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ORIGINAL INVESTIGATION

Tumor cells infected with oncolytic influenza A virus prime natural killer cells for lysis of resistant tumor cells

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Abstract Tumor resistance to lysis by resting natural killer (NK) cells may be overcome by priming of NK cells with cytokines or by binding of NK activating receptors to ligands expressed on target cells. In this study, major histocompatibility complex class I (MHC-I)-negative LNCaP and MHC-I-positive DU145 cells were infected with genetically modified influenza A virus lacking the non-structural gene 1 (ΔNS1 IAV). The cells were used to investigate the influence of ΔNS1 IAV infection on NK cell lysis of tumor cells as well as to prime NK cells for lysis of LNCaP and DU145 cells. While LNCaP cells infected with ΔNS1 IAV showed enhanced lysis when compared with mock-infected cells (93% ± 1.47 vs. 52% ± 0.74), both mock-infected and ΔNS1 IAV-infected DU145 cells were resistant to NK cell lysis. Moreover, NK cells primed with ΔNS1 IAV-infected LNCaP/DU145 cells effectively lysed resistant DU145 and sensitive LNCaP cells to a greater extent than NK cells primed with mock-infected LNCaP/DU145 or non-primed NK cells. Also, NK cell priming with ΔNS1 IAV-infected tumor cells enhanced extracellular signal-regulated kinase phosphorylation and increased granule release in NK cells. The increased granule release was specifically mediated by NKp46, which eventually potentiated NK cells primed with ΔNS1 IAV-infected tumor cells to overcome the inhibitory effects posed by MHC-I expression on DU145 cells. These findings show that in addition to direct lytic activity of NK cells, ΔNS1 IAV may influence anti-tumoral responses by priming NK cells.

Keywords Cytotoxicity · NK cell priming · Major histocompatibility complex class I · Degranulation · Oncolytic influenza A virus

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Introduction

Human natural killer (NK) cells as components of the innate immunity substantially contribute to the elimination of virus-infected cells as well as anti-tumor immune response [1]. Although NK cells can kill target cells spontaneously without prior stimulation, a delicate balance between inhibitory [killer immunoglobulin-like receptors (KIR), CD94-NK group 2, member A (NKG2A)] and activating receptor signals [natural cytotoxic receptors (NCRs-NKp30, NKp44 and NKp46), NK group 2, member D (NKG2D) and DNAX accessory molecule-1 (DNAM-1)] tightly regulates their activation [1]. Target cells are recognized by their diminished expression of major histocompatibility complex class I (MHC-I) molecules, which normally interact with inhibitory receptors on the NK cell surface [2–7]. The down-regulation of MHC-I molecules on some tumor cells and some virus-infected cells is believed to lower this inhibition below a target threshold, making the target cell susceptible to NK cell-mediated lysis [8]. Some tumor cell lines are NK resistant through constitutive expression of MHC-I molecules. However, resistance to NK-mediated lysis is overcome by pre-incubation of NK cells with IL-2, thus generating "lymphokine-activated killer cells" that are capable of multiple lysis. In addition, priming or activation of NK cells through interaction with

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susceptible target cell was shown to provide sufficient stimulus to overcome MHC-I-mediated inhibition in a manner analogous to that mediated by IL-2 [8]. Also, the up-regulation/induction of ligands [influenza/parainfluenza virus hemagglutinin (HA) binds NKp44 and NKp46; UL16 binding proteins (ULBP1-3) and MHC class I-related chain A and B (MICA/B) bind NKG2D; poliovirus receptor (PVR) binds DNAM-1] for NK cell activating receptors on tumor cells and virus-infected cells correlates with their sensitivity to NK-mediated killing [9, 10]. In this regard, Mandelboim et al. 2001 [11] elegantly showed using target cells infected with wild type influenza A virus (IAV) that NK cell lysis was enhanced in IAV-infected target cells mainly via binding of NKp46 to viral HA expressed on target cells.

IAV infection can induce apoptosis in host cells, in vivo and in vitro [12, 13]. IAV can infect and replicate both in tumor and untransformed cells. They encode a non-structural (NS1) protein involved in virulence and inhibition of IFN-α/β system during virus infection [14]. Interestingly, mutant IAV lacking the NS1 gene (ΔNS1 IAV) does not replicate in non-malignant cell lines but grows efficiently in oncogenic Ras-expressing cells [15] as well as in IFN-resistant cancers [16]. The apathogenic nature of ΔNS1 IAV combined with the selective replication properties of this virus in both oncogenic Ras-expressing and IFN-resistant tumor cells renders this virus an attractive candidate for use in immunovirotherapy and may be interesting for induction of NK cell activity. In this regard, the activation of the adaptive immune system by infection of tumor cells with oncolytic viruses reportedly led to the elimination of tumor cells [17, 18]. Also, direct injection of established Renca tumors with inactivated, replication-defective Sendai virus particles was shown to cause systemic activation of NK cells and enhanced their cytotoxicity against tumor cells [19].

In this study, we have used MHC-I-negative LNCaP and MHC-I-positive DU145 cells that have been infected with ΔNS1 IAV to investigate the influence of ΔNS1 IAV on NK lysis of tumor cells as well as to prime/activate NK cells for lysis of target cells. We demonstrate that infection of prostate cancer cells with ΔNS1 IAV renders the cells to become more sensitive to NK lysis. More importantly, our results reveal that NK cells that are primed with ΔNS1 IAV-infected tumor cells degranulate and lyse their targets more than non-primed or mock-infected tumor-primed NK cells, even to the extent of overriding MHC-I inhibition.

Materials and methods

Cell lines

DU145 and LNCaP (clone FGC) cell lines were obtained from the German Cancer Research Center (DKFZ, Heidelberg, Germany). The cell lines were grown in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal calf serum (FCS). Media and supplement were from Seromed (Berlin, Germany).

Virus preparation

ΔNS1 IAV [containing: PB2, PA, nucleoprotein (NP), M genes from H1N1 A/PR8/34; surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) genes from H1N1 A/New Caledonia/20/99; PB1 gene from H3N2 A/Texas/1/77] was prepared by reverse genetics using methods as previously described [20].

Reagents and monoclonal antibodies

Unconjugated influenza A virus HA antibody (IVC102) and PVR were purchased from Abcam (Cambridge, UK); unconjugated ULBP1-3 were from R&D systems (Wiesbaden, Germany); PE-conjugated MICA/B was from BD Pharmingen (San Diego, CA); purified NKp46 was from Miltenyi Biotec (Bergisch Gladbach, Germany). Fluorescein isothiocyanate-conjugated HLA-Class 1 mAb was from Biosource (Camarillo, CA), mouse anti-human HLA-class 1 antigen ABC was from US Biological (Swampscott, MA).

Cell viability assay

Cell viability was investigated using the modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium-bromide (MTT) assay. Briefly, LNCaP and DU145 cells were grown in 96-well plates after which they were either mock infected or ΔNS1 IAV infected. At 20-h post-infection (p.i.), MTT reagent was added for 4 h. Thereafter, 100 µl of sodiumdodecylsulfate (SDS) solution (20% SDS in a 1:1 dimethyformamide (DMF)/H_2O solution) was added, and the plate was incubated overnight at 37°C in a CO_2 incubator. Plates were read on a multiwell scanning spectrophotometer (Tecan, Crailsheim, Germany) at a wavelength of 560 nm and a reference wavelength of 620 nm. Cell viability was determined as the relative reduction in the amount of MTT reduced by cells to its purple formazan derivative, which correlates with the amount of viable cells in relation to cell control.

Cytotoxicity assay and flow cytofluorometric analysis

Cytotoxicity of NK cells was determined by a 4-h coupled luminescent method using the "aCella-Tox" kit (Cell Technology, Mountain View, CA), as previously described [21]. LNCaP and DU145 cell lines, both mock- and ΔNS1 IAV-infected 20-h p.i., were used as target cells. For monoclonal

antibody (mAb) blocking experiments, 10 µg/ml of the corresponding purified mAb was used. Flow cytometry (FACS Calibur; Becton Dickinson, Mountain View, CA) was used for cell surface expression analysis, while cellular DNA content was measured using propidium iodide staining [22]. In case of unconjugated antibodies, PE-conjugated isotype-specific goat anti-mouse second reagent (R&D Systems, Wiesbaden, Germany) was used.

Polyclonal NK cell preparation

Human peripheral blood mononuclear cells were isolated from the blood of healthy volunteers by Ficoll–Hypaque centrifugation followed by separation using the MACS NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. Flow cytometric analysis to determine purity of NK cells showed that more than 95% of the cells were CD56 + CD3 − (not presented).

Immune staining

LNCaP and DU145 cells grown on culture flasks were mock or ΔNS1 IAV infected. Twenty-h p.i. cells were fixed with ice-cold acetone/methanol for 15 min at room temperature and blocked with blocking solution for 30 min at 37°C. Thereafter, cells were stained for 1 h at 37°C with mouse anti-influenza A mAb (1:1,500 dilution) (Millipore; Schwalbach, Germany) for the detection of NP antigen. Cells were then washed and incubated in Biotin-SP-conjugated AffiniPure F(ab')$_2$-fragment goat anti-mouse IgG (1:1,000 dilution) (Jackson ImmunoResearch, West Grove, PA) for 45 min at 37°C. After incubation, cells were washed again and incubated in a streptavidin–peroxidase conjugate (1:3,000 dilution) (Calbiochem; Darmstadt, Germany) for 30 min at 37°C. After washing, cells were incubated for 10 min in AEC substrate, washed and photographed.

NK cell priming

LNCaP and DU145 cell lines, both mock- and ΔNS1 IAV-infected 20-h p.i,, were cocultured with resting NK cells for 3 h at 37°C. After this period, the primed NK cells were separated from the coculture using CD56 microbeads (Miltenyi Biotec). Separated cells were used for cytotoxicity experiments against target cells.

NK cell degranulation experiment

After 3-h priming of NK cells with mock- or virus-infected prostate cancer cell lines, NK cells were stimulated by mAb cross-linking as previously described [23, 24]. Briefly, cells were labeled with 1 µg/ml appropriate mAbs for 30 min at 4°C. After washing, cells were stimulated with 10 µg/ml AffiniPure F(ab')$_2$ fragment goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 5 min at 37°C. Reaction was stopped with ice-cold PBS. After overnight incubation at 37°C, supernatants were collected for analysis and quantification of granule release by ELISA assay (Perforin/Granzyme B-ELISA kit, Diaclone Research, Besancon Cedex, France) according to manufacturer's instructions. Additionally, after 3-h priming of NK cells with mock- or virus-infected prostate cancer cell lines, primed and non-primed NK cells were cocultured with LNCaP for a further 4 h. Supernatants were collected for analysis and quantification of granule release by ELISA assay.

Western blot

After 30-min priming of NK cells with mock- or virus-infected prostate cancer cell lines, Western blot analysis was performed as previously described [25]. Briefly, cell lysates were subjected to SDS–PAGE before transfer to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using the Mini-Protean II system (Bio-Rad, Munich, Germany). After transfer, blots were blocked in tris-buffered saline (TBS) blocking buffer containing 3% bovine serum albumin for 1 h at room temperature to saturate the non-specific protein-binding sites on the nitrocellulose membrane. The following primary rabbit polyclonal Abs were used: ERK1/2, phospho-ERK1/2, all from cell signaling (Beverly, MA, USA); mouse polyclonal beta actin Ab was from Sigma–Aldrich (Taufkirchen, Germany). The blots were incubated overnight with the primary Ab diluted in TBS at 4°C with gentle agitation. Following a 1-h incubation period with peroxidase-conjugated secondary Ab at room temperature, visualization was performed by enhanced chemiluminescence using a commercially available kit (Amersham, Liverpool, UK).

Measurement of IFN-γ production

A total of 2×10^4 target cells (mock-infected and ΔNS1 IAV-infected DU145 and LNCaP cells; 20-h p.i.) were cocultured with 1×10^5 NK cells for 24 h. For mAb blocking, HA or IgG Ab was added to the target cells 1 h prior coculture with NK cells. NK cells alone or mock-infected and ΔNS1 IAV-infected DU145 and LNCaP were used as control. ΔNS1 IAV directly infected 1×10^5 NK cells. Supernatants were collected and tested for the production of IFN-γ. The amounts of IFN-γ were determined using the Quantikine Human IFN-γ ELISA kit (R&D Systems, Wiesbaden, Germany) according to manufacturer's protocol.

Statistics

Values presented are the mean ±SE of mean (SEM) of at least three experiments.

Results

Influence of ΔNS1 IAV infection on cell viability and cell cycle progression of LNCaP and DU145 cells

Oncolytic properties of ΔNS1 IAV were tested by MTT on DU145 and LNCaP cells infected at different multiplicity of infection (MOI) ranging from 0.02 to 200, (20-h p.i.). Results show that the percentage of viable cells decreases with increasing MOI. At MOI 2, no significant cell death occured in LNCaP-infected cells compared with over 30% cell death in DU145-infected cells. LNCaP-infected cells only showed significant cell death at MOIs 20 (12%) and 200 (47%). DU145 cells were almost completely eradicated (82% cell death) at MOI 20 (Fig. 1a). The same percentage of viable cells was obtained for DU145 and LNCaP at MOIs 0.2 and 2, respectively. To further investigate the oncolytic effect of ΔNS1 IAV, cell cycle analyses of mock-infected and ΔNS1 IAV-infected LNCaP and DU145 cells (20-h p.i.) were performed to address whether ΔNS1 IAV infection at MOI ranging from 0.02 to 2 modified their cell cycle. As shown in Fig. 1b ΔNS1 IAV infection even at MOI 2 did not result in cell death of LNCaP cells. This is revealed as the fractional DNA content in the sub-G1 (M1) phase (indicates induction of cell death) of the cell cycle. There was, however, a G2/M (M4) block (17 vs. 13.9%, respectively, for ΔNS1 IAV-infected LNCaP cells and mock-infected LNCaP cells). Moreover, infection with ΔNS1 IAV at MOI 20 resulted in 19.3% and 85% cell death of LNCaP and DU145 cells, respectively. On the other

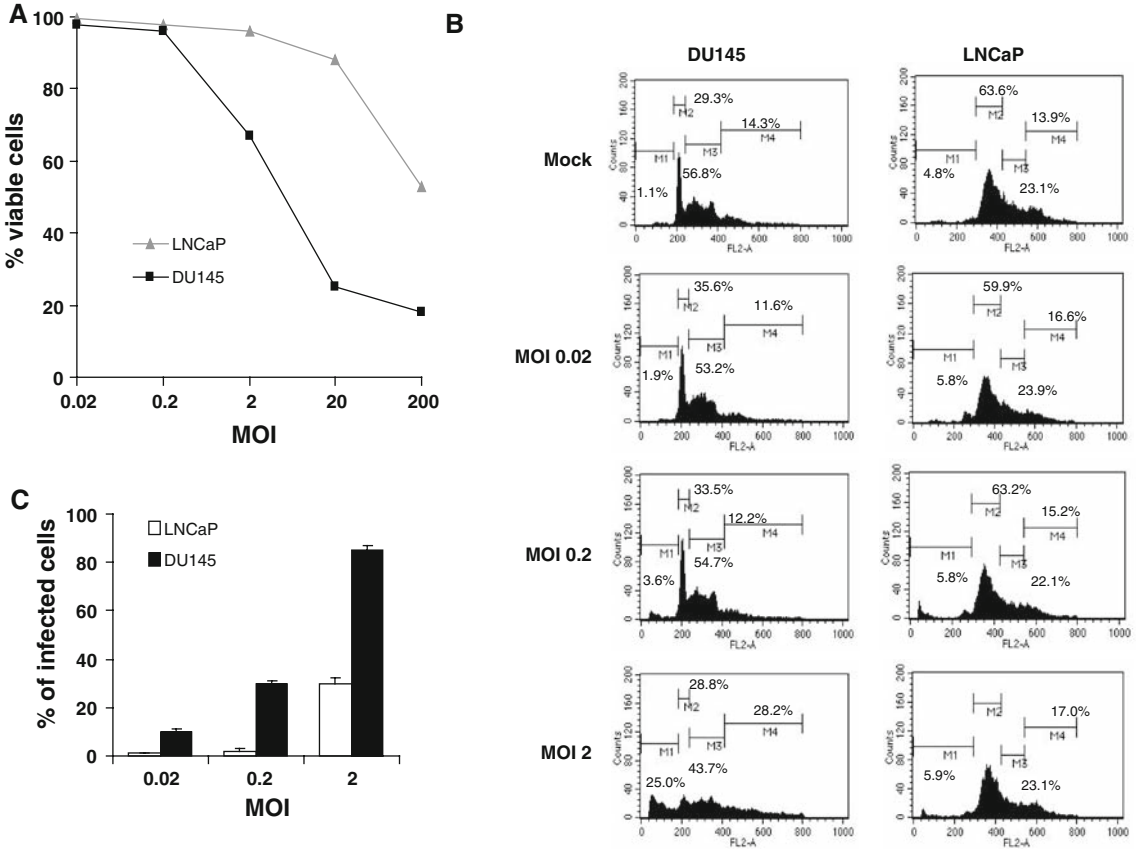

Fig. 1 Cell viability and cell cycle progression of ΔNS1 IAV-infected prostate cancer cells. LNCaP and DU145 cells were infected with ΔNS1 IAV for 20 h at indicated MOI. Mock-infected LNCaP and DU145 cells were used as control. **a** Cell viability was determined by MTT. Percentage of viable cells was deduced from that of mock-infected cells (set to 100%). Results are representative of three different experiments. **b** Cell cycle was determined by staining cells with propidium iodide. M1, M2, M3 and M4 indicate sub-G1, G0/G1, S and G2/M phases, respectively. *Values* indicate percentage of cells at the different phases. The percentages of cells in G0/G1, S and G2/M phases were deduced from the number of viable cells (set to 100%) after deducting the dead cells (sub-G1) from total gated cells. Results are representative of three different experiments. **c** Immune staining of infected cells for NP antigen expression using influenza A mAb. *Columns* indicate percentage of infected cells; *error bars* indicate ±SEM

hand, apoptotic cells in ΔNS1 IAV-infected DU145 cells at MOI 2 represented 25% of the tumor population compared with 1.1% of mock-infected cells. Here, a G2/M (M4) block (28.2% vs. 14.3%, respectively, for ΔNS1 IAV-infected DU145 cells and mock-infected DU145 cells) was observed. Infection of DU145 cells with ΔNS1 IAV at MOI 0.2 resulted in minimal cell death (3.6%) and no significant block in the cell cycle. Furthermore, immune staining of infected cells for influenza A antigen revealed that DU145 cells are highly permissive to infection by ΔNS1 IAV, while LNCaP cells are less permissive to ΔNS1 IAV infection. In cultures infected at MOI 2, 85% and 30% of DU145 and LNCaP were, respectively, infected (Fig. 1c). Based on the fact that comparable number (about 30%) of LNCaP and DU145 cells were infected by ΔNS1 IAV at MOI 2 and 0.2, respectively, and that at these MOIs, similar percentage of viable LNCaP and DU145 cells occurred, MOI 2 for infection of LNCaP cells and MOI 0.2 for infection of DU145 cells were therefore used for further experiments.

Infection of LNCaP but not DU145 cells with ΔNS1 IAV enhances NK cell cytolytic activity

A 4-h cytolytic experiment to determine NK cell lysis of virus-infected and mock-infected LNCaP and DU145 cells 20-h p.i. was performed. NK cells were observed to more efficiently kill ΔNS1 IAV-infected LNCaP cells than mock-infected LNCaP cells in an E/T ratio dependent manner (32 vs.80%, E/T 5:1; 52 vs. 93%, E/T 10:1, respectively, for mock-infected LNCaP cells and ΔNS1 IAV-infected LNCaP cells; Fig. 2a). On the other hand, DU145 cells were resistant to NK cell lysis. Infection of DU145 cells with ΔNS1 IAV did not increase their sensitivity to NK cell lysis (Fig. 2b).

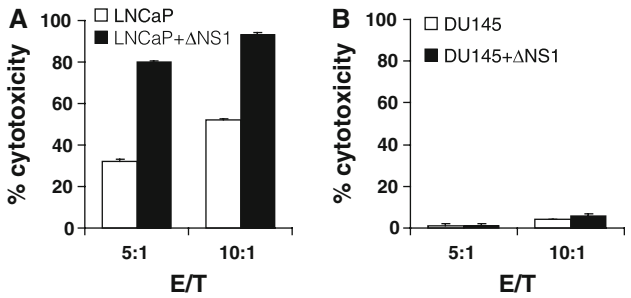

Fig. 2 Influence of ΔNS1 IAV infection of prostate cancer cells on NK cell cytotoxicity. (**a** and **b**) LNCaP and DU145 cells were infected with ΔNS1 IAV for 20 h at MOI 2 and 0.2, respectively. Primary NK cells from healthy donors were used in a 4-h NK cell cytotoxicity assay against mock-infected and virus-infected target cells at indicated effector to target (E/T) ratios. *Columns* represent means of triplicate of one representative experiment; *error bars* indicate ±SEM. One representative of at least three different experiments is shown

Ligand expression pattern in ΔNS1 IAV-infected and uninfected LNCaP and DU145 cells

Natural killer (NK) cells destroy virus-infected cells and tumor cells partly by recognizing on target cells the diminished expression of major histocompatibility complex class I (MHC-I) molecules, which normally interact with killer inhibitory receptors (KIR) on the NK cell surface [2–7]. NK cells also express triggering receptors like NKp30, NKp44, NKp46 and NKG2D [7] that are specific for non-MHC ligands expressed on target cells. Based on these, the expression pattern of ligands for NK cell triggering receptors as well as ligands for the NK cell inhibitory receptor (MICA/B, ULBP1-3, PVR, HA, and MHC-I) in LNCaP and DU145 cells was investigated. The results obtained show that viral infection of LNCaP and DU145 cells induces no changes in the ligand expression pattern for activating NK cell receptor, except for induction of HA expression (Fig. 3a and b). LNCaP expressed very little or no MHC-I, which was also not influenced by viral infection (Fig. 3c). On the other hand, DU145 cells were seen to express high amounts of MHC-I on their surface. Infection of DU145 cells with ΔNS1 IAV resulted in increased MHC-I expression (relative expression: 171 ± 5 for ΔNS1 IAV-infected DU145 cells and 98 ± 2 for mock-infected DU145 cells; Fig. 3d). Taken together, the results indicate that infection of prostate cancer cell lines with ΔNS1 IAV leads to the induction of HA expression on the cell surface of infected cells and increases the expression of MHC-I on cells that already express NK cell inhibitory receptor ligand.

Possible mechanism of enhanced NK cell killing of IAV-infected prostate cancer cells

In order to verify whether influenza virus HA was responsible for the enhanced NK lysis, NKp46 expression on NK cells as well as HA expression on prostate cancer cells was blocked using mAbs. Results obtained indicate that blocking HA on virus-infected LNCaP cells reduced the enhanced killing from 79 to 36%, while a slight increase rather than inhibition of NK cell lysis was observed in mock-infected LNCaP cells (from 30 to 37%) upon HA blocking. This slight increase is non-specific since blocking mock-infected LNCaP cells with control IgG Ab showed similar result (from 30 to 35%). NKp46 blocking on NK cells also inhibited lysis of both virus-infected and mock-infected LNCaP cells from 79 and 30 to 34 and 14%, respectively (Fig. 4a). DU145 cells expressed high levels of MHC-I and were resistant to NK cell killing. Since NK cell killing is regulated by a balance of inhibitory and activating receptors, MHC-I expression was blocked with mAb to see if NK cell killing may be attained. Accordingly, NK cells

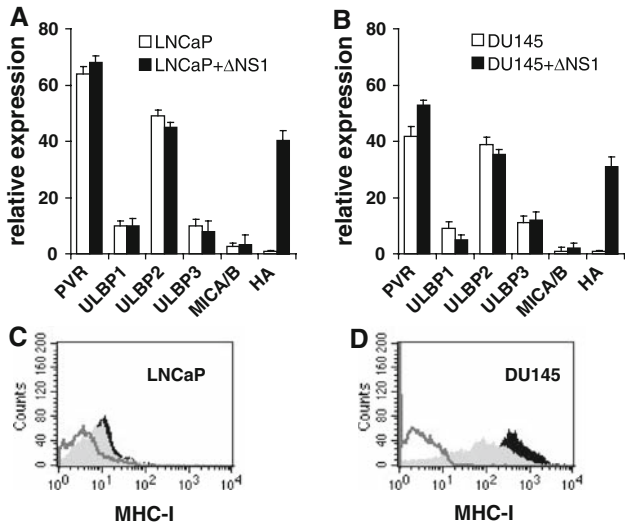

Fig. 3 Ligand expression pattern of prostate cancer cells. Flow cytometric analysis for the expression of indicated NK cell activating receptors in mock-infected and ΔNS1 IAV-infected **a** LNCaP, at MOI 2 for 20 h **b** DU145 at MOI 0.2 for 20 h. *Columns* represent means of triplicate of one representative experiment; error bars indicate ±SEM. Expression of NK cell inhibitory receptor ligand, MHC-I in **c** LNCaP, **d** DU145. *Empty histograms* show isotype control, *gray scale-filled histograms* show mock-infected cells, black scale-filled histograms show ΔNS1 IAV-infected cells. One representative of at least five separate experiments is shown

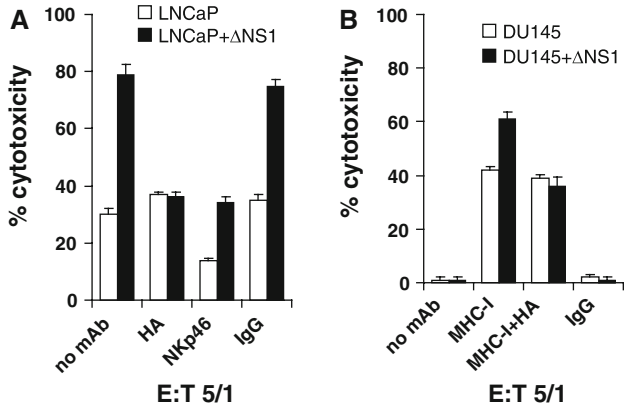

Fig. 4 Possible mechanism of enhanced NK cell killing of IAV-infected prostate cancer cells. **a** LNCaP and **b** DU145 cells were infected with ΔNS1 IAV for 20 h at MOI 2 and 0.2, respectively. Cells were treated with indicated mAb prior to coculture with primary NK cells. IgG isotype control Ab was used as negative control. A 4-h NK cell cytotoxicity assay against mock-infected and virus-infected target cells at indicated E/T ratios. Columns represent means of triplicate of one representative experiment; error bars indicate ±SEM

were able to lyse DU145 cell upon MHC-I blocking, with enhanced lysis observed in virus-infected DU145 cells (42 vs 61%, Fig. 4b). Combined blocking of MHC-I and HA on the virus-infected DU145 cells resulted in lysis inhibition (Fig. 4b). These results indicate that infection of prostate cancer cells with ΔNS1 IAV enhances NK cell killing of target cells possibly through the viral-induced HA expression.

NK cells primed with ΔNS1 IAV-infected tumor cells overcome MHC-I inhibition

Two NK evasion strategies have been described for tumor cells, namely the prevention of priming (type 1 evasion) and failure to trigger (type 2 evasion) [8]. Most NK-resistant cell lines are type 1 and fail to prime resting NK cells but are lysed by IL-2-primed NK cells [8]. Since DU145 cells are effectively lysed by IL-2-primed NK cells (Fig. 5), we hypothesized that DU145 cells may either fail to prime and/or trigger resting NK cells. To investigate this hypothesis, we primed NK cells by coculturing resting NK cells with mock or ΔNS1 IAV-infected LNCaP and mock or ΔNS1 IAV-DU145 cells for 3 h. After 3 h of coculture, about 50% of NK cells were recovered from coculture with ΔNS1 IAV-infected LNCaP or DU145 cells, while about 80% of NK cells were recovered from coculture with LNCaP or DU145 cells. The rest NK cells were retained in the immunological synapse. The recovered (primed) NK cells were used as effector cells in cytotoxicity experiments against DU145 and LNCaP targets. The results obtained show that NK cells primed with ΔNS1 IAV-infected LNCaP cells resulted in 2.5- and 35-fold increased lysis of LNCaP and DU145 target cells, respectively, while NK cells primed with ΔNS1 IAV-infected DU145 cells resulted in 2.2- and 14-fold increased lysis of LNCaP and DU145 target cells, respectively, when compared to resting NK cells. Priming of NK cells with mock-infected LNCaP/DU145 did not enhance NK lysis of target cells (Fig. 5a and b).

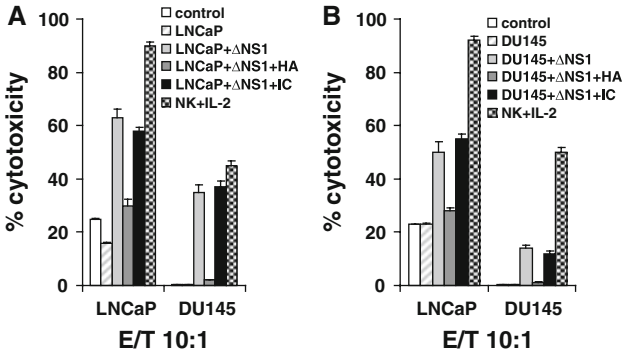

Fig. 5 NK cells primed with ΔNS1 IAV-infected tumor cells overcome MHC-I inhibition. NK cells were cocultured (primed) with mock-infected or ΔNS1 IAV-infected **a** LNCaP and **b** DU145 cells for 3 h. After 3-h coculture, non-primed resting NK cells (control), tumor-primed NK cells were used for cytotoxicity experiments against LNCaP and DU145 target cells. IL-2 activated NK cells were used as positive control. For blocking experiments, ΔNS1 IAV-infected cells were incubated with HA or isotype control (IC) mAb for 1 h prior to coculture with NK cells. Columns represent means of duplicate of one representative experiment; error bars indicate ±SEM

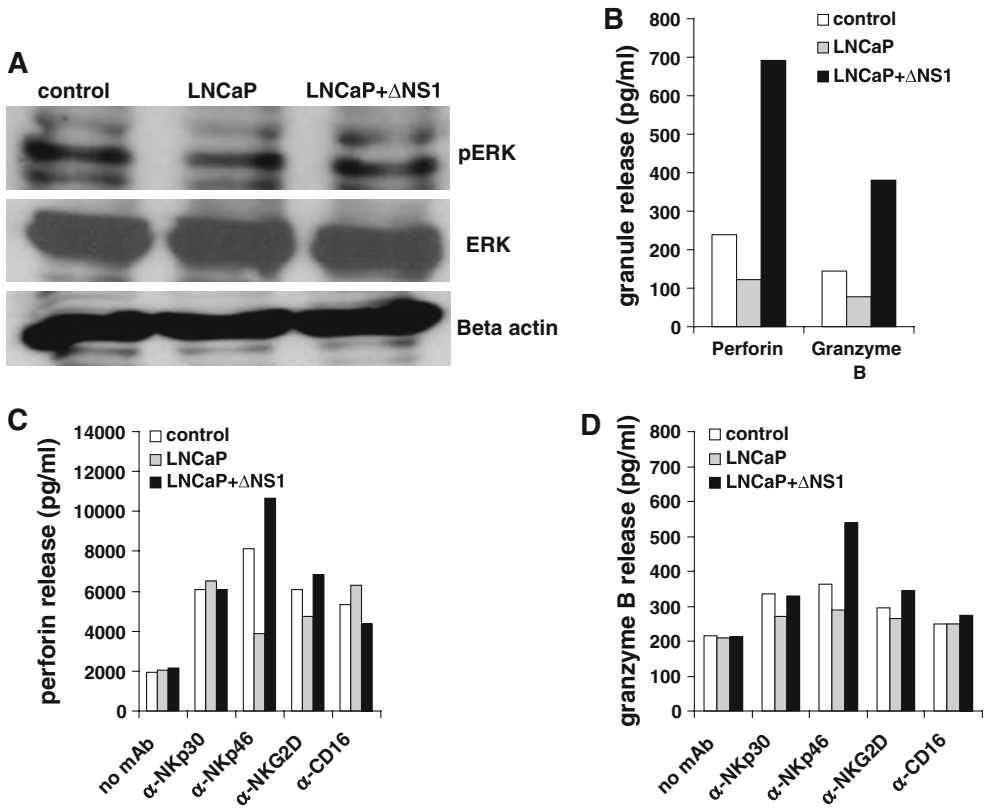

Fig. 6 NK cells primed with ΔNS1 IAV-infected tumors degranulate more rapidly than non-primed or those primed with mock-infected tumor cells. **a** NK cells were cocultured (primed) with mock-infected or ΔNS1 IAV-infected LNCaP for 30 min. Primed NK cells were collected from coculture and purified using CD56 microbead. Proteins were isolated from NK cells and analyzed by Western blot for ERK activation. **b** NK cells primed with mock-infected or ΔNS1 IAV-infected LNCaP cells were cocultured for 4 h with LNCaP target. Afterward, supernatant was collected and analyzed by ELISA for granule release. Non-primed NK cells were used as control. **c** Perforin and **d** granzyme B release following mAb cross-linking of NK cell receptors in non-primed NK control as well as in NK cells primed for 3 h with mock-infected and ΔNS1 IAV-infected LNCaP cells. Columns represent means of duplicate of one representative experiment

Blocking of ΔNS1 IAV-infected LNCaP cells with HA before NK coculture inhibited lysis. This indicates that ΔNS1 IAV induces the ability of prostate cancer cells to prime and trigger NK cells for enhanced lysis of NK-sensitive (LNCaP) and NK-resistant (DU145) targets in a HA-dependent fashion.

NK cells primed with ΔNS1 IAV-infected tumors degranulate more rapidly than non-primed or those primed with mock-infected tumor cells

The ligation of NK cells with their targets is known to rapidly cause a transient activation of ERK, which apparently control lytic granule movement [26]. To study this phenomenon, Western blot experiments were performed after 30 min coculture of mock- or ΔNS1 IAV-infected LNCaP cells with resting NK cells. Resting NK cells were used as control. The results obtained show a stronger ERK phosphorylation in NK cells primed with ΔNS1 IAV-infected LNCaP cells than in mock-infected LNCaP-primed or control NK cells (Fig. 6a). We also verified whether enhanced ERK phosphorylation correlated with granule release. For this purpose, NK cells were primed for 3 h as described and cocultured with LNCaP for a further 4 h. Supernatants were collected and analyzed for granule release. The results reveal that NK cells primed with ΔNS1 IAV-infected LNCaP cells clearly released more perforin and granzyme B granules than control NK cells or NK cells primed with mock-infected LNCaP cells (Fig. 6b). The activation of ERK by NK cell activating receptors like NKp46 and CD16 was reported to play a pivotal role in NK cell cytotoxicity and granule polarization [22]. The NK cell activating receptors that might have been activated upon NK cell priming were therefore studied. For this, following 3-h priming of NK cells, perforin and granzyme B release after stimulation of primed and control NK cells with mAb as described was analyzed. Stimulation of NK cells with NKp46, a receptor for HA expressed on ΔNS1 IAV-infected cells, specifically mediated enhanced granule release in ΔNS1 IAV-infected LNCaP cells (Fig. 6c and d).

Discussion

The activation of the immune system, with particular reference to T cells, by infection of LNCaP cells with oncolytic influenza virus expressing truncated NS1 protein was reported to eliminate tumor cells [27]. The influence of oncolytic influenza virus on NK cell killing of prostate cancer cells was investigated for the first time in the present study. LNCaP cells infected with ΔNS1 IAV showed enhanced lysis by NK cell when compared to mock-infected LNCaP cells. Blocking HA expression inhibited lysis of ΔNS1 IAV-infected LNCaP cells. Blocking NKp46 on NK cells inhibited lysis of both ΔNS1 IAV-infected and mock-infected LNCaP cells. This indicates that apart from HA, other yet unidentified cellular ligands for NKp46 are expressed by prostate cancer cells in addition to the NKG2D ligands, ULBP1-3. On the other hand, both mock-infected and ΔNS1 IAV-infected DU145 cells were resistant to NK cell lysis as they express high levels of MHC-I, with ΔNS1 IAV infection further increasing MHC-I expression. Blocking MHC-I expression on DU145 cells rendered them sensitive to NK cell lysis and significantly enhanced lysis was observed for ΔNS1 IAV-infected DU145 cells. The combined blocking of MHC-I and HA in DU145 cells inhibited the enhanced NK cell lysis of ΔNS1 IAV-infected DU145 cells to levels comparable to that observed for blocking MHC-I alone.

This is in concordance with previous studies demonstrating that cells infected with wild-type IAV are more sensitive to NK lysis in an HA-dependent manner [11] and that different IAV isolates considerably differ in their ability to stimulate NK cell through HA binding [28]. Interestingly, it was suggested that evolutionary changes (which occurred concurrently with the acquisition of two new potential glycosylation site motifs in HA) observed in HA receptor binding properties of H3N2 viruses may be associated with a reduction in their ability to activate NK cells [28]. This knowledge may be valuable to construct novel oncolytic IAV that exert enhanced activity to stimulate NK cells.

Lytic activity of resting NK cells may be induced by tumor targets in two steps, namely priming and triggering [8, 29]. Consequently, NK evasion strategies for tumor cells depend largely on the prevention of priming (type 1 evasion) and/or failure to trigger (type 2 evasion) [8]. North et al. 2006 [8] proposed that the activation through interaction with a susceptible target cell may provide sufficient stimulus to overcome MHC-I-mediated inhibition in a manner analogous to that mediated by IL-2. The particularly used NK-resistant acute lymphoid leukemia cell line (CTV-1) was able to prime but failed to trigger resting NK cells. The resultant activated NK cells were shown to lyse a broad spectrum of tumor cells [8].

LNCaP cells used in the present study were able to both prime and trigger resting NK cells for target cell lysis. The infection of LNCaP cells with ΔNS1 IAV further enhanced lytic activity of NK cells. However, due to considerable overlap between mechanisms involved in priming and triggering, it was difficult to determine at which step virus infection influenced NK cell activation. In fact, ligands on the surface of CTV-1 cells had to activate/up-regulate CD69, a known triggering receptor on the surface of NK cells, to prime NK cells for lysis of tumor targets [8]. DU145 cells exerted type 1 evasion (fail to prime) but possessed the ability to trigger lytic activity of IL-2-stimulated NK cells. DU145 cells infected with ΔNS1 IAV were able to prime and trigger resting NK cells through binding of virus HA to NKp46, another triggering receptor on the surface of resting NK cells. It is worthy to mention that priming of NK cells with either mock- or ΔNS1 IAV-infected LNCaP/DU145 did not change surface expression of NK cell activating and inhibitory receptors or intracellular expression of perforin and granzyme B. The increased lysis of target cells by NK cells primed with ΔNS1 IAV-infected tumor cells was associated with a rapid ERK activation and increased perforin and granzyme B granule release. Also, the enhanced perforin and granzyme B granule release was specifically mediated by NKp46.

Although it is not altogether new that HA can enhance lysis of virus-infected target cells by NK cells as well as activate NK cells directly [30–32], we have shown for the first time in the context of NK cell priming that the interaction of HA with NKp46 following pre-incubation of NK cells with ΔNS1 IAV-infected tumor cells stimulates NK cells to override MHC-I inhibitory signals.

ΔNS1 IAV is currently being evaluated as an anti-influenza vaccine in clinical phase II trials with early results showing no side effects [33]. The results presented in this report introduce a new perspective for the use of oncolytic ΔNS1 IAV in combination with NK cells for prostate cancer treatment.

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