Modulation of NKp30- and NKp46-Mediated Natural Killer Cell Responses by Poxviral Hemagglutinin

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Abstract

Natural killer (NK) cells are an important element in the immune defense against the orthopox family members vaccinia virus (VV) and ectromelia virus (ECTV). NK cells are regulated through inhibitory and activating signaling receptors, the latter involving NKG2D and the natural cytotoxicity receptors (NCR), NKp46, NKp44 and NKp30. Here we report that VV infection results in an upregulation of ligand structures for NKp30 and NKp46 on infected cells, whereas the binding of NKp44 and NKG2D was not significantly affected. Likewise, infection with ectromelia virus (ECTV), the mousepox agent, enhanced binding of NKp30 and, to a lesser extent, NKp46. The hemagglutinin (HA) molecules from VV and ECTV, which are known virulence factors, were identified as novel ligands for NKp30 and NKp46. Using NK cells with selectively silenced NCR expression and NCR-CD3ε reporter cells, we observed that HA present on the surface of VV-infected cells, or in the form of recombinant soluble protein, was able to block NKp30-triggered activation, whereas it stimulated the activation through NKp46. The net effect of this complex influence on NK cell activity resulted in a decreased NK lysis susceptibility of infected cells at late time points of VV infection when HA was expression was pronounced. We conclude that poxviral HA represents a conserved ligand of NCR, exerting a novel immune escape mechanism through its blocking effect on NKp30-mediated activation at a late stage of infection.

Introduction

Vaccinia virus (VV) is an extensively studied, prototypic member of the Poxviridae family. It is a large virus with a double-stranded DNA genome of ~200 kbp encoding ~250 genes [1]. VV has a broad cellular tropism and infects almost any cell line in culture [1]. VV is highly immunogenic and has been successfully used to vaccinate against smallpox [2]. Vaccinia-derived vectors have also been extensively used as expression vectors for foreign genes and as recombinant vaccines [3]. In spite of various immune evasion mechanisms [4,5], VV and other poxviruses elicit strong humoral and cellular immune responses [6–10].

Natural killer (NK) cells play an important role in protective immune responses against VV [6,11,12] and the ectromelia mousepox virus (ECTV) [13,14]. Interferon(IFN)-γ secretion by NK and non-NK cells appears to be involved in the antiviral effect [6,14,15]. Type I interferons are essential for the activation of NK cells against VV [16,17]. Recently, it has been reported that VV infection induces ligands for the activating natural cytotoxicity receptors (NCR), NKp46, NKp44 and NKp30, and increases susceptibility to lysis by NK cells [18]. VV-induced NCR ligand(s) were described to appear early during infection but have not been identified on a molecular level. Furthermore, it was shown that the activating NK cell receptor NKG2D is involved in the NK-cell mediated resistance to poxvirus disease in C57BL/6 mice [19]. Expression of NKG2D ligands was reported to be enhanced by ECTV infection [19].

The functions of NK cells are regulated through a balance of activating and inhibitory signals, which are transmitted through particular receptors binding cytokines or ligand structures on interacting target cells and pathogens [20,21]. Most inhibitory receptors recognize particular MHC class I isoforms and thereby ensure tolerance of NK cells against self antigens [22]. CD16, NKG2D, the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46, as well as Nkp80, DNAM-1, and various costimulatory receptors are involved in the activation of human NK cells [20,21].

NCR are important activating receptors for the anti-tumor and anti-viral activity of NK cells [20,21,23]. Heparan sulfate proteoglycans have been described as ligand structures for NKp46, NKp44 and NKp30 [24–26]. Nuclear factor BAT3, which is released from tumor cells under stress conditions, and a
Author Summary

Natural killer (NK) cells, which belong to the innate immune system, play a critical role in the defence against viruses. The orthopoxvirus family member vaccinia virus is not only used for vaccinations of humans, but is also presently considered as a promising agent for oncolytic virotherapy of tumors. It is, therefore, of importance to better understand mechanisms involved in recognition and lysis of vaccinia virus-infected cells by NK cells. In this study, we have identified the hemagglutinin molecule from vaccinia virus and the related mousepox virus as a novel interaction partner for two activating receptor molecules on NK cells. As one of these receptors was blocked by hemagglutinin, vaccinia-infected cells were less effectively killed by human NK cells than uninfected cells. Our findings support the use of hemagglutinin-deficient VV variants for oncolytic therapy.

Results

Poxviral infection induces ligands for NKp30 and NKp46

Using soluble NCR ectodomains fused to the Fc portion of human IgG1 [33] for immunofluorescence stainings, we observed that upon infection of the human cancer line HeLa with the poxviruses, vaccinia virus (VV) or ectromelia virus (ECTV), surface expression of ligand structures for the NK receptors NKp30 and NKp46 was strongly induced as compared with uninfected cells (Figure 1A,B). The efficiency of infection was assessed using the monoclonal antibody (mAb) VI-4G9 to VV HA, which cross-reacted with the closely related, ECTV-derived HA molecule. NKp30 and NKp46 ligands were more markedly induced by VV than by ECTV infection. Poxviral infection, however, only weakly enhanced the surface expression of ligands for NKp44. Similar results were obtained when infecting other human carcinoma cell lines such as A549 and PANC-1 or melanoma lines with VV or ECTV (see below and data not shown). We also confirmed previous findings [18], that VV infection induced NKp30 and NKp46 ligand expression in MRC-5 human fetal lung fibroblasts as well as in primary, human foreskin fibroblasts (Figure S1), noted, however, that the permissivity to VV infection as judged by HA expression, and the upregulation of NKp30/NKp46 ligands was much lower than in HeLa cells used in the same experiment. The murine lung tumor line TC-1 was labeled by NKp30-Fc and NKp46-Fc after VV infection (Figure 1C), too, suggesting that involved ligand structures were of viral origin rather than being human host cell-derived proteins.

HA-deficient VV fails to induce NCR ligands

When analyzing VV deletion mutants we observed that deletion of the HA gene in VV resulted in a complete abrogation of the NKp30-Fc and NKp46-Fc surface stainings by infected cells (Figure 2A), indicating that either HA alone represented the ligand for NKp30 and NKp46, or constituted part of a complex ligand structure. Reinserion of the HA genes from VV or ECTV into this HA-deficient VV mutant rescued the expression of NKp30 and NKp46 ligands in HeLa cells as shown by infection with such revertant viruses (Figure 2A). The efficiency of infection by the HA-deficient mutant virus was monitored by EGF, which was introduced into the HA locus of VV strain WR, and expression levels of reintroduced HA were monitored using the anti-HA mAb VV1-I4G9. In accordance with the comparatively weaker staining of ECTV-infected cells (Figure 1B), infection with the VV(WR);HA-HA/ECTV/flag revertant virus reconstituted the staining by NKp30-Fc and NKp46-Fc less efficiently than VV(WR);HA-HA/HA/flag, suggesting that HA from VV was more efficiently bound by soluble NCR than HA from ECTV. The nonmembrane-bound viral serine protease inhibitor SPI-3 is associated with HA on the plasma membrane of infected cells [42]. To analyze whether SPI-3 contributed to the ligand structure we analyzed the staining of HeLa cells infected with ΔSPI-3 and ΔHA/ΔSPI-3 VV mutants. While the ΔHA/ΔSPI-3 double mutant produced a complete loss of NKp30 and NKp46 staining similar to the ΔHA mutant, deletion of SPI-3 alone had no effect on NCR binding. Thus, SPI-3 does not appear to be part of the NCR ligand structure. Bimodal stainings for HA, NKp30-Fc and NKp46-Fc in Figure 2B were likely due to a non-synchronized VV infection in this experiment resulting in different HA expression levels in two subpopulations. Staining of VV-infected cells by NKp44-Fc was reduced to background levels after infection with HA-deficient VV suggesting that the weak binding of this NCR was also HA-dependent.

To obtain additional evidence for the involvement of HA in binding of NKp30 and NKp46, HeLa cells were infected with VV strain WR in the absence or the presence of the viral replication inhibitor cytotoxic arabinoside (AraC). AraC treatment efficiently reduced expression of the early and late-phase product HA [43], which was accompanied by a major reduction of the reactivity of infected cells with NKp30 and NKp46 (Figure 2C). Here we compared for the first time the canonical long ectodomain of NKp30 (contained in the 1C7a, b and c isoforms of NKp30) with the short isoform that lacks 25 amino acids within the Ig-like domain (contained in the isoforms 1C7d, e and f). NKp30(short)-Fc showed a weaker reactivity with VV-infected cells than NKp30(long)-Fc, but also its binding was strongly diminished by AraC treatment (Figure 2C).
Poxviral HA binds Nkp30 and Nkp46

As shown in Figure 3A, the preincubation of VV-infected HeLa cells with an excess of the anti-HA mAb VVI-4G9 fully blocked the stainings with NKp30-Fc and NKp46-Fc soluble receptors. Also, the weak binding of NKp44-Fc was cancelled by anti-HA. The binding of NKG2D-Fc, which was used as a control, was not affected by preincubation with anti-HA (Figure 3A). In the same line, preincubation of NKp30-Fc with soluble monomeric HA ectodomains from VV reduced their reactivity with VV-infected PANC-1 cells (Figure 3B). This is probably due to complex formation and saturation of HA binding sites in NKp30-Fc chimeras preventing subsequent binding to cell-expressed HA. Furthermore, preincubation of NKp30-Fc with soluble monomeric HA ectodomains from VV reduced their reactivity with VV-infected PANC-1 cells (Figure 3B). This is probably due to complex formation and saturation of HA binding sites in NKp30-Fc chimeras preventing subsequent binding to cell-expressed HA.

While VV infection of HeLa/B7-H6 transfectants resulted in an enhanced labeling with NKp30, the B7-H6 surface expression of infected cells, which was detected with a novel B7-H6 reactive mAb, was even slightly reduced (Figure 3C). These results indicate that poxviral HA and B7-H6 molecules are independent ligands for NKp30. The nuclear protein BAT3 has been reported to mediate NKp30 binding after stress-induced transfer to the cell surface [27]. While we detected a slightly increased surface staining for BAT3 in VV-infected HeLa cells, the BAT3 staining of VV:HA-infected cells was reduced (Figure 3B). Thus, a potential BAT3-mediated triggering of NKp30 is unlikely to explain the enhanced cytolytic activity against VV:HA-infected cells (see below).

To study NCR binding to poxviral HA independent of other viral proteins, HeLa cells were stably transfected with HA(VV) and HA(ECTV) genes (Figure 3D). We observed increased binding of NKp30-Fc and NKp46-Fc to HA-expressing HeLa cells. The enhancement of NCR binding was, however, limited, which was likely due to ~10 times lower cell surface expression levels of transfected HA as compared with VV-infected cells.

In enzyme-linked immunosorbent assays (ELISA), NKp30-Fc and NKp46-Fc also reacted with plate-bound VV particles in a HA-dependent manner but did not recognize HA-deficient VV particles (Figure S2). Moreover, soluble recombinant HA(VV) and HA(ECTV) molecules adsorbed to ELISA plates were recognized by NKp30-Fc and NKp46-Fc.
Figure 2. Increased binding of NKp30-Fc and NKp46-Fc is dependent on HA expression. (A) HeLa cells were infected for 18 h with wild-type VV(WR) (black), ECTV (gray), a HA-deletion mutant of VV (pink) as well as with this ΔHA mutant reconstituted with the HA genes from VV(WR) (green) and ECTV (orange). Stainings of uninfected cells are shown as filled gray histograms. Infection efficiencies were monitored by staining against the late-phase viral product HA (top row) or EGFP (second row), which was inserted into the HA locus of the ΔHA mutant. Infected and uninfected cells were labelled with NKp30-Fc, NKp46-Fc and NKp44-Fc. (B) Stainings with anti-HA and NCR-Fc chimeras as in A. Cells were infected with wild-type VV (gray), VV:ΔSPI-3 (black), VV:ΔHA (pink), and VV:ΔHA/ΔSPI-3 (green) mutant viruses, respectively. (C) Analysis of NCR binding to HeLa cells infected with VV(WR) for 18 h in the absence (gray lines) or presence (black lines) of 100 μg/ml cytosine arabinoside (AraC). AraC prevents entry into the late phase of the viral replication cycle. Uninfected (filled curves) and infected cells were reacted with anti-HA, NKp46-Fc, NKp44-Fc, or chimeric receptors containing the long or the short ectodomain of NKp30 fused to hIgG1-Fc as indicated.

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Role of NCR-linked glycans and glycosylated proteins on VV-infected and uninfected cells

An O-linked carbohydrate attached to Thr 225 has been described to regulate the binding of NKp46 to influenza virus HA [44]. Moreover, desialylation abolished the binding of NKp46 and NKp44 ectodomains to Sendai and Newcastle disease virus hemagglutinin-neuraminidase [30,31,33]. To better understand molecular mechanisms involved in the recognition of VV-infected cells by NKp30 and NKp46, we studied the involvement of N- and O-linked glycans in this interaction (Figure S2 and data not shown), suggesting that NKp30 had a higher affinity for HA than NKp46. The ELISA results fully supported the notion that HA serves as NCR ligand structure.

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Analysis of NKGD2 ligands

Previously it has been reported that the expression of ligands for the activating NK receptor NKGD2 was not affected by VV infection of human fetal foreskin fibroblasts [18]. In this work, we essentially confirmed this finding by using NKGD2-Fc chimeric soluble receptors and VV-infected HeLa cells. A detailed analysis of the NKGD2 ligands MICA, MICB, ULBP1, ULBP2, ULBP3, and ULBP4 revealed, however, previously unnoted differential down- or upmodulation of these ligands (Figure S4). In contrast to VV infection, expression of HeLa cells with ECTV infection of mouse cells [19].

VH infection and HA expression modulates NK lysis susceptibility and cytokine secretion

Next we assessed the functional impact of VH infection on the susceptibility to lysis by primary NK cells from different donors. This is a complex issue due to the involvement of several activating and inhibitory receptors on responding NK cells. A representative assay shows the lysis of HHF(WR)- and VH(WR,DHA)-infected HeLa cells side by side and after different periods of VH infection (Figure S5A, HHF expression in the course of infection and, in case of VH:DHA, expression of EGFP were monitored along with the infection time-dependent binding of NKp30-Fc and NKp46-Fc (Figure S5A). NK cells from donor #2 expressed higher levels of NKp30, NKp46 and NKp44, but lower levels of NKGD2, than NK cells from donor #1 (Figure S5B). This phenotype correlated with a more efficient lysis of infected and VH-infected HeLa cells by NK cells from donor #2 (Figure S5A). At time points of infection (12 h, 18 h), when HA expression was high (Figure S5A), wild-type

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VV infection resulted in an inhibition of lysis as compared with uninfected or VV:ΔHA-infected targets, suggesting that high HA expression levels had an inhibitory rather than an activating effect on NK cells. At 6 h post infection with wild-type VV, and at 6 and 12 h post infection with VV:ΔHA, we observed a slightly enhanced lysis susceptibility compared with uninfected targets, suggesting that HA-independent early viral factors promoted lysis susceptibility. These assumptions were confirmed in experiments using AraC to block the entry into the late phase of viral replication. AraC treatment during VV infection diminished HA

**Figure 4. Deglycosylation differentially influences the binding of NKp30-Fc and NKp46-Fc.** (A) Uninfected HeLa cells and VV(WR)-infected HeLa cells were stained with anti-HA to monitor infection efficiency. Uninfected (gray lines) and infected (black lines) were labelled with NKp30-Fc (left column) and NKp46-Fc (right column) fusion proteins, that were N-deglycosylated in vitro using protein N-glycanase F (PNGase) for 16 h, or treated with α2-3,6,8-neuraminidase (NAase) for 1 h, or with a cocktail of O-deglycosylating enzymes (α2-3,6,8-neuraminidase, β1,4-galactosidase, endo-α-N-acetylgalactosaminidase, β1,2,3,4,6-N-acetylglucosaminidase) for 1 h at 37°C, respectively. (B) Western blots of enzymatically deglycosylated NKp46-Fc and NKp30-Fc fusion proteins that were detected by NKp30- and NKp46-specific mAbs.

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Figure 5. Reduced lysis susceptibility of target cells after infection with wild-type VV. (A) Cytotoxicity assay using primary NK cells from two different donors as effectors and HeLa cells as targets. HeLa cells were either left uninfected or infected with wild-type VV(WR) or HA-deficient VV(WR) for 6 h, 12 h and 18 h, respectively, and labeled with $^{51}$Cr. Different effector-to-target cell (E:T) ratios are shown. HA surface expression of VV-infected cells, NCR-Fc binding to wild-type VV and VV:ΔHA infected cells as well as the expression levels of NKp30, NKp46, NKp44 and NKG2D on NK cells are shown.
expression and NCR binding (see Figure 2C). While VV infection resulted in a reduced lysis of HeLa cells by the human cell line NK-92 and by primary NK cells, infection with VV:ΔHA or infection with VV in the presence of AraC enhanced the lysis susceptibility as compared with uninfected targets (Figure 5B).

To further investigate the potential inhibitory function of poxviral HA on NK cells, we utilized HeLa and MaMel-8A cells transfected with either HA(VV) or HA(ECTV) in cytotoxicity assays with NK-92 or primary NK cells from different donors. As compared with vector control transfectants, ectopic expression of HA(VV) or HA(ECTV) by these tumor cell lines (see Figure 3D) correlated with a reduced kill by NK-92 and primary NK cells (Figure 5C). In the same line, inclusion of soluble recombinant HA(VV) in the cytotoxicity assay reduced the lysis of uninfected and VV-infected HeLa cells (Figure 5A). Conversely, the preincubation of VV-infected, but not uninfected, HeLa cells with anti-HA mAb enhanced their lysis by primary NK cells (Figure 5B). A partial rescue of the lysis susceptibility of VV-infected targets was achieved when the target cells were preincubated with soluble NKP30-Fc (Figure 5C), but not with NKP46-Fc (data not shown). Furthermore, a blocking anti-NKP30 mAb added to the assay mixture partially inhibited the lysis of uninfected or VV:ΔHA-infected, but not VV-infected targets (data not shown), suggesting that in the latter case, HA(VV) had outcompeted antibody binding to NKP30 on NK cells.

In addition, we studied the effect of VV infection on the secretion of the effector cytokines TNF-α and IFN-γ by primary IL-2 activated NK cells after cocultivation with VV-infected cells. HeLa stimulator cells were either left uninfected or infected with wild-type VV(WR) or VV(WR):ΔHA, respectively, before UV irradiation to prevent viral spread to responding NK cells. Coculture of NK cells with UV-irradiated, uninfected HeLa cells stimulated the secretion of TNF-α into the culture supernatant, even though they underwent UV-induced apoptosis. VV:ΔHA-infected HeLa also enhanced TNF-α production, whereas VV-infected HeLa cells blocked TNF-α secretion (Figure 7A). Primary NK cells cultured alone, or cocultured with uninfected HeLa cells, produced large amounts of IFN-γ (Figure 7B). Coculture with VV:ΔHA-infected HeLa cells reduced IFN-γ secretion to ~80% and coculture with wild-type VV-infected HeLa cells to ~30% of these levels. We conclude that HA-proficient VV is able to inhibit the secretion of effector cytokines by NK cells.

**NCR triggering is differentially affected by HA and VV infection**

We sought to characterize the individual contributions of the three NCR to lysis of VV-infected targets. To this end, we transfected the NK cell line NK-92 with lentiviral vectors encoding shRNAs specific for NKP30, NKP46 and NKP44. NK-92 cells express significant levels of NKP30, NKP46 and NKP44, yet low levels of NKP46 (Figure 6A). The successful downmodulation of NCR expression in stably shRNA-transfected NK-92 cells was demonstrated by staining with anti-NCR antibodies (Figure 6A). In the same way, off-target effects of shRNA transfection on the expression levels of non-targeted NCR and NKG2D were excluded. NK-92 lines with silenced NCR expression were used in cytotoxicity assays with uninfected and VV-infected HeLa/B7-H6 targets (Figure 6B). Consistent with B7-H6 being a major cellular ligand for NKP30, we found that knockdown of NKP30 significantly reduced the lytic capacity against uninfected HeLa/B7-H6. NKP46-silenced NK-92 exerted a reduced kill of uninfected HeLa/B7-H6 suggesting that a NKP46 ligand was expressed by these targets. NKP44 silencing reduced the lytic activity only slightly. VV infection resulted in a strongly reduced lysis of HeLa/B7-H6 by untransduced NK-92 (Figure 6B). Silencing of NKP30 partially rescued this reduced lysis indicating that it was, in part, caused by inhibition through the NKP30 receptor. By contrast, NKP46 shRNA transfection abolished the lysis of VV-infected cells suggesting that cytotoxicity was triggered by NKP46. Also NKP44-silenced NK-92 showed a reduced kill of VV-infected HeLa/B7-H6 targets as compared with regular NK-92. VV-infected and uninfected HeLa/CLEC12B control transfectants as well as BaF3/B7-H6 targets, which were not sufficiently infectable by VV, showed similar patterns of lysis susceptibility to NCR-silenced NK-92 effector cells (Figure 6C).

NKP30, NKP46 and NKP44 have been reported to occur in molecular complexes facilitating synergistic signaling [48]. This cross-talk might obscure the functional outcome of individual interactions in trans between HA and NCR. To selectively study triggering by NKP30, NKP46 and NKP44 receptors we utilized LacZ-inducible BWZ.36 NCR-CD3 reporter cells as previously published by us [33]. Infection of HeLa cells with VV, but not VV:ΔHA, blocked the basic stimulation of BWZ.36/NKP30− cells by uninfected HeLa cells (Figure 7A). By contrast, VV:ΔHA cells blocked the basic stimulation of BWZ.36/NKP30− reporter cells (Figure 7B). In the same way, infection with VV:ΔHA induction of BWZ.36/NKP46− reporter cells was minor (Figure 7A). We also used HeLa cells stably transfected with HA from VV or ECTV and vector control transfectants as stimulator cells. HA expression inhibited the activation of NKP30− and stimulated the activation of NKP46− cells, whereas we noted no significant HA-specific response by NKP44− cells (Figure 7C). In another approach, we plate-coated anti-NCR antibodies to provide for a basic stimulation of BWZ.36/NCR− reporter cells. Crude plasma membrane preparations from VV-infected, VV:ΔHA-infected and uninfected HeLa cells were added to the assay wells. As shown in Figure 7D, membranes from VV-infected cells interfered with the activation of NKP30− while they acted cooperatively with anti-NKP46 for the activation of NKP46− reporter cells. Membranes from VV:ΔHA-infected and uninfected HeLa cells had no effect. Likewise, solubilized recombinant HA(VV)-Fc partially blocked the activation of NKP30− cells by anti-NKP30, but enhanced the activation of NKP46− cells (Figure 7E). Surface-adsorbed HA(VV)-Fc stimulated NKP46−, but not NKP30− or NKP44− reporter cells (Figure 7E). An inhibition by supernatants from VV-infected cells was also seen when HeLa/B7-H6 cells were used for the activation of NKP30− reporter cells.
NK Cell Activity Altered by Vaccinia Hemagglutinin

A

anti-NKG2D

anti-NKp30

anti-NKp46

anti-NKp44

NK-92

NK-92/NKp30 shRNA

NK-92/NKp46 shRNA

NK-92/NKp44 shRNA

B

% Specific lysis

NK-92

NK-92 shRNA NKp30

NK-92 shRNA NKp46

NK-92 shRNA NKp44

HeLa/B7-H6

NK-92

NK-92 shRNA NKp30

NK-92 shRNA NKp46

NK-92 shRNA NKp44

HeLa/B7-H6 VV-infected

C

% Specific lysis

NK-92

NK-92 shRNA NKp30

NK-92 shRNA NKp46

NK-92 shRNA NKp44

HeLa/CLEC12

NK-92

NK-92 shRNA NKp30

NK-92 shRNA NKp46

NK-92 shRNA NKp44

HeLa/CLEC12 VV-infected

NK-92

NK-92 shRNA NKp30

NK-92 shRNA NKp46

NK-92 shRNA NKp44

BaF3/B7-H6

* * ** **
NK Cell Activity Altered by Vaccinia Hemagglutinin

Figure 6. NKp30, NKp46 and NKp44 are differentially involved in the lysis of VV-infected targets. (A) Cytofluorometric analysis of NK-92 cells after retroviral transduction with vectors expressing shRNAs specifically silencing NKp30, NKp46 or NKp44 expression. PE-conjugated anti-NCR mAbs as well as an anti-NKGD2-PE mAb were used for the staining of normal and shRNA-transduced NK-92 cells. (B) Cytotoxicity assay using NCR-silenced and normal NK-92 cells as effectors as indicated. Uninfected and VV-infected HeLa/B7-H6 cells were used as targets. Different E:T ratios are shown. (C) Cytotoxicity assay using NCR-silenced and normal NK-92 cells as effector cells as indicated. Uninfected and VV-infected HeLa/CLEC8B as well as uninfected BaF3/B7-H6 cells were used as targets. Statistical comparison of kills with shRNA-transduced versus normal NK-92 cells was done by Student’s t-test (paired, 2-tailed). *, p<0.05, **, p<0.01.
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Discussion

Natural killer cells represent an important element in protective immune responses against vaccinia and mousepox virus. This is mainly evidenced by mouse models in which NK cells were depleted by anti-asialo-GM1 antibodies [6,11,13,14,19,49]. Low NK cell cytotoxicity in humans is associated with an increased susceptibility towards severe and recurrent herpesvirus infections [50], whereas little is known about the role of human NK cells in the defense of poxviral infections.

VV infection sensitizes human tumor cell lines and autologous T cells for NK-mediated lysis in vitro [51]. More recently, it has been shown that VV-infected human fibroblasts are recognized by NK cells through the NCR NKp46, NKp44 and NKp30 [18]. VV-induced NCR ligand structures were, however, not identified in that study. Genetically engineered forms of the highly immunogenic poxvirus VV have been successfully employed for vaccinations of humans [3,52]. Moreover, attenuated VV strains are presently considered for oncolytic virotherapy in humans [41]. Therefore, it is of importance to identify NK cell ligand structures induced by poxviruses, particularly in the course of VV infection.

In this study we have identified VV hemagglutinin as a novel virus-encoded ligand for the activating NK cell receptors NKp30 and NKp46. Several lines of evidence presented herein led to this conclusion; i, the induction of ligand structures on VV-infected human carcinoma cells, as detected by staining with soluble recombinant NKp30 and NKp46 receptors, was fully abolished after deletion of HA in the viral genome and reconstituted after reintersion, ii, anti-HA antibodies blocked the enhanced staining of VV-infected cells by NKp30- and NKp46-Fc, iii, soluble recombinant HA interfered with the binding of NKp30 to VV-infected cells, to VV particles, and to its cellular ligand B7-H6; and iv, HA-transfected cells showed an increased reactivity with NKp30-Fc and NKp46-Fc. Furthermore, the binding of NKp30 and NKp46 to plate-bound VV, but not VV:HA particles, as well as the blocking effects of anti-HA antibodies and soluble recombinant HA, confirmed the staining of VV-infected cells. In the same line, infection of HeLa cells with the mouse poxvirus ECTV resulted in a significant induction of ligands for NKp30 and NKp46. HA(ECTV) reconstituted NKp30 and NKp46 binding either after infection with a VV:HA(ECTV) virus revertant (see Figure 2A) or in HeLa cells stably expressing HA(ECTV) (see Figure 3D). Our results indicate that the HA molecule from ECTV also serves as a ligand for human NKp30 and NKp46, however, a HA deletion mutant of ECTV still needs to be investigated. Mouse NK cells express NKp46 [53], but not NKp30 [54]. It will be interesting to analyze in future studies whether ECTV-infected cells are recognized by the murine homologue of NKp46. Furthermore, the possible interactions of human NKp30 and NKp46 with variola virus HA, whose extracytoplasmic domain shares ~84% amino acids with vaccinia HA, will be of interest since variola is able to establish a systemic infection of immunocompetent human hosts and might thus be able to overcome a first-line defence by NK cells more readily.

In accordance with a previous study using human fibroblasts for infection [18], we detected an induction of NKp44-Fc binding after infection with VV or ECTV (see Figure 1), which was, however, weak as compared with the staining of infected cells by NKp30-Fc and NKp46-Fc. This slightly increased binding of NKp44-Fc was not observed when cells were infected with the HA-deficient mutants VV:ΔHA and VV:ΔHA/ΔSPI-3. Therefore, it seems possible that poxviral HA represents a minor ligand for NKp44 as well. Binding of NKp44-Fc to viral particles in ELISA and to HA-transfected HeLa cells was, however, negligible. The latter result may be explained by the comparatively low expression levels of HA(VV) and HA(ECTV) on the surface of HA-transfected cells.

In keeping with previous work [18], the entirety of NKG2D ligands as detected by NKG2D-Fc soluble receptors were not significantly altered after VV infection. Upon detailed inspection we noted, however, a differential behavior of the six NKG2D ligands tested, with ULBP1, ULBP2 and ULBP4 being slightly upregulated, MICA and ULBP3 unaltered, and MICB slightly downregulated. Presently, we have no explanation for this complexity. Here we show that ECTV infection leads to a partial loss of staining with human NKG2D-Fc which is reflected in a decreased reactivity of antibodies against MICB, MICA and ULBP3 with ECTV-infected human cells. This finding contrasts with a recent report showing enhanced expression of mouse NKG2D ligands upon ECTV infection of mouse embryo fibroblasts and enhanced NKG2D-mediated cytotoxicity [19]. It is unexpected as humans are not the natural host for ECTV.

Recently, the B7-H6 molecule has been identified as cellular ligand for NKp30 [28]. The B7 family member B7-H6 contains a membrane-distal IgV-like domain and a membrane-proximal IgC-like domain. VV(HA) and NKp30 both contain a membrane-distal IgV-like domain linked to the membrane through a short glycosylated stalk region [46,55]. HA from ECTV and VV show 92.5% sequence homology within the N-terminal Ig-like domain. In light of these structural similarities it seems reasonable to assume that homotypic interactions within IgV-like domains play a role in the binding of NKp30 to B7-H6 and poxviral HA, respectively. As no Ig superfamily member has so far been reported as ligand structure for NKp46, the interaction of NKp46 with poxviral HA remains elusive at present.

Unexpectedly, we observed that after 12–18 hrs of VV infection, target cells became less susceptible to lysis by polyclonal NK cells or by the NK cell line NK-92. At early time points of infection, when HA expression was low or when the late viral phase was blocked by AraC, the susceptibility of infected HeLa cells to NK lysis was, however, slightly increased. Regarding these modulating effects of VV infection, we noted minor differences among primary NK cells from different donors. Since infection with the HA-deficient virus did not reduce lysis susceptibility as compared with uninfected targets, we reasoned that HA, which is mainly expressed as a late-phase product [43], had an inhibitory effect on NK cells, whereas some early phase products may exert a stimulatory effect on NK cells. This infection-time dependent
Figure 7. Inhibition of NKp30 triggering and enhancement of NKp46 triggering by poxviral HA. LacZ-inducible BWZ.36 reporter cells expressing the ectodomains of NKp30, NKp46, NKp44 or CD16, linked to the transmembrane and cytoplasmic domains of mouse CD3ζ, were used as indicated. Induction of LacZ expression is analyzed using the colorigenic substrate CPRG in an ELISA reader as absorption at 595 nm. (A) Reporter cells were cocultured for 18 h with uninfected HeLa cells, or HeLa cells infected with wild-type VV or HA-deficient VV (5 \times 10^4 stimulator cells/well). Uninfected and infected stimulator cells were pretreated with 254 nm UV light. (B) BWZ.36/NKp46-CD3ζ reporter cells were cocultured for 20 h with uninfected HeLa cells, or HeLa cells infected with wild-type VV or HA-deficient VV using the indicated numbers of stimulator cells. Uninfected and
infected stimulator cells were pretreated with UV light. (C) NCR reporter cells were cocultured for 21 h with HeLa cells stably transfected with either the empty pcDNA3.1+ expression vector, pcDNA3.1+/HA(WR) or pcDNA3.1+/HA(ECTV), respectively. For a negative control, BWZ.36/NCR-CD3ζ cells were cultured without stimulator cells. (D) NCR reporter cells were cultured for 18 h in 96-wells coated with either anti-NKp30, anti-NKp46 or anti-NKp44 mAbs alone, or together with UV-irradiated membranes prepared from uninfected HeLa cells, or from HeLa cells after infection with either wild-type VV or HA-deficient VV. For a negative control, BWZ.36/NCR-CD3ζ cells were cultured without prior coating of anti-NCR mAbs. (E) NKp30ζ and NKp46ζ reporter cells were cultured for 18 h in 96-wells coated solely with anti-NKp30 or anti-NKp46 mAbs, or coated with mAbs together with recombinant soluble HA(VV)-Fc fusion protein. For stimulation of reporter cells, HA(VV)-Fc was also coated alone, and non-coated wells were used for a negative control. (F) NKp30ζ and, for control, NKp44ζ reporter cells were cocultured for 18 h with HeLa/B7-H6 cells in the absence or the presence of UV-inactivated supernatants from HeLa cells after infection with either wild-type VV(WR) or VV(WR)/HA. All assays were performed in triplicates. For statistical comparisons of virus-infected cells and syncitia formation [58,59]. HA is a proviral virulence factor as HA-competent virus replicates much more rapidly in hosts than HA-deficient virus [60,61]. Our herein presented results substantiate in this study by using i, VV(WR)- and VV(WR):AHA-infected targets for NK-92 effectors, ii, using transfectants expressing HA from VV or ECTV, and iii, using anti-HA antibodies and soluble NKp30-Fc to enhance the lysis of VV-infected cells (see Figures 5 and S6). The poxviral HA(A56R) molecule, which has no lectin-binding properties and is not essential for viral infection [56], is strongly expressed on the surface of VV-infected cells and in a major proportion of EEV membranes [57]. Together with the HA-associated SPI-3 serpin (K2L), HA blocks the entry of superinfecting virus into VV-infected cells and syncitia formation [58,59]. HA is a proviral virulence factor as HA-competent virus replicates much more rapidly in hosts than HA-deficient virus [60,61]. Our herein reported finding that NK cell-mediated lysis of wild-type VV-infected and HA-transfected target cells was reduced is consistent with the higher virulence of HA-proficient VV and constitutes a novel immune escape mechanism of VV, and possibly other poxviral family members, during the late phase of infection. Our data also showed that SPI-3 was not required for the inhibitory effect of HA (see Figure 2).

Coculture of preactivated primary NK cells with VV-infected, UV-irradiated tumor cells resulted in a significant reduction of IFN-γ and TNF-α secretion compared to stimulation by uninfected, UV-irradiated cells. This functional blockade of NK cytotoxicity secretion was considerably more pronounced in the presence of HA-competent than HA-deficient VV, suggesting that HA exerted an inhibitory effect. As the inhibition of cytotoxicity secretion in vitro may require a direct contact between infected cells and NK cells, it does not exclude a systemic activation of NK cells by type I interferons in secondary lymphoid organs or other non-infected tissues [17], and is therefore not in conflict with protective NK-mediated IFN-γ responses during poxviral infection as reported earlier [6,14,15].

Using NK-92 effectors with selectively silenced NCR expression and Lucζ-inducible, NCR-CD3ζ reporter cells we dissected differential effects of VV infection and HA expression on NKp30 and NKp46 triggering. Collectively, our results clearly document an inhibition of NKp30-mediated activation and a stimulation of NKp46-mediated activation. This was observed using VV-infected cells as targets for NCR-silenced NK-92, and also when NCR-CD3ζ reporter cells were cultured with UV-inactivated infected cells, membranes or supernatants derived from infected cells, HA(VV) and HA(ECTV) transfectants, or soluble recombinant HA, respectively. Presented are the results for poxviral HA inhibitory effect on NKp30-mediated NK activation, regardless of NKp30 being present in its natural conformation on NK cells or being expressed as a CD3ζ-linked protein in BWZ.36 reporter cells. In preliminary experiments using primary NK cells, we failed to detect a reduced CD3ζ association with NKp30 after interaction with VV-infected Hela cells. Such a mechanism has been proposed for the inhibitory effect of HCMV pp65 protein on NK cells activation through NKp30 [34]. We rather assume that poxviral HA binds to NKp30 in such a way that cis-interactions of NKp30 with itself or other proteins and thereby induced conformational changes required for signal transduction may be prevented. Conversely, the interaction of HA with NKp46 appears to be productive as NKp46-CD3ζ reporter cell triggering is stimulated. Because the net effect of NK cell interaction with VV-infected cells or soluble recombinant HA appears to be inhibitory, we assume that interaction of HA with NKp30 functionally dominates the interaction with NKp46.

As cells infected with VV(WR)/HA showed an increased lysis susceptibility (see Figure 5) we searched for potential HA-independent activating ligands. The known NKp30 ligand B7-H6 could be ruled out (see Figure 3), and we also could not pinpoint MHC class I downregulation as being responsible for enhanced NK activation (unpublished results). Consistent with earlier work [62], regulators of complement activation, CD46, CD55 and CD59, were slightly enhanced after VV and VV:HA infection (unpublished results). CD59 might thus play a role for VV-enhanced, HA-independent NK cell activation [63]. The surface expression of LFA-1, LFA-3, CD44, CD24, and in particular of CD62L, molecules, was induced after VV and VV:HA infection (unpublished results). The functional significance of these findings in terms of augmented NK cell adhesion to VV-infected cells remains open at present.

In summary, the herein presented results characterize poxviral, and in particular vaccinia viral, hemagglutinin as novel ligand structures for the natural cytotoxicity receptors NKp30 and NKp46, with a possible additional involvement of NKp44. While NCR reporter cells indicated a blockade of NKp30 triggering through HA and a stimulation of NKp46 triggering, an inhibitory effect of VV infection on lysis susceptibility of cancer cells became dominant at late time points of infection when HA expression was pronounced. In poxvirus oncolytic therapy, NK cell-mediated cytotoxicity is discussed to play an important role. Our findings would support the use of HA-deficient VV variants in therapeutic approaches employing oncolytic poxviruses.
Materials and Methods

Cell lines

The human cervix carcinoma HeLa, the human pancreatic adenocarcinoma line PANC-1, the murine mastocytoma line P815, and the fetal lung fibroblast line MRC-5 were obtained from the American Type Culture Collection (ATCC). The human foreskin fibroblast line VH7 has been described [64]. B7-H6:GFp transduced HeLa and BaF3 cells are described in Supporting Information Text S1. HeLa/CLECBl2 transfectants have been described [65]. The metastatic melanoma cell line Ma-Meli-h8a [66] was a kind gift from Dr. A. Paschen, Department of Dermatology, University Clinics Essen. The human lung carcinoma line TC-1/A2 [67] was provided by Dr. A. Cid, DKFZ, Heidelberg. Cell lines were cultured in RPMI-1640 (Invitrogen, Karlsruhe, Germany) supplemented with 2 mM glutamine and 10% fetal calf serum. NK-92CI (hereafter referred to as NK-92) is a human natural killer tumor cell line that has been transfected with human IL-2 cdNA and grows independently of IL-2.

Plasmids and transfections

The hemagglutinin genes of VV strain WR and ECTV strain MP-Nu were cloned from mRNA of virus-infected cells using following primers HA(WR)-5':: GAGGAATTCCTACAAAGTCCTTGGTTTTATAA- TGGATTAGTATAAAAAGTG, and WR181-GAGGGTACCCTCGTTCTAATTGTGGGGGACTG / WR-181ATG-Nco: CTGGATCCGTATTGCTTGTTTTTGTGATTCCGGGAGGGTACCAATTGATACATTTTG, HA(ECTV)-5':: GAGGAATTCCTACAAAGTCCTTGGTTTTATAATTTACG, and inserted in frame with the FLAG epitope in the vector p7.5k-131A-FLAG. From these plasmids the coding sequences for HA(VV) and HA(ECTV)/flag and empty pCDNA3.1(+) (Invitrogen). 2.5 x 10^7 HeLa cells cultured in 6-well plates were transfected with 4 μg pCDNA3.1(+)/HA(VV)/flag, pCDNA3.1(+)/HA(ECTV)/flag or empty pCDNA3.1(+) vector and 10 μl lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 2 days, cells were selected with geneticin (1 mg/ml in Dulbecco’s phosphate-buffered saline [DPBS]; Sigma-Aldrich, Taufkirchen, Germany) and, after cell sorting for high HA expression, maintained at 0.5 mg/ml geneticin.

The cloning and expression of B7-H6 cdNA is described in Supplemental Materials and Methods (Text S1).

Preparation of VV stocks

The VV strains Western Reserve (WR), ectromelia virus (ECTV) strain MP-Nu (kindly provided by Dr. H. Ellerbrock, Robert-Koch-Institut, Berlin, Germany) were used. VV(WR) was parent of the mutants VV(WR):ΔHA, VV(WR):ΔHA-HA(VV)/flag and VV(WR):ΔHA-HA(ECTV)/flag (see Construction of VV HA mutants). Crude stocks of VV and ECTV were prepared from supernatants of sonicated infected cells. Cell debris were removed by centrifugation at 1,680 x g for 10 min. To produce purified VV stocks supernatants of infected cells were subjected to two additional centrifugation steps (1,680 x g for 10 min), followed by centrifugation through a sucrose cushion (36% sucrose, 31,000 x g, 90 min). VV titers were determined by a standard plaque assay using CV-1 cells.

The SPI-3 and HA-deficient VV mutants VV-T7ΔSPI-3, VV-ΔSPI-5 and VV-T7ΔSPI-3/ΔSPI-5 have been described [58] and were amplified and purified as described above. Following infection of HeLa cells, HA and/or SPI-3 deficiency were confirmed using the HA-specific mAb VVI-4G9 and the SPI-3-specific mAb 4A11-4A3 (see Antibodies).

Construction of VV HA mutants

The VV HA deletion mutant was cloned by replacing the HA gene with an EGFp cassette through homologous recombination. The 5' and 3' flanking regions of the VV HA gene were cloned in the pUC18 vector using the following primer pairs, WR180-kpnI: GAGGGTACCCTCGTTCTAATTGGCGGACTG / WR-181ATG-Nco: CTGGATCCGTATTGCTTGTTTTTGTGATTCCGGGAGGGTACCAATTGATACATTTTG, and WR101-BamH: GAGGATCCGGGCCGCAATTTTGGACATGCTAATGCTTGGGATAG / WR182-SphI: CTGGATCCGTATTGCTTGTTTTTGTGATTCCGGGAGGGTACCAATTGATACATTTTG. An EGFp cassette was cloned between the flanking regions. For HA deletion, African green monkey CV-1 cells (ATCC CCL-70) were infected with wild-type VV (multiplicity of infection [m.o.i.] 0.05) and after 2 h transfected with the pUC plasmid containing the EGFp gene between HA flanking regions using the SuperFect transfection reagent (Qiagen, Hilden, Germany). Infected cells were harvested 2 days later and lysates produced by freezing/thawing. CV-1 cells were infected with the lysate in limited dilution. EGFp-expressing viruses were selected in two rounds of infection. HA deficiency was confirmed by cytofluorometric analysis using the anti-HA mAb VVI-4G9.

FLAG epitope-tagged HA genes from VV and ECTV were reinserted in the thymidine kinase (Tk) locus of the VV(WR):ΔHA mutant. For homologous recombination, p7.5k-HA(WR)/flag and p7.5k-HA(ECTV)/flag plasmids containing HA cDNAs under control of the Tk promoter and homologous flanking regions of the Tk gene were used. The recombinant viruses VV(WR):ΔHA-HA(WR)/flag and VV(WR):ΔHA-HA(ECTV)/flag were selected by infecting 143 tk− cells in the presence of bromodeoxycytidine.

Viral infection of tumor cells

Tumor cells were seeded in 6-well plates at ~1 x 10^6 cells/well. Adherent cells were washed once in D-PBS and m.o.i. 4 of VV or ECTV were added in 500 μl RPMI-1640 without FCS for 1–2 h at 37° in a CO2 incubator. Medium was replaced by 2.5 ml complete RPMI-1640 medium in incubated at 37°C for up to 20 h before harvesting in D-PBS/0.05% trypsin/5 mM EDTA or enzyme-free cell dissociation buffer (Invitrogen). In some experiments, cytotoxic D-arabinofuranoside ( AraC) (Sigma-Aldrich), which prevents viral DNA replication and synthesis of late-phase proteins, was present throughout the infection period at 100 μg/ml.

Soluble recombinant HA and NCR-Fc fusion proteins

The ectodomains of HA from VV(WR) and ECTV were amplified by using the primer pairs HA(WR)-5': GAGGAAGGTACCATGC- GATCTCTCTAATATGACACGATTACCAATTGATACATTTTG / sHA(WR)-3': GAGGAATTCCTACAAAGTGCGTATTGATTCTATTTGATACATTTTG, and HA(ECTV)-5': GAGGAATTCCTACAAAGTGCGTATTGATTCTATTTGATACATTTTG / sHA(ECTV)-3': GAGGAATTCCTCTCGAAGATGTTATATATTACCTATTTGATACATTTTG and subcloned in the vector pGene/V5-His. From these plasmids, the coding sequences for V5-His-tagged, soluble HA(VV) and HA(ECTV)/flag molecules were subcloned in the plasmid pRES2-EGFP (BD Biosciences/Clontech, Heidelberg, Germany). To boost gene expression, a β-globin intron II (derived from plasmid pSG5, Stratagene, Amsterdam, The Netherlands) was inserted between the promoter and coding sequences. Supernatants of HEK293T cells transiently transfected using the SuperFect transfection reagent (Qiagen) were harvested and recombinant and HA(VV)-V5-His0 and
HA/EVT-V5-His6 proteins were purified using His-SpinTrap columns (GE Healthcare). The correct molecular weights of recombinant proteins were verified by Western blot using a V5 epitope-specific mAb (Invitrogen).

To produce recombinant, Fc-linked HA molecules, the ectodomain of VV(WR) (Met1-Val277) was excised out of pRES2-EGFP/VV(WR)-V5-His6, and cloned in frame with PCR-amplified cDNA coding for the Fc portion of mouse IgG2a (Fab’2-Ka2) (accession no. BC031470), using a short EcoRI/BamHI-flanked linker sequence (GILQPGGS) derived from pBluescript KS II+ (Invitrogen). The HA-Fc fusion protein was subcloned into the expression vector pMT2mcs for transient transfection of HEK293T cells and purification by protein A Sepharose CL-4B (GE Healthcare Life Sciences, Freiburg, Germany) as described [33,69]. The integrity of purified HA-Fc was checked on Western blot using peroxidase-labeled goat anti-mouse IgG/Fc and enhanced chemiluminescence as described [69].

NCR-Fc fusion proteins were produced in HEK293T cells as described [26,33]. Using the calcium phosphate transfection method, 293T cells were transiently transfected with NKP46-hIgG1-Fc, NKP44-hIgG1-Fc, and NKP30-hIgG1-Fc cDNAs [31] subcloned into the expression vector pMT2mcs [69]. The ectodomain of the NKP30 isoform 1C7d/e/f with a shorter IgC2-like domain (accession no. Y14768; AE65–H89/Q90E with regard to the Ig-like, V-set domain of the long isoform) has been kindly provided by Dr. Ofer Mandelboim, (Hebrew University, Jerusalem, Israel). The short NKP30 ectodomain was also Fc-linked and produced in HEK293T cells. NCR-Fc chimeric proteins were purified using protein A Sepharose CL-4B (GE Healthcare Life Sciences, Freiburg, Germany). The integrity of purified NCR-Ig fusion proteins was confirmed by Western blots using peroxidase-labeled goat anti-human IgG/Fc. Enzymatic N-/O-deglycosylations of NCR fusion proteins were done after binding to protein A Sepharose beads to allow for enzyme removal by washing. Deglycosylations were carried out as described for VV-infected and uninfected cells (Text S1), except that PBS [pH 6.0] was used as reaction buffer, and PNGase F digestions were done for 1 or 16 h at 37°C. The integrity of fusion proteins and molecular weight shifts after deglycosylation were confirmed by Western blot using mAbs specific for NKP46 and NKP30 and peroxidase-labeled goat anti-mouse IgG secondary antibodies.

Antibodies
Monoclonal antibodies (mAbs) to VVI-4G9 to and B2D10 to VV hemagglutinin were kindly provided by J.W. Hooper (Virology Division, USAMRIID, Fort Detrick, MD), and H. Shida (Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan), respectively. Rabbit polyclonal antibody to vaccinia virus was from quartett Immunodiagnostika, Berlin, Germany. The SPI-3 reactive mAb 4A11-4A3 has been described [70]. Additional antibodies specific for NK and tumor cell surface proteins and secondary antibodies are described in Supplemental Materials and Methods (Text S1).

Flow cytometry
For cell surface immunofluorescence stainings, ~0.5 × 10⁶ cells were washed once in ice-cold FACS buffer (D-PBS/2% FCS) followed by incubation with a saturating amount of the primary mouse mAb antibody (0.5–1 µg purified antibodies, 100 µl hybridoma culture supernatants) for 45 min on ice. After 2 washes, cells were incubated with PE-labeled goat anti-mouse Ig for 20 min on ice. NCR-Fc fusion proteins (1–2 µg per staining) were complexed with PE-labeled goat anti-human IgG/Fc (Dianova; 1 µl in 100 µl FACS buffer) for 30 min before addition to cells for 45 min on ice. Cells were washed twice with 1 ml FACS buffer and resuspended in 100 µl FACS buffer with 1.5 µg/ml propidium iodide (Sigma-Aldrich) to label dead cells. Cytofluorometric analyses were done using a FACS Calibur flow cytometer and CellQuest software (Becton Dickinson, Heidelberg, Germany). For all FACS stainings, representative examples from at least 3 repeats with similar results are shown.

Ethics statement
Primary human NK cells were exclusively isolated from buffy coats, which were purchased from the Institute for Clinical Transfusion Medicine and Cellular Therapy, Heidelberg, and had been made anonymous. The Ethics Commission of the University of Heidelberg permitted the use of buffy coats for research purposes without an informed consent by the anonymous blood donor.

Human primary NK cells
Human polyclonal NK cells were isolated from buffy coats using the NK cell negative isolation kit (Dyna/Invitrogen). 95-99% of the purified NK cells were CD3- /CD56+. Cells were grown in Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen), supplemented with 10% human serum, penicillin/streptomycin, 100 U/ml IL-2 (National Institutes of Health cytokine repository), 1 µg/ml phytohemagglutinin P (PHA-P) and irradiated JY lymphoblastoid cells as feeder cells.

shRNA silencing of NCR
For production of lentiviruses expressing NCR-specific shRNAs, 5 × 10⁶ HEK293T cells at exponential growth phase (70–80% confluent) in DMEM/10% FCS medium were transfected in a 150 cm² flask by the calcium phosphate precipitation method with the following plasmids: 30 µg pLKO.1 shRNA plasmid (1 µg/µl), 15 µg packaging plasmid (psPAX2, 1 µg/µl), 6 µg VSV-G envelope plasmid (pMD2.G, 0.5 µg/µl). To the plasmid mixture, first 1260 µl H₂O and 163 µl 2.5 M CaCl₂, and then 1500 µl 2× HBS (280 mM NaCl, 100 mM HEPES, 1.5 mM Na₃HPO₄ [pH 7.12]) were added under vortexing and aeration for 30 sec. The DNA precipitate was added slowly to HEK293T cells. The packaging and envelope plasmids were purchased from Invitrogen. Human pLKO.1 lentiviral shRNA target gene sets for NCR1, NCR2 and NCR3 were from ABgene (Epsom, UK). Clones TRC0000063537, TRC0000063500 and TRC0000063270 yielded the best silencing effects for NKP46, NKP44 and NKP30, respectively.

24 h after transfection, culture supernatants were collected from transfected cells and filtered through a 0.22-µm sterile filter. The medium was replaced and collected again after 48 h. Cell medium was stored in 3 ml aliquots at −80°C. For spinfection of 0.5 × 10⁶ NK-92 cells, 3 ml of lentivirus-containing medium (with 0.5 µg/ml Polybrene [Sigma-Aldrich]) was used. Transduced cells were selected with 1 µg/ml puromycin. The transduction efficiency was assessed by staining with NCR-specific mAbs. Transduced NK-92 cells were selected with 1 µg/ml puromycin.

Chromium release assays
Target cells (0.5 × 10⁶) were labeled in 100 µl assay medium (IMDM with 10% FCS and 1% penicillin/streptomycin) with 100 µCi (3.7 MBq) of sodium chromate [³⁵Cr] for 1 h at 37°C. Cells were washed thrice and resuspended at 5 × 10⁶ cells/ml in 100 µl assay medium. Effector cells were resuspended in assay medium (100 µl/well supplemented with 200 U/ml IL-2) and mixed at different effector to target (E:T) ratios with 5000 labeled target cells/well in a 96-well V-bottom plate. In some experiments recombinant soluble V5-His6-tagged HA(VV) or ICOS-L for
control was included at ~1 μg/well. In other experiments, uninfected and VV-infected target cells were preincubated during the labeling time with the anti-HA mAb VVI-4G9, the irrelevant mAb MOPC-21, or NKp30-Fc at ~2 μg/well. In these experiments, primary NK cell effectors were preincubated with the FcγRIII-blocking antibody CLB-FcR gran/1 (2 μg/1.2×10^6 effector cells for 20 min). Maximum release was determined by incubation of target cells in 1% Triton X-100 solution. Spontaneous ^51^Cr release was measured by incubating target cells in the absence of effector cells. All samples were done in triplicates. Plates were incubated for 4 h at 37°C. Supernatant was harvested and ^51^Cr release was measured in a γ-counter. The percentage of cytotoxicity was calculated according to the following formula: ([experimental release – spontaneous release] / [maximum release – spontaneous release]) x 100. The ratio between maximum and spontaneous release was at least 3 in all experiments.

**CD3ε-reporter cells and LacZ assays**

The generation of LacZ-inducible BWZ.36 reporter cells expressing NKp46-CD3ε, NKp44-CD3ε, NKp30-CD3ε, or CD16-CD3ε and the procedure of the LacZ assay has been described [33]. Briefly, HA-transfected or normal HeLa cells were plated in a 96-well round-bottom plate (1.25×10^4 cells/well). Adherent HeLa cells were infected using m.o.i. 4 in 96-well flat-bottomed cell culture plates and cultured for 20 h in 100 μl RPMI-1640/10% FCS medium followed by UV inactivation (254 nm) of viral particles for 10 min. In some experiments, ELISA plates were coated with 0.5 μg mAbs against NKp30, NKp46 and NKp44 (from R&D Systems) in 0.05 M carbonate buffer (pH 9.6) overnight before washing and addition of reporter cells. In other experiments, ELISA plate wells were coated with ~1 μg of recombinant soluble HA(VV)-Fc alone or in combination with anti-NCR mAbs. 50 μl culture supernatant from VV(WR)- or VV(WR):HA-infected HeLa cells (m.o.i. 4, 20 h), that had been cleared from cellular debris by centrifugation at 2300 x g for 5 min and inactivated by exposure to 254 nm UV light for 10 min, was added to adherent HeLa/B7-H6 cells in triplicate wells. In another approach, crude membranes were produced from VV-infected and uninfected HeLa cells by three cycles of freezing in liquid N2 and thawing, followed by clearing of intact cells and nuclei at 1000 x g for 5 min, and pelleting of membranes at 16000 x g for 10 min at 4°C in a microcentrifuge. Crude membranes were resuspended in 250 μl and 10 μl added to assay wells in triplicates.

Adherent tumor cells were co-cultured with 10^5 NCR-CD3ε reporter cells for 18 h at 37°C. PMA (phorbol 12-myristate 13-acetate; 5 ng/ml; Sigma-Aldrich) plus ionomycin (0.5 μg/ml; Merck/Kalbiochem, Darmstadt, Germany) was used as positive control for the LacZ inducibility in reporter cells. The stimulation of NCR-CD3ε cells was determined by addition of LacZ substrate buffer (9 mM MgCl2, 0.15 mM CPRG, 100 mM 2-ME, 0.125% Nonidet P-40 in PBS [pH 7.5]) for 4 h at 37°C. The cleaved CPRG was measured in an ELISA reader with an absorbance at 595 nm with 630 nm as the reference wavelength. The data are shown as means of triplicate values and s.e.m. from representative examples of three similar experiments.

Additional experimental methods can be found in Supplemental Materials and Methods (Text S1).

**Supporting Information**

**Figure S1** Induction of NKp30 and NKp46 ligands on VV-infected human fibroblasts. Human MRC-5 fetal lung fibroblasts (A) and human VH7 foreskin fibroblasts (B) were infected with VV(WR) using m.o.i. 8 for 20 h, or were mock-infected before harvesting and staining with the indicated NCR-Fc fusion protein or the anti-HA mAb VVI-4G9 to monitor infection efficiencies. (C) For comparison, HeLa cells were infected using the same conditions and also stained with NKp30-Fc, NKp46-Fc or anti-HA.

(EPS)

**Figure S2** NKp30-Fc and NKp46-Fc bind to plate-coated viral particles and recombinant HA. (A, B) Enzyme-linked immunoassays using 96-well plates coated with viral particles (VV strains WR and Lister, HA-deficient VV(WR)), with recombinant His6-tagged HA ectodomains from VV and ECTV, or with recombinant ICOS-L as an irrelevant protein. Coated and washed ELISA plates were reacted with NKp30-Fc, NKp46-Fc and NKp44-Fc, followed by incubation with goat anti-hIgG horseradish peroxidase(HRP)-conjugated secondary antibodies. The binding of viral particles was confirmed using the anti-HA mAb VVI-4G9 and goat anti-mIgG horseradish peroxidase(HRP)-conjugated secondary antibodies or polyclonal rabbit anti-VV for VV:AHA particles, respectively. In (A) and (B), some wells were incubated for 15 min with the anti-HA mAb VVI-4G9 before addition of NCR-Fc. In (B), NKp30-Fc was also preincubated with soluble recombinant HA(VV):V5-His6 proteins before addition to the virus-coated ELISA plate ("NKp30-Fc + sVV(HA)"). The lack of binding of NKp30/NKp46-Fc to VV:AHA particles and the partial inhibitions observed in the presence of anti-HA or soluble HA underpin the HA-specific binding. (C) Western blot showing SDS-PAGE-separated, recombinant V5-His6-tagged HA(VV) and HA(ECTV) ectodomains, and V5-His6-tagged ICOS-L as a control protein, reacted with a mAb specific for the V5 epitope. (EPS)

**Figure S3** Analysis of glycan-based NCR ligands on VV-infected and uninfected cells. Uninfected (A) and VV-infected (B) HeLa cells were left untreated or treated with a mixture of heparanase I and III, or with hyaluronidase (left column), or deglycosylated with protein N-glycanase F for removal of N-glycans, a cocktail of O-deglycosylating enzymes, or with neuraminidase alone (right column), as described in Supplemental Materials and Methods (Text S1). After enzymatic treatment and washing, cells were immediately stained on ice with NKp30/Fc, NKp46-Fc or NKp44-Fc fusion proteins complexed with goat anti-hIgG-PE secondary antibodies. Heparanase I/III and hyaluronidase treatment reduced the staining of uninfected cells by the three NCR, but enhanced the staining of infected cells by NKp30-Fc and NKp46-Fc. Desialylation caused a strong enhancement of NKp44 labeling, whereas it had a reducing effect on NKp30-Fc binding to infected cells. (C) NP-40 lysates of VV-infected HeLa ("H") cells and murine NIH-3T3 ("3") cells, were either left untreated or digested with endoglycosidase H (Endo H), N-glycosidase F, neuraminidase or O-glycosidase, respectively. The lysates were separated by SDS-PAGE and a Western blot reacted with anti-HA mAb VVI-4G9 and hIgG-conjugated anti-mIgG antibodies. The dominant, fully glycosylated, long form of HA (68 kDa) are labeled with "L" and "S". The major HA form contained ~5 N-glycans and ~2 O-glycans as estimated from respective shifts in molecular weight after enzymatic deglycosylations. After neuraminidase treatment a minor portion of anti-HA-reactive molecules from HeLa lysates migrated at an apparently higher molecular weight (asterisks). These bands probably represent unseparated aggregates of HA. (EPS)
Figure S4  ECTV infection results in reduced NKG2D ligand expression. HeLa cells infected for 20 h with VV (black line) or ECTV (gray line) were stained with mAb VVI-4G9 to monitor HA surface expression, with soluble NKG2D IgG-Fc fusion protein and with mAbs recognizing the NKG2D ligands MICA, MICB, ULBP1, ULBP2, ULBP3, or ULBP4, as indicated. Uninfected controls are shown with filled histograms. Infection with ECTV, but not VV, caused a downmodulation of the NKG2D ligands MICA, MICB and ULBP3.

EPS

Figure S5 Cytofluorometric analysis of target and effector cells used in the cytotoxicity assay of Figure 4A. (A) Infection time-dependent increase of HA surface and intracellular EGFP expression in HeLa cells infected for 6, 12 and 18 h, respectively, with wild-type VV(WR) or VV(WR):ΔHA[EGFP] in the top panel. The time-dependent increase of NKp30-Fc and NKp46-Fc binding to HeLa cells infected with wild-type VV for 12 and 18 h, but not to cells infected with VV:ΔHA, is shown below. (B) Stainings of primary NK cells from donor 1 (black line) and donor 2 (gray line) with PE-conjugated mAbs against NKG2D, NKp30, NKp46 or NKp44. The binding of a PE-labeled control antibody (MOPC-21) is presented as filled histogram.

EPS

Figure S6 Inhibition of NK lysis by poxviral HA. (A) Uninfected or VV-infected HeLa cells were ^11Cr-labeled and used in a cytotoxicity assay with primary NK cells preincubated with either soluble recombinant HA-V5-His, derived from VV(WR), or an irrelevant recombinant protein (ICOSL-V5-His). Different E:T ratios are shown. (B) Lysis assay with primary NK cells as effectors and uninfected or VV-infected HeLa cells which were preincubated with either an irrelevant mAb (MOPC21) or with anti-HA mAb VVI-4G9. NK cells were preincubated with an excess of the blocking anti-CD16 mAb CLB-FcR gran/1 in order to prevent Fc receptor binding. (C) HeLa transfected either with the empty pcDNA3.1(+) vector for control, or with pcDNA3.1(+) (HA):ECTV, were used in a lysis assay using NK-92 as effector cells. Where indicated, target cells were preincubated with an excess of soluble NKp30-Fc. Statistical comparison of samples with recombinant soluble HA-V5-His, versus samples with control protein (A), samples with anti-HA versus samples with control mAb (B), and samples with NKp30-Fc versus without NKp30-Fc (C) were done using Student’s t-test (paired, 2-tailed), * p<0.05, ** p<0.01, *** p<0.001.

EPS

Figure S7  VV-infected stimulator cells reduce secretion of IFN-γ and TNF-α by primary NK cells. Enzyme-linked immunoassays for human TNF-α (A) and IFN-γ (B) secreted into the culture medium of primary IL-2-activated NK cells cultivated together with uninfected HeLa cells, HeLa cells infected with wild-type VV(WR), or HA-deficient VV(WR), respectively, for 24 h. Prior to incubation with NK cells, the stimulator cells were treated with 254 nm UV light to prevent viral spread. For control, NK cells were also cultured alone. Statistical comparison of infected versus non-infected HeLa cells was done using Student’s t-test (2-tailed), n.s., not significant, ***, p<0.001.

EPS

Text S1  Supplemental materials and methods.

(DOC)

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References

1. Moss B (2007) Poxviridae: The viruses and their replication. In Knipe DM, Howley PM, eds. Fields Virology Philadelphia: Lippincott Williams & Wilkins 2: 2905–2946.
2. Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID (1988) Smallpox and its Eradication. Geneva: World Health Organization.
3. Moss B (1996) Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A 93: 11341–11348.
4. Smith GL, Symons JA, Khanna A, Vanderplasschen A, Barrett JW, et al. (2003) Poxviruses and immune evasion. Annu Rev Immunol 21: 377–423.
5. Seet BT, Johnston JB, Brunetti CR, Barrett JW, Everett H, et al. (2003) Vaccinia virus immune evasion. Immunol Rev 159: 137–154.
6. Karupiah G, Coupar B, Ramshaw I, Boyle D, Blanden R, et al. (1990) Vaccinia virus infection of mice. J Immunol 172: 6265–6271.
7. Karupiah G, Buller RM, Van Rooijen N, Duarte CJ, Chen J (1996) Different roles for CD4^+ and CD8^+ T cells in the protection against vaccinia virus infection of mice. J Immunol 156: 4070–4079.
8. Brutkiewicz RR, Klaus SJ, Welsh RM (1992) Window of vulnerability of vaccinia virus-infected cells to natural killer (NK) cell-mediated cytolysis correlates with enhanced NK cell triggering and is concomitant with a decrease in H-2 class I antigen expression. Nat Immunol 11: 203–214.
9. Bukowski JF, Woda BA, Habu S, Okumura K, Welsh RM (1983) Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. J Immunol 131: 1531–1537.
10. Belyakov IM, Earl P, Dzutsev A, Kuznetsov VA, Lemon S, et al. (2003) Effector cell-based vaccine induced tumor rejection in a murine model of metastatic breast cancer. J Immunol 171: 6784–6794.
11. Chisholm SE, Reyburn HT (2006) Recognition of vaccinia virus-infected cells by natural killer (NK) cells. J Immunol 176: 11348.
12. Bukowski JF, Woda BA, Habu S, Okumura K, Welsh RM (1983) Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. J Immunol 131: 1531–1537.
13. Ytterberg S, Blatt PN, Brownstein DG (1989) Evidence that NK cells and interferon are required for genetic resistance to lethal infection with ectromelia virus. Arch Virol 108: 49–58.
14. Parker AK, Parker S, Yokoyama WM, Corbett JA, Buller RM (2007) Induction of natural killer cell responses by ectromelia virus controls infections. J Virol 81: 4070–4079.
15. Huang S, Hendriks W, Althage A, Hennmi S, Bluthmann H, et al. (1993) Immune response in mice that lack the interferon-gamma receptor. Science 259: 1742–1745.
16. Deonarain R, Alcami A, Alexiou M, Dallman MJ, Gewert DR, et al. (2000) Poxviruses and immune evasion. Annu Rev Immunol 21: 377–423.
17. Cerwenka A, Hooper, H. Shida, and D. Cosman are acknowledged for their kind gifts of antibodies.

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20. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, et al. (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 19: 197–223.
21. Lanier LL (2005) NK cell recognition. Annu Rev Immunol 23: 225–274.
22. Moretta L, Moretta A (2004) Killer immunoglobulin-like receptors. Curr Opin Immunol 16: 626–633.
23. Arnon TI, Markel G, Mandelboim O (2006) Tumor and viral recognition by natural killer cells. Semin Cancer Biol 16: 348–358.
24. Blouw Nt, Qin Q, Bar-Ilan A, Hershkovitz O, Gazit R, et al. (2004) Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. J Immunol 173: 2392–2401.
25. Hershkovitz O, Jove S, Blouw Nt, Zilka A, Landau G, et al. (2007) Characterization of the recognition of tumor cells by the natural cytotoxicity receptor, NKp44. Biochemistry 46: 7436–7436.
26. Hershkovitz O, Jarahian M, Zilka A, Bar-Ilan A, Landau G, et al. (2008) Altered glycosylation of recombinant NKp30 hampers binding to heparan sulfate: a lesson for the use of recombinant immuno-receptors as an immunological tool. Glycobiology 18: 28–41.
27. Fagg G, Stramandinin E, Simhadri VR, von Treskow B, Sasse S, Reiners KS, et al. (2007) Human leukocyte antigen-B-associated transcript 3 is expressed from tumor cells and engages the NKp30 receptor on natural killer cells. Immunity 29: 965–974.
28. Brandt CS, Barzin M, Yi EC, Kennedy J, Gao Z, Fox B, et al. (2009) The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J Exp Med 206: 1495–1503.
29. Byrd A, Hoffmann SC, Jarahian M, Momburg F, Watzl C (2007) Expression analysis of the ligands for the natural killer cell receptors NKp30 and NKp46: Plas One 12: e1339.
30. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, et al. (2001) Recognition of viral hemagglutinins on infected cells by NKp46 activates lytic NK cytotoxicity. J Virol 75: 2680–2689.
31. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinin by NKp44 but not by NKp30. Eur J Immunol 31: 2391–2392.
32. Arnon TI, Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, et al. (2001) Recognition of viral hemagglutinin by NKp44 but not by NKp30. Eur J Immunol 31: 2391–2392.
33. Jarahian M, Watzl C, Fournier P, Arnold A, Djandji D, et al. (2009) Activation of natural killer cells by newcastle disease virus hemagglutinin. J Immunol 183: 120–127.
34. Arnon TI, Achdout H, Levi O, Markel G, Saleh N, et al. (2005) Inhibition of the ligands for the natural killer cell receptors NKp30 and NKp46: Plas One 12: e1339.
35. Chisholm SE, Howard K, Gomez MV, Reyburn HT (2007) Expression of ICP0 sufficient to trigger natural killer cell recognition of herpes simplex virus-1 infected cells. Nat Immunol 6: 515–523.
36. Baraz L, Haazanov E, Condiotti R, Kotler M, Nagler A (1999) Natural killer (NK) cells prevent virus production in cell culture. Bone Marrow Transplant 2: 179–180.
37. Zhang Q, Yu YA, Wang E, Chen N, Danner RL, et al. (2007) Eradication of solid human breast tumors in nude mice with an intravenously injected light-attenuating oncolytic vaccinia virus. Cancer Res 67: 10038–10046.