

microscope. Images were captured with a RT-Slider Spot digital camera (Diagnostic Instruments).

### Whole-cell voltage clamp recordings

Rat olfactory epithelia were used for whole-cell voltage clamp recordings<sup>25</sup>. Fragments of septal olfactory mucosa were placed in the perfusion chamber such that the basal portions were immersed in physiologic buffer (136.9 mM NaCl, 5.3 mM KCl, 4.2 mM NaHCO<sub>3</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 3.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.6 mM D-glucose, pH 7.4), whereas the upper epithelial surfaces with olfactory cilia were exposed to the air. The patch electrode (resistance of 8–16 MΩ) was filled with a solution containing 110 mM KCl, 4 mM NaCl, 2 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub> and 2 mM MOPS at pH 7.4 in the presence or absence of affinity purified RGS2 antibodies (4 μg ml<sup>-1</sup>), RGS2 antibodies (4 μg ml<sup>-1</sup>) plus recombinant RGS2 (4 μg ml<sup>-1</sup>) or control antibodies. After stable contact with an olfactory neuron, 1-s puffs of odorant mixture containing 1.6 mM ethyl butyrate, eugenol and (+) and (-) carvones were applied. The whole-cell response over the time course of several minutes was recorded after being amplified by a voltage-clamp amplifier (Axopatch 200B, Axon Inst.) and filtered at 2–5 kHz. After compensation the series resistance was always lower than 20 MΩ. In some experiments the epithelia were incubated for 5 min in buffer containing tetrodotoxin.

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1. Sunahara, R. K., Dessauer, C. W. & Gilman, A. G. Complexity and diversity of mammalian adenylyl cyclases. *Annu. Rev. Pharmacol. Toxicol.* **36**, 461–480 (1996).
2. Hurley, J. H. Structure, mechanism, and regulation of mammalian adenylyl cyclase. *J. Biol. Chem.* **274**, 7599–7602 (1999).
3. Hepler, J. R. Emerging roles for RGS proteins in cell signaling. *Trends Pharmacol. Sci.* **20**, 376–382 (1999).
4. Kehrl, J. H. Heterotrimeric G protein signaling: roles in immune function and fine-tuning by RGS proteins. *Immunity* **8**, 1–10 (1998).
5. Sunahara, R. K., Tesmer, J. J., Gilman, A. G. & Sprang, S. R. Crystal structure of the adenylyl cyclase activator Gsα. *Science* **278**, 1943–1947 (1997).
6. Natochin, M. & Artemyev, N. O. A single mutation Asp229→Ser confers upon Gsα the ability to interact with regulators of G protein signaling. *Biochemistry* **37**, 13776–13780 (1998).
7. Chatterjee, T. K., Eapen, A. K. & Fisher, R. A. A truncated form of RGS3 negatively regulates G protein-coupled receptor stimulation of adenylyl cyclase and phosphoinositide phospholipase C. *J. Biol. Chem.* **272**, 15481–15487 (1997).
8. Tseng, C. C. & Zhang, X. Y. Role of regulator of G protein signaling in desensitization of the glucose-dependent insulinotropic peptide receptor. *Endocrinology* **139**, 4470–4475 (1998).
9. Schild, D. & Restrepo, D. Transduction mechanisms in vertebrate olfactory receptor cells. *Physiol. Rev.* **78**, 429–466 (1998).
10. Reed, R. R. Signaling pathways in odorant detection. *Neuron* **8**, 205–209 (1992).
11. Pace, U., Hanski, E., Salomon, Y. & Lancet, D. Odorant sensitive adenylyl cyclase may mediate olfactory reception. *Nature* **316**, 255–258 (1985).
12. Sklar, P. B., Anholt, R. R. & Synder, S. H. The odorant-sensitive adenylyl cyclase of olfactory receptor cells. Differential stimulation by distinct classes of odorants. *J. Biol. Chem.* **261**, 15538–15543 (1986).
13. Dessauer, C. W., Tesmer, J. J., Sprang, S. R. & Gilman, A. G. Identification of a Gα binding site on type V adenylyl cyclase. *J. Biol. Chem.* **273**, 25831–25839 (1998).
14. Landis, C. A. *et al.* GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature* **340**, 692–696 (1989).
15. Bakalyar, H. A. & Reed, R. R. Identification of a specialized adenylyl cyclase that may mediate odorant detection. *Science* **250**, 1403–1406 (1990).
16. Firestein, S. & Weblin, F. Odor-induced membrane currents in vertebrate olfactory receptor neurons. *Science* **244**, 79–82 (1989).
17. Kleene, S. J. & Gesteland, R. C. Calcium-activated chloride conductance in frog olfactory cilia. *J. Neurosci.* **6**, 3624–3629 (1991).
18. Kurahashi, T. & Taw, K.-W. Co-existence of cationic and chloride components in odorant-induced current of vertebrate olfactory receptor cells. *Nature* **363**, 71–74 (1993).
19. Pepperl, D. J., Shah-Basu, S., VanLeeuwen, D., Granneman, J. G. & MacKenzie, R. G. Regulation of RGS mRNAs by cAMP in PC12 cells. *Biochem. Biophys. Res. Commun.* **243**, 52–55 (1998).
20. Beadling, C., Druey, K. M., Richter, G., Kehrl, J. H. & Smith, K. A. Regulators of G protein signaling exhibit distinct patterns of gene expression and target G protein specificity in human lymphocytes. *J. Immunol.* **162**, 2677–2682 (1999).
21. Wei, J. *et al.* Phosphorylation and inhibition of olfactory adenylyl cyclase by CaM kinase II in Neurons: a mechanism for attenuation of olfactory signals. *Neuron* **21**, 495–504 (1998).
22. Sinnarajah, S. *et al.* Inhibition and enhancement of odorant-induced cAMP accumulation in rat olfactory cilia by antibodies directed against Gαs/olf- and Gαi-protein subunits. *FEBS Lett.* **426**, 377–380 (1998).
23. Summers, M. D. & Smith, G. E. in *Texas Agricultural Experiment Station Bulletin* 1,555 (College Station, Texas, 1987).
24. Salomon, Y., Londos, C. & Rodbell, M. A highly sensitive adenylyl cyclase assay. *Anal. Biochem.* **58**, 541–548 (1974).
25. Firestein, S. & Werblin, F. in *Chemical Senses Vol. 1, Receptor Events and Transduction in Taste and Olfaction* (eds Brand, J. G. *et al.*) 449–467 (Marcel Dekker, New York, 1989).

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## Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells

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Natural killer (NK) cells destroy virus-infected and tumour cells, apparently without the need for previous antigen stimulation<sup>1</sup>. In part, target cells are recognized by their diminished expression of major histocompatibility complex (MHC) class I molecules, which normally interact with inhibitory receptors on the NK cell surface<sup>2–8</sup>. NK cells also express triggering receptors that are specific for non-MHC ligands; but the nature of the ligands recognized on target cells is undefined<sup>9–14</sup>. NKp46 is thought to be the main activating receptor for human NK cells<sup>9,15</sup>. Here we show that a soluble NKp46-immunoglobulin fusion protein binds to both the haemagglutinin of influenza virus and the haemagglutinin-neuraminidase of parainfluenza virus. In a substantial subset of NK cells, recognition by NKp46 is required to lyse cells expressing the corresponding viral glycoproteins. The binding requires the sialylation of NKp46 oligosaccharides, which is consistent with the known sialic binding capacity of the viral glycoproteins. These findings indicate how NKp46-expressing NK cells may recognize target cells infected by influenza or parainfluenza without the decreased expression of target-cell MHC class I protein.

We studied the role of NKp46 in NK recognition by producing a fusion protein in which the extracellular domain of NKp46 is fused to the Fc portion of immunoglobulin (Ig). Previous reports suggested that NKp46, together with the NKp44 activating receptor, is involved in the lysis of MHC class-I-negative 721.221 cells<sup>12,14,15</sup>. We observed little staining of 721.221 cells when cells were incubated with the NKp46-Ig fusion protein (Table 1). As NK cells can effectively lyse virus-infected cells<sup>1</sup>, we tested whether infection with Sendai virus (SV; a mouse paramyxovirus) increased the binding of NKp46-Ig. Remarkably, a 10-fold increase in the staining by NKp46-Ig was observed (Table 1). This effect is specific for NKp46, as SV infection did not alter the binding of other NK receptor-Ig fusion proteins tested (NKAT-8-Ig (KIR2DS4-Ig), KIR-1-Ig (KIR2DL1-Ig), or CD16-Ig; data not shown).

To identify the putative NKp46 ligand, we immunized mice with SV-infected 721.221 cells, and screened spleen-derived B-cell hybridoma supernatants for increased staining of virus-infected cells relative to non-infected cells. The supernatants of one of the hybridoma clones tested (135.7) efficiently blocked the binding of NKp46-Ig to SV-infected cells (Table 1). Enzyme-linked immunosorbent assays (ELISAs) using SV as immunoadsorbent indicated that monoclonal antibody (mAb) 135.7 recognizes a viral gene

product (data not shown).

SDS–polyacrylamide gel electrophoresis (PAGE) analysis of 135.7-reactive proteins immunoprecipitated from detergent lysates of <sup>125</sup>I cell-surface-labelled SV-infected 721.221 cells revealed that 135.7 binds a protein with an apparent relative molecular mass of 70,000 (*M<sub>r</sub>* 70K), similar to the reported mobility of the haemagglutinin–neuraminidase (HN) glycoprotein. Indeed, NKp46–Ig binding was blocked when infected cells were first incubated with anti-HN mAbs TC-1D6 or TC-9C1 but not with the TC-9A1 mAb that is specific for the other principal SV glycoprotein—the fusion (F) protein (Table 1).

Despite the dependency on HN of NKp46–Ig binding to SV-infected 721.221 cells, we failed to detect an increased susceptibility of these cells to NK-mediated lysis associated with SV infection (data not shown), perhaps because non-infected 721.221 cells are already sensitive to NK-mediated lysis and/or receptors other than

NKp46 are dominant in triggering the lysis of 721.221. Thus, to test the role of NKp46 recognition of HN in NK lysis, we transiently transfected 293T cells with a plasmid encoding HN. Forty-eight hours after transfection, cell-surface HN expression was confirmed using mAbs TC-1D6 (Fig. 1a) or 135.7 (data not shown). Notably, transfection resulted in a twofold increase in NKp46–Ig staining, without enhancing the staining with other immunoglobulin fusion proteins, KIR-1–Ig, NKAT-8–Ig or CD16–Ig (Fig. 1a). Moreover, NK GAL, an NK line derived from healthy donor peripheral blood lymphocytes (PBLs), lysed HN-transfected 293T cells at least fourfold more efficiently than non-transfected or mock-transfected cells (Fig. 1b, c). Pre-incubation of NK GAL with a mouse antiserum raised against NKp46–Ig inhibited the increased killing of HN-transfected 293T cells, whereas incubation with a control serum had little effect (Fig. 1b). The same experiment revealed that each of the three HN-specific mAbs could block NK-mediated lysis, but a control mAb specific for CD99 had no significant effect (Fig. 1c).

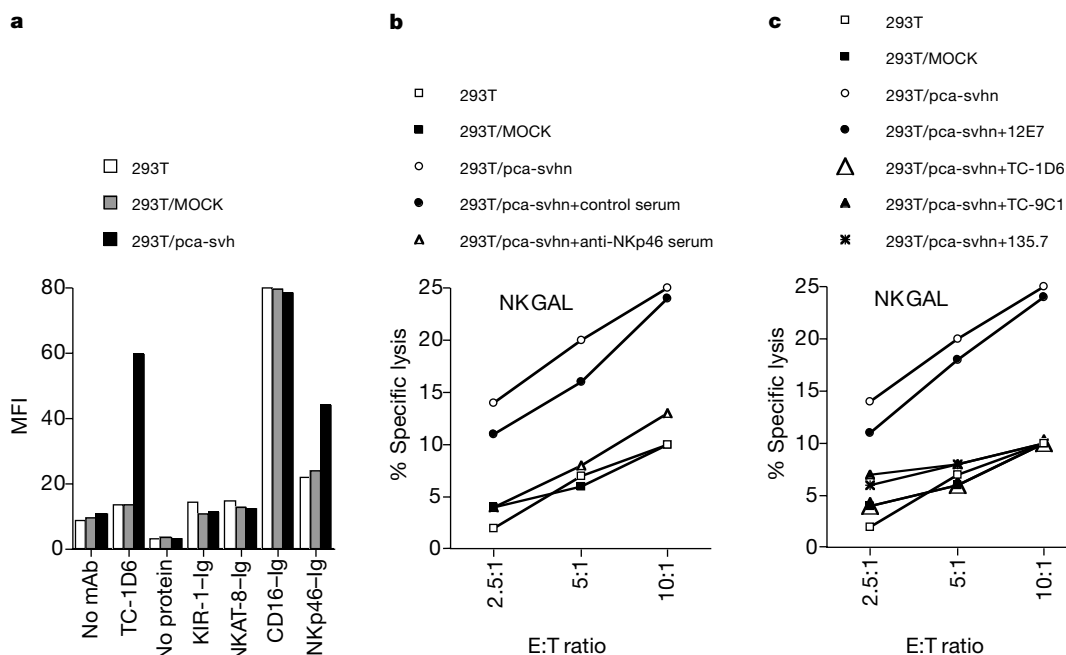
One of the hallmarks of NK recognition is its lack of antigen specificity. Having shown that NKp46 interacts with the SV HN in NK-mediated lysis, we next tested whether it could interact with the influenza virus (IV) haemagglutinin (HA). IV infection of 1106mel cells (a class-I-deficient cell line) resulted in a fourfold increase in NKp46–Ig binding (Table 2). As above, the specific nature of the enhanced binding is shown by the constant binding of other immunoglobulin fusion proteins (data not shown). Notably, the increased NKp46–Ig binding was completely or partially blocked, respectively, by the HA-specific mAbs H28-E23 or H17-L2 (Table 2). In contrast, HN-specific mAbs TC-1D6, TC-9C1 or 135.7 had no effect on binding (data not shown).

We next showed that IV infection of 1106mel cells enhances NK-GAL-mediated lysis, and that the enhanced killing is blocked

**Table 1 Anti-haemagglutinin antibodies inhibit NKp46–Ig binding to Sendai-virus-infected cells**

mAb specificity	mAb binding (MFI)		NKp46–Ig binding (MFI)	
	721.221	721.221 SV	721.221	721.221 SV
No mAb	–	5	5	6
TC-9A1	Anti-Fusion	5	82	6
TC-1D6	Anti-HN	7	141	7
TC-9C1	Anti-HN	13	179	7
135.7	Anti-HN	5	95	6

721.221 cells (10<sup>6</sup> per ml) were incubated overnight with 100 μl ml<sup>-1</sup> of SV-containing supernatant. Cells (infected or uninfected) were washed, incubated with the indicated mAbs and stained either with FITC-labelled goat anti-mouse immunoglobulin, or with NKp46–Ig fusion protein followed by PE-conjugated goat anti-human Fc. MFI, median fluorescence intensity; the MFI was rounded to the nearest whole number. Background staining of SV-infected 721.221 and 721.221 cells with the PE-conjugated anti-human Fc was 4 and 2, respectively. Results are representative of five independent experiments. Similar results were obtained with the B-cell line RPMI 8866.



**Figure 1** Lysis of HN-transfected 293T cells by NK GAL. **a**, NKp46–Ig binding to 293T cells transfected with Sendai virus haemagglutinin cDNA. 293T cells were transiently transfected with a control PCDNA3 plasmid (293T/MOCK) or with a cDNA encoding for SV HN (293T/pca-svhn) using Fugene reagent (Boehringer Mannheim). After 48 h, cells were stained either with TC-1D6 mAb or with KIR-1, NKAT-8, CD16 and NKp46 immunoglobulin fusion proteins. MFI, median fluorescence intensity. Controls were the same cells stained either with FITC-conjugated anti-mouse antibodies (no mAb), or with PE-conjugated anti-human Fc antibodies (no protein). Results represent one experiment of three performed. **b, c**, Enhanced lysis of 293T cells transfected with the Sendai virus

HN cDNA is blocked by anti-NKp46 and anti-HN mAb. Forty-eight hours after transfection, 293T, 293T/MOCK and 293T/pca-svhn cells were labelled with [<sup>35</sup>S]methionine and washed. Cells were then incubated at the effector to target (E:T) ratios indicated; with NK GAL pre-incubated with either control serum or with anti-NKp46 serum (**b**). Alternatively, cells were incubated with the indicated mAb for 1 h on ice, washed, and incubated with NK GAL (**c**). In all experiments, NK cells were pre-incubated with 50% human serum for 1 h on ice and washed to block Fc receptors. Results represent one experiments of three performed in both **b** and **c**.

by pre-incubation of cells with anti-NKp46 serum but not with control serum or mAbs 12E7 and 3G8, which are specific for CD99 and CD16, respectively (Fig. 2a). Incubation of IV-infected 1106mel cells with mAb H28-E23 resulted in complete inhibition of the increased lysis, whereas mAb H17-L2 had a partial inhibitory effect, mirroring the results of the blocking experiment (Fig. 2b, Table 2).

We also examined the recognition of IV-infected 1106mel cells by clones prepared from NK GAL by limiting dilution. All 28 clones tested were positively stained with the anti-NKp46 serum. Twenty-one of the clones exhibited enhanced recognition of IV-infected cells relative to uninfected cells (Fig. 2c); pre-incubation of IV-infected 1106mel cells with the HA-specific mAb H28-E23 completely inhibited the IV-enhanced lysis by all 21 NK clones (data not shown). Pre-incubation of NK clones with anti-NKp46 serum largely inhibited the IV-enhanced lysis of 13 of these clones, (for example clone 6, Fig. 2c), whereas 6 of the clones were partially inhibited (for example clone 15, Fig. 2c). The average median fluorescence intensity (MFI) (median fluorescence intensity) after staining with anti-NKp46 serum for these two groups was 28.9 and 32.8, respectively.

**Table 2 Anti-haemagglutinin antibodies inhibit NKp46-Ig binding to influenza-virus-infected cells**

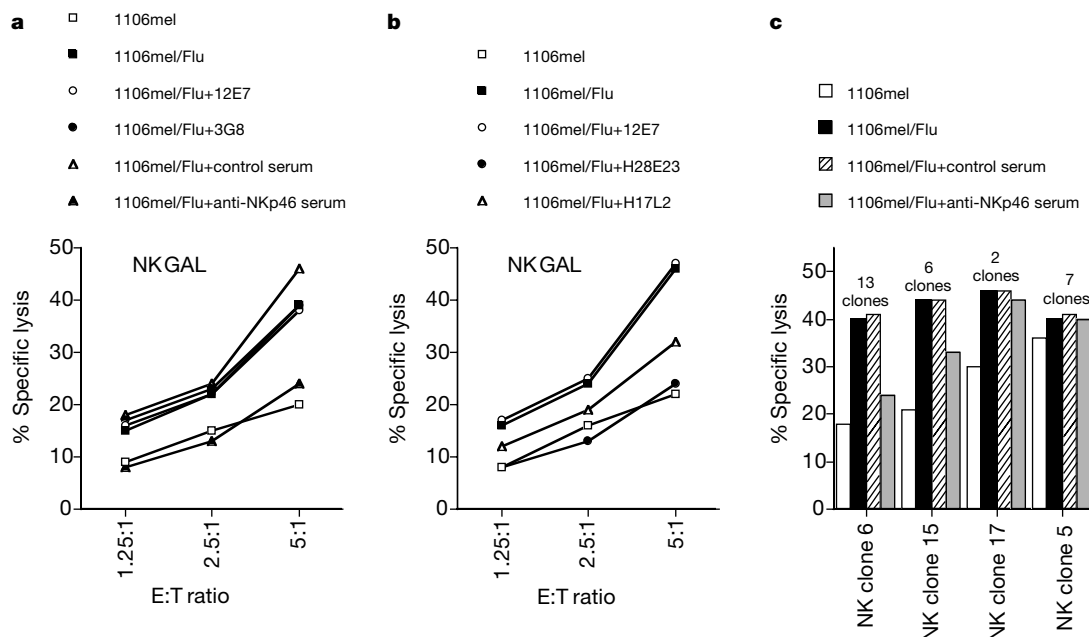
mAb specificity	mAb binding (MFI)		NKp46-Ig binding (MFI)	
	1106mel	1106mel IV	1106mel	1106mel IV
No mAb	-	18	22	112
H28-E23	Anti-HA	18	2,016	100
H17-L2	Anti-HA	11	2,100	110
NA2-1C1	Anti-NA	10	2,000	109

1106mel cells ( $10^6$  per ml) were incubated overnight with  $1,000 \text{ U ml}^{-1}$  of IV. Cells (infected or uninfected) were washed, incubated with the indicated mAbs, and stained with either FITC-conjugated goat anti-mouse immunoglobulin or the NKp46-Ig fusion protein followed by PE-conjugated goat anti-human Fc. MFI, median fluorescence intensity; the MFI was rounded to the nearest whole number. Background staining of IV-infected 1106mel and 1106mel cells with the PE-conjugated anti-human Fc was 7 and 6, respectively. Results are representative of eight independent experiments

Two clones that showed enhanced lysis of IV-infected cells were not affected by the NKp46 antiserum (for example clone 17, Fig. 2c). The average MFI for anti-NKp46 staining for this group of two clones was 17.5. Finally, no IV-associated enhancement in lysis was observed in seven of the clones tested (for example clone 5, Fig. 2c), and the MFI for anti-NKp46 staining of this group was 31.3. Similar results were obtained when the same NK clones were tested with HN-transfected 293T cells (data not shown). These findings indicate, first, that NKp46 is required for the recognition of HA- and HN-expressing cells by a substantial subset of NK cells; and second, that other populations of NK cells can lyse these cells in an NKp46-independent manner.

Previous reports indicated that haemagglutinin can enhance lysis of virus-infected target cells by NK cells, as well as activate NK cells directly<sup>1,16,17</sup>. Our observations argue that this is mediated by interaction with the triggering receptor NKp46, rather than by a nonspecifically increased conjugation effect. NKp46-Ig binds to 293T transfectants expressing HN (Fig. 1a), as well as to IV-infected 1106mel cells (Table 2). The enhancement in binding of NKp46-Ig to 1106mel after IV infection can be blocked with purified HA (Fig. 3a). In addition, purified HA binds to peripheral blood NK cells, and this binding can be blocked by NKp46-Ig but not by control CD99-Ig (data not shown). These data suggest that there is a direct interaction between NKp46 and haemagglutinin.

To test further this direct activation through the interaction of HA and cellular NKp46, we transfected BW cells with constructs encoding chimaeric proteins in which extracellular portion of the NKp46 (BW-NKp46) or CD16 (BW-CD16) are fused to the transmembrane and tail domains of the CD3 $\zeta$  chain. We incubated the transfectants or untransfected control cells with influenza virus for 20 h and tested supernatants for the presence of interleukin (IL)-2. This revealed that virus induced IL-2 secretion from BW-NKp46 but not from BW-CD16 or untransfected BW cells (Fig. 3b). This suggests that HA, presented in a multivalent state on IV, can ligate NKp46 in such a manner that may activate effector function.



**Figure 2** Lysis of IV-infected 1106mel cells by NK GAL and derived clones. **a**, NK cells were incubated with no antibody, control anti-CD99 mAb (12E7), anti-CD16 mAb (3G8), control serum, or anti-NKp46 serum for 1 h on ice. NK cells were then washed and incubated with either 1106mel or IV-infected 1106mel cells at the indicated E:T ratios. **b**, IV-infected 1106mel cells were incubated with various mAbs for 1 h on ice, and then with NK GAL at the indicated E:T ratios. Results represent one experiment of three

performed in both **a** and **b**. **c**, Twenty-eight NK clones were derived from the NK GAL by limiting dilution. Blocking experiments with serum containing polyclonal antibodies were as in Fig. 1. The E:T ratio was 5:1. Numbers indicate the number of clones that behaved similarly to the one NK clone presented. Results represent one experiment of two performed. In all experiments, NK cells were pre-incubated with 50% human serum for 1 h on ice and washed to block Fc receptors.

Thus, although on a molar basis NKp46 may not be the chief haemagglutinin receptor on NK cells, it can be a functional haemagglutinin receptor.

Sendai virus HN and influenza virus HA both recognize terminal *N*-acetylneuraminic acid residues (sialic acids) attached to galactose, suggesting that they use a common mechanism for binding to NKp46. The involvement of sialic acid in the interaction of NKp46 with HA is indicated by a number of findings.

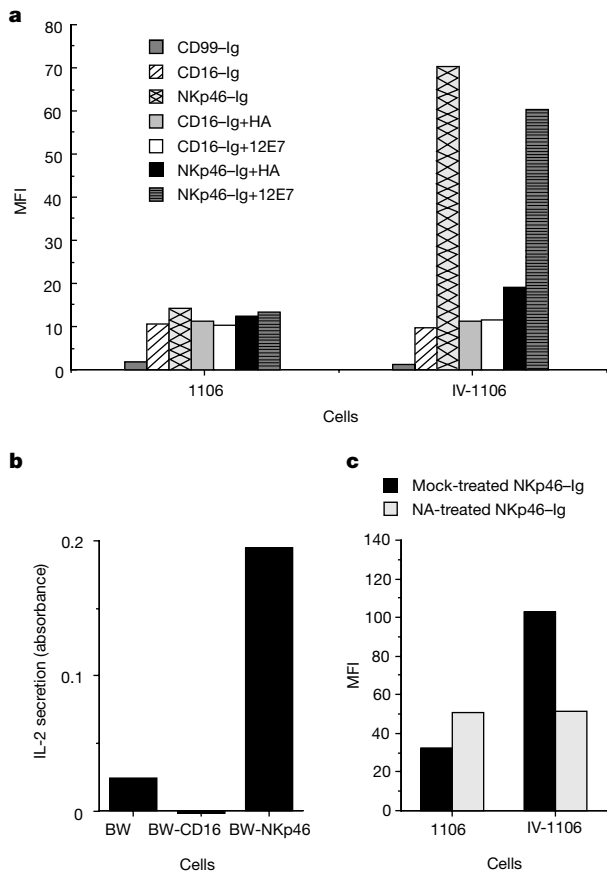
First, NKp46-Ig can completely block IV-mediated agglutination of sheep erythrocytes at a protein concentration as low as 2  $\mu$ M (data not shown). Second, pre-incubation of IV-infected cells with a mAb (NA2-1C21) that blocks the enzymatic activity of IV neuraminidase (NA, the other principal IV glycoprotein expressed on the surface of infected cells and virions) significantly enhances NKp46-Ig binding to IV-infected cells (Table 2). Third, NKp46-Ig does not stain target cells infected with measles (data not shown) whose HA does not bind sialic acid<sup>18</sup>. Last, and most directly, treatment of NKp46-Ig with bacterial neuraminidase reduced its binding to IV-infected cells without reducing its binding to uninfected cells (Fig. 3c).

Treatment of NKp46-Ig did not effect staining of non-infected 1106mel cells, measured by flow cytometry (Fig. 3c), nor alter the integrity of the protein tested by SDS-PAGE analysis (data not

shown). As de-sialylation often increases interactions by reducing negative charge repulsion of receptor-ligand pairs<sup>19</sup>, this strongly supports the direct interaction of NKp46 with the sialic-binding site of HA. Haemagglutinins of different influenza viruses have different specificities for sialic acid linked to galactose by  $\alpha$ 2,3 or  $\alpha$ 2,6 linkages<sup>20</sup>, both expressed by NK cells<sup>21,22</sup>.

We interpret these findings to indicate that NKp46 binds to target cells in two ways: first, through the interaction of NKp46-associated sialic acid with viral sialic acid receptors; second, in a sialic-acid-independent interaction with undefined cellular ligands. The former is clearly responsible for the enhanced killing of IV-infected cells by the NK cells that we have studied. It cannot be absolutely ruled out that the interaction of HA with NKp46 could principally augment NK cytotoxicity by facilitating binding of NKp46 to a second cellular receptor. It is also unclear whether the interaction of NKp46 with viral HA is sufficient for triggering lysis. The role of non-viral ligands to NKp46 and the role of other NK-triggering receptors, for recognition of viral infected cells, is clearly an important area of further research.

The dissociation constant of HA for sialic acids is in the millimolar range—too low for a monomeric interaction to account for either the stable binding of NKp46-Ig detected by flow cytometry (which requires dissociation constants less than 0.1  $\mu$ M), or the



**Figure 3** NKp46 binding to and activation by haemagglutinin. **a**, NKp46-Ig was incubated with or without 40  $\mu$ g purified HA protein (no blocking of NKp46-Ig binding was evident when less than 40  $\mu$ g of purified HA protein was used). Mixtures were next incubated with IV-infected or non-infected 1106mel cells and stained with PE-conjugated goat anti-human Fc. Results represent one experiment of two performed. MFI, median fluorescence intensity. Similar results were obtained when SV-infected 721.221 cells were used. **b**, Cells ( $10^6$  in 1 ml) were incubated with 1,000 U IV for 20 h in 24-well plates. IL-2 levels in the supernatants were measured by ELISA, and results are given as absorbance (optical density 650). Absorbances from ELISAs of supernatants of untreated cells (0.144, 0.127 and 0.238 for BW, BW-CD16 and BW-NKp46, respectively) were

subtracted from those of treated cells. BW cells and transfectants were not susceptible to IV infection in these conditions, as shown by the negative staining of cells with anti-NA mAb. Results represent one experiment of three performed. **c**, NKp46-Ig was incubated with 0.01 U of insoluble neuraminidase attached to beaded agarose (N-5254; Sigma) or with PBS (control) for 1 h at 17  $^{\circ}$ C on a roller. IV-infected, or non-infected 1106mel cells were washed, and stained either with NA-treated or mock-treated NKp46-Ig, followed by PE-conjugated anti-human Fc. Results represent one experiment of eight performed. The activity of NA was confirmed by SDS-PAGE analysis of NA-treated fetuin, a highly sialylated protein.

potency of NKp46-Ig in blocking viral haemagglutination. This implies either a multimeric interaction of HA with NKp46 or that NKp46 interacts more intimately with HA after contact is initiated by the sialic-acid binding. Even in the former case, NKp46 would need special properties to distinguish it from other cellular glycoproteins, as terminal sialic residues are ubiquitous on N- and O-linked oligosaccharides found on glycoproteins. One possibility is that NKp46 (which is thought to have a single N-linked and two O-linked oligosaccharides<sup>14</sup>) oligomerizes at the cell surface to enable multivalent interaction of sialic acid with a single HA complex, which as a trimer possesses three sialic-acid-binding sites.

The existence of NK clones that recognize IV-infected cells in an NKp46-independent manner (for example NK GAL clone 17, Fig. 2c) infers that other lysis receptors are involved in the recognition of virus-infected cells (for example NKp44; our unpublished data). Other receptors may also recognize HA, as the enhanced lysis associated with virus infection can be completely blocked by anti-HA mAb. Given that members of at least seven virus families use sialic acid as a receptor for virus entry into host cells, this suggests a general strategy for NK cell recognition of a substantial subset of viruses. □

## Methods

### Cells and viruses

We used the following cell lines: the MHC class-I-negative human EBV-transformed B-cell line 721.221; the MHC class-I-negative human melanoma line 1106mel; and the adenovirus-transformed, SV40-large T-antigen-transfected, human fibroblast kidney cell line 293T. NK cells (lines and clones) were isolated from PBLs using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec Inc); NK cells were kept in culture as described<sup>23</sup>. SV and the IVA/PR/8/34 (H1N1) were purchased from Spafas (Preston City, CT, USA).

### Monoclonal antibodies

SV-specific mAb<sup>24,25</sup> and IV-specific mAb<sup>26</sup> have been described. Anti-CD99 mAb 12E7 was a gift from A. Bernard (Hospital de L'Archet, Nice, France). The hybridoma-producing mAb 3G8 was given by J. Unkeless (Mt Sinai School of Medicine, New York, USA).

### Cytotoxicity assays

The cytotoxic activity of NK lines and clones against the various targets was assessed in 5-h <sup>35</sup>S-release assays<sup>27</sup>. In experiments where mAbs were included, NK cells were first incubated with 50% human serum (to prevent binding of the mAb to the various Fc receptors expressed on the surface of human NK cells) and washed. The final mAb concentration was 20 µg ml<sup>-1</sup>, or a 1:100 dilution in cases where the mAbs were present in ascites from hybridoma-bearing mice. In all experiments, the spontaneous release was less than 25% of maximal release. Each point represents the average of duplicate values. The range of the duplicates was within 5% of their mean.

### Immunoglobulin fusion proteins

The generation of CD16-Ig fusion protein has been described<sup>10</sup>. DNA fragments encoding the extracellular portions of NKp46 (isoform accession number AJ006121), KIR-1 (accession number L41267) and NKAT-8 (NM\_012314) were amplified by polymerase chain reaction (PCR) from complementary DNA isolated from human NK clones. These PCR-generated fragments were cloned into the mammalian expression vector containing the Fc portion of human IgG1, and immunoglobulin fusion proteins were produced as described<sup>10</sup>. Sequencing of the constructs revealed that the cDNAs of all immunoglobulin fusion proteins were in frame with the human Fc genomic DNA and were identical to the reported sequences. All immunoglobulin fusion proteins used in this work migrate as a single band on standard non-reduced SDS-PAGE gels, and each was regularly assayed by SDS-PAGE to ensure that the proteins had not degraded. The procedure for staining cells with immunoglobulin fusion proteins has been described<sup>10</sup>.

### Production of anti-NKp46 serum

BALB/c mice were injected in their foot pads with 40 µg of NKp46-Ig or KIR-1-Ig fusion proteins emulsified in complete Freund's adjuvant (CFA). Mice were boosted after 6 weeks, and sera were collected 12 d later. For control sera, mice were immunized as above with PBS emulsified in CFA. Sera were tested for NKp46 antigen specificity on YTS, KIR-1-transfected YTS cells, various NK lines and clones, and non-NK lines. The anti-NKp46 serum was used at a 1:100 dilution, a concentration at which binding was saturated, as measured by flow cytometry. In agreement with the pattern of expression of NKp46 reported<sup>14</sup>, only NK cells were positively stained with the anti-NKp46 serum, and control cells 721.221, RPMI 8866, Jurkat and others remained unstained. All NK cells (4 lines and more than 50 clones) were stained to various degrees with this serum. Most directly, sera

were tested on NKp46-, CD16- and CD99-stable transfectants of BW cells, and data clearly show the specificity of the anti-NKp46 serum. In addition, re-directed lysis of P815 cells could be induced when the cells were coated with the anti-NKp46 serum and incubated with various NK lines and clones.

### Blocking of NKp46-Ig binding to virus-infected cells

Cells (721.221 and 1106mel) were infected either with 100 µl of SV supernatant (for 721.221), or with 1,000 units ml<sup>-1</sup> of IV (for 1106mel). After overnight incubation, infected cells were washed and incubated with the various mAbs for 1 h on ice. Next, cells were washed and assayed for staining with 10 µg of the appropriate immunoglobulin fusion protein as described<sup>10</sup>.

### Blocking of NKp46-Ig binding using purified HA

Ten micrograms of various immunoglobulin fusion proteins were incubated with 40 µg of purified HA protein<sup>28</sup> at final volume of 100 µl PBS/BSA/azide, for 2 h on ice. These mixtures were then incubated with cells for 2 h on ice and stained for the presence of immunoglobulin fusion proteins using described staining procedures<sup>10</sup>.

### Activation of NKp46-CD3ζ chimaera

Murine BW thymoma cells (TCRαβ negative) were transfected with constructs encoding chimaeric proteins in which the extracellular portion of NKp46 or CD16 is fused to the transmembrane and tail domains of the CD3ζ chain. The cytoplasmic domain of the T-cell receptor ζ-chain is sufficient to trigger IL-2 secretion on ligation of extracellular portion<sup>29</sup>. BW and stable BW transfectants were incubated with influenza virus for 20 h, and cell supernatants were assayed for the presence of IL-2 by ELISA kit (PharMingen, San Diego, CA).

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1. Trinchieri, G. Biology of natural killer cells. *Adv. Immunol.* **47**, 187–376 (1989).
2. Lanier, L. L. NK cell receptors. *Annu. Rev. Immunol.* **16**, 359–393 (1998).
3. Ljunggren, H. G. & Karre, K. In search of the 'missing self': MHC molecules and NK recognition. *Immunol. Today* **11**, 7–10 (1990).
4. Davis, D. M. *et al.* The human natural killer cell immune synapse. *Proc. Natl Acad. Sci. USA* **96**, 15062–15067 (1999).
5. Braud, V. M. *et al.* HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391**, 795–799 (1998).
6. Colonna, M. & Samaridis, J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* **268**, 405–408 (1995).
7. Wagtmann, N. *et al.* Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity* **2**, 439–449 (1995).
8. D'Andrea, A. *et al.* Molecular cloning of NK1. A natural killer cell receptor for HLA-B allotypes. *J. Immunol.* **155**, 2306–2310 (1995).
9. Bottino, C., Biondi, R., Millo, R., Moretta, L. & Moretta, A. The human natural cytotoxicity receptors (NCR) that induce HLA class I independent NK cell triggering. *Hum. Immunol.* **61**, 1–6 (2000).
10. Mandelboim, O. *et al.* Human CD16 as a lysis receptor mediating direct natural killer (NK) cell cytotoxicity. *Proc. Natl Acad. Sci. USA* **96**, 5640–5644 (1999).
11. Pende, D. *et al.* Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J. Exp. Med.* **190**, 1505–1516 (1999).
12. Cantoni, C. *et al.* NKp44, A triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J. Exp. Med.* **189**, 787–796 (1999).
13. Biondi, R. *et al.* The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur. J. Immunol.* **29**, 1014–1020 (1999).
14. Pessino, A. *et al.* Molecular cloning of NKp46: novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J. Exp. Med.* **188**, 953–960 (1998).
15. Sivori, S. *et al.* NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur. J. Immunol.* **29**, 1656–1666 (1999).
16. Alsheikhly, A. *et al.* Sendai-virus-induced cell-mediated cytotoxicity *in vitro*. The role of viral glycoproteins in cell-mediated cytotoxicity. *Scand. J. Immunol.* **17**, 129–138 (1983).
17. Alsheikhly, A. R., Orvell, C., Andersson, T. & Perlmann, P. The role of serologically defined epitopes on mumps virus HN-glycoprotein in the induction of virus-dependent cell-mediated cytotoxicity. Analysis with monoclonal antibodies. *Scand. J. Immunol.* **22**, 529–538 (1985).
18. Maisner, A. & Herrler, G. Membrane cofactor protein with different types of N-glycans can serve as measles virus receptor. *Virology* **210**, 479–481 (1995).
19. Varki, A. Sialic acids as ligands in recognition phenomena. *FASEB J.* **11**, 248–255 (1997).
20. Weis, W. *et al.* Structure of the influenza virus hemagglutinin complexed with its receptor, sialic acid. *Nature* **333**, 426–431 (1988).
21. De Lau, W. B., Kuipers, J., Voshol, H., Clevers, H. & Bast, B. J. HB4 antibody recognizes a carbohydrate structure on lymphocyte surface proteins related to HB6, CDw75, and CD76 antigens. *J. Immunol.* **150**, 4911–4919 (1993).
22. Pinola, M., Renkonen, R., Majuri, M. L., Tiisala, S. & Saksela, E. Characterization of the E-selectin ligand on NK cells. *J. Immunol.* **152**, 3586–3594 (1994).
23. Mandelboim, O. *et al.* Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen C alleles and also occurs with empty major histocompatibility complex molecules. *J. Exp. Med.* **184**, 913–922 (1996).
24. Peterhans, E., Bachi, T. & Yewdell, J. W. Evidence for different receptor sites in mouse spleen cells for the SV hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. *Virology* **128**, 366–376 (1983).
25. Yewdell, J. W. & Gerhard, W. Delineation of four antigenic sites on a paramyxovirus glycoprotein via which antibodies mediate distinct antiviral activities. *J. Immunol.* **128**, 2670–2675 (1982).

26. Yewdell, J. W., Gerhard, W. & Bachi, T. Monoclonal anti-hemagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid activation of influenza virus A/PR/8/34-mediated hemolysis. *J. Virol.* **48**, 239–248 (1983).
27. Porgador, A., Mandelboim, O., Restifo, N. P., Strominger, J. L. Natural killer cell lines kill autologous beta2-microglobulin-deficient melanoma cells: implications for cancer immunotherapy. *Proc. Natl Acad. Sci. USA* **94**, 13140–13145 (1997).
28. Brand, C. M. & Skehel, J. J. Crystalline antigen from the influenza virus envelope. *Nature* **238**, 145–147 (1972).
29. Irving, B. A. & Weiss, A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell* **64**, 891–901 (1991).

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## Identification of CRE1 as a cytokinin receptor from *Arabidopsis*

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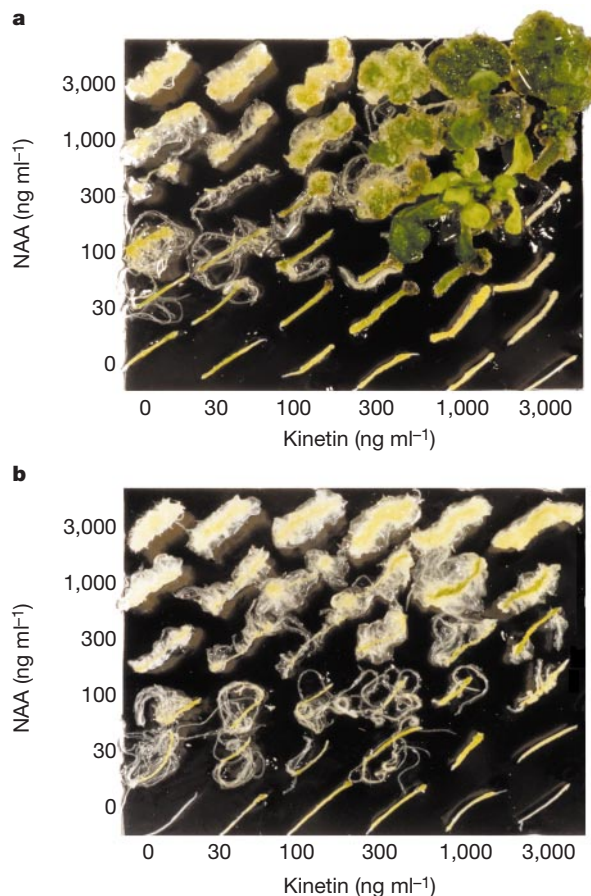
Cytokinins are a class of plant hormones that are central to the regulation of cell division and differentiation in plants<sup>1,2</sup>. It has been proposed that they are detected by a two-component system, because overexpression of the histidine kinase gene *CKII* induces typical cytokinin responses<sup>3</sup> and genes for a set of response regulators of two-component systems can be induced by cytokinins<sup>4,5</sup>. Two-component systems use a histidine kinase as an environmental sensor and rely on a phosphorelay for signal transduction. They are common in microorganisms, and are also emerging as important signal detection routes in plants<sup>6–9</sup>. Here we report the identification of a cytokinin receptor. We identified *Arabidopsis cre1* (cytokinin response 1) mutants, which exhibited reduced responses to cytokinins. The mutated gene *CRE1* encodes a histidine kinase. *CRE1* expression conferred a cytokinin-dependent growth phenotype on a yeast mutant that lacked the endogenous histidine kinase *SLN1* (ref. 10), providing direct evidence that *CRE1* is a cytokinin receptor. We also provide evidence that cytokinins can activate *CRE1* to initiate phosphorelay signalling.

Generally, cytokinins induce cell division, chloroplast development and formation of shoots (buds)<sup>1</sup>. We screened mutagenized *Arabidopsis* for mutants that were impaired in cytokinin responses, including rapid cell proliferation and shoot formation in tissue culture. We isolated a mutant designated *cytokinin response 1-1* (*cre1-1*). We tested the responses of *cre1-1* to auxin and cytokinin in tissue culture, using naphthalene acetic acid (NAA) as an auxin and kinetin as a cytokinin (Fig. 1). Wild-type explants responded to increasing levels of kinetin with rapid proliferation, greening and formation of shoots (Fig. 1a). By contrast, such cytokinin responses were not evident in *cre1-1* (Fig. 1b). The mutant was also less

responsive to other cytokinins, including *trans*-zeatin, isopentenyladenine, benzyl adenine and the phenylurea-type synthetic cytokinin thidiazuron (see Supplementary Information).

Next we tested the responses of *cre1-1* to various plant hormones in a root elongation assay. External application of cytokinins<sup>11</sup>, ethylene<sup>12</sup>, auxins<sup>13</sup> or abscisic acid<sup>14</sup> inhibits root elongation. The root of the *cre1-1* mutant was less sensitive to benzyl adenine than that of wild-type plants, but it responded normally to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the auxin indole-3-acetic acid (IAA) (Fig. 2a–c). The responses of *cre1-1* to low levels of abscisic acid (ABA) were slightly higher than normal (Fig. 2d). The cytokinin responses of *cre1-1* heterozygotes were intermediate between those of *cre1-1* homozygotes and the wild type (see Supplementary Information).

We mapped the *CRE1* locus to the top of chromosome 2 between the *rga* and *nga1145* markers (see Supplementary Information). We searched the genome sequence of *Arabidopsis* between these markers for genes that could code for proteins involved in signal transduction. Among them was the hypothetical gene *At2g01830*, possibly coding for a histidine kinase. The nucleotide sequence of *At2g01830* revealed that this gene was mutated in the *cre1-1* mutant. Hereafter we refer to this gene as *CRE1*. *CRE1* is identical to *WOL*<sup>15</sup> (see below) and *AHK4* (ref. 16). A genomic fragment containing *CRE1* was introduced into *cre1-1* mutant calli. Wild-type calli that had been transformed with the control vector regenerated shoots when cultured in the presence of the



**Figure 1** Callus growth of the cytokinin-resistant mutant *cre1-1* in different auxin and cytokinin concentrations. Hypocotyl segments were excised and cultured on media containing different levels of kinetin and NAA. After 21 days in culture, the induced calli were arranged and photographed. Wild-type explants (a) proliferated rapidly, turned green, and produced shoots in the presence of high concentrations of cytokinins. The *cre1-1* explants (b) did not.