proteins in cell lines. Although, this system has disadvantages, one of its major advantages is that if an increase in Helios expression is observed, this can be attributed solely to the presence of the Ncr1-Noe proteins, as it does not involve the mouse environment present in bone marrow chimeras. Strikingly, using two transduced cell systems, an NK tumor line and a cell line, we observed that Helios expression is upregulated in response to the Ncr1-Noe proteins only. These results suggest that the upregulation of Helios observed in the Ncr1-Noe mouse and possibly the hyperactivity of the NK cells observed in these mice might be attributed to the presence of the Ncr1-Noe protein and to the different signals it generates as compared with WT Ncr1.

Disclosures

The authors have no financial conflicts of interest.

References


