Selective gene transfer to tumor cells by recombinant Newcastle Disease Virus via a bispecific fusion protein

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Abstract. Much interest exists presently in development of vectors for gene therapy of tumors based on RNA viruses because these viruses replicate in the cytoplasm and do not integrate into DNA. The negative stranded paramyxovirus, Newcastle Disease Virus (NDV) from chicken has the additional advantages of preferential replication in tumor cells and of oncolytic and immunostimulatory properties. We here describe the bispecific fusion protein αHN-IL-2 which binds to NDV, inhibits its normal cell binding property and introduces a new binding specificity for the interleukin-2 receptor (IL-2R). We demonstrate selective gene transfer to tumor cells expressing IL-2R via the bispecific fusion protein when using recombinant NDV carrying as marker gene the enhanced green fluorescence protein (NDFL-EGFP). Hemadsorption (HA) and neuraminidase activities (NA) of the HN protein of NDV were shown to be blocked by αHN-IL-2 simultaneously and the absence of HA-activity of modified NDV was confirmed *in vivo*. Retargeted virus-binding to IL-2R positive tumor cells was not sufficient for the process of cellular infection. It required in addition membrane fusion via the viral F-protein. By modification of recombinant NDV with a bispecific molecule, our results demonstrate a novel and safe strategy for selective gene transfer to targeted tumor cells.

Introduction

RNA viruses are very promising vectors for gene therapy or for oncolytic virotherapy (1,2). Integral to the life cycle of all RNA viruses is the formation of double-stranded RNA (dsRNA). dsRNA activates Toll-like receptor 3 (3) and a spectrum of cellular defence mechanisms including the activation of RNA binding protein kinase (PKR) and the release of interferon α and ß. Tumors are frequently defective in their PKR signaling and interferon response pathways. They are therefore relatively permissive for infection by RNA viruses.

Newcastle Disease Virus (NDV), a negative-strand RNA virus belonging to the family Paramyxoviridae, shows tumor selective replication, which leads to tumor apoptosis and death (4-6). Two main properties of this virus are very interesting. First, NDV has immune stimulatory properties. Clinical post-operative anti-tumor vaccination studies in breast cancer (7) and glioblastoma patients (8) employing NDV-modified autologous tumor cell vaccine (9) showed significant improvement of patient survival (10). Secondly, some NDV strains have oncolytic activity. This can be used to induce tumor lysis (oncolysis) in a very efficient way. A phase I trial of intravenous administration of PV701, an oncolytic NDV strain, in patients with advanced solid cancers was reported recently and a phase II clinical trial has already started (11). Recent studies based on reverse genetics showed that foreign genes can be cloned into this virus (12). A recombinant NDV expressing the enhanced green fluorescent protein (EGFP) was generated independently by two groups by applying reverse genetics techniques (13,14). This suggests that new therapeutic genes can also be cloned into this virus for efficient gene expression in tumor tissues.

Two glycoproteins are expressed at the surface of NDV: the hemagglutinin-neuraminidase (HN) protein and the fusion (F) protein. The HN protein mediates the attachment of NDV to cells via sialic acid-containing receptors at cell surfaces and interacts with the F protein (15) which is responsible for the fusion between the viral envelope and the cellular membrane (16).

Since the sialic acid-containing receptors for NDV are ubiquitously expressed on cell surfaces, NDV binds to every cell, whether it is a normal or a tumor cell. Although NDV is selectively replicating in tumor cells, its binding to normal cells might compromise its therapeutic effect and cause side-effects when administered systemically *in vivo*. To avoid the binding of NDV to normal cells and to selectively target it to tumor cells, we modified the virus by adding a bispecific fusion protein to it. This bispecific protein (named αHN-IL-2) contains a single-chain antibody (scFv) specific for the HN protein linked to the cytokine human interleukin-2. αHN-

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IL-2 blocks the native cell binding activity of NDV and simultaneously provides a new binding specificity for the interleukin-2 receptor (IL-2R) (here used as tumor target). Using IL-2R- cells (Jurkat cells) and IL-2R$^+$ cells (MT-2 cells), we showed previously *in vitro* that the modification of NDV with αHN-IL-2 blocks NDV binding to Jurkat cells and redirects it to bind to MT-2 cells (17). In this study, we investigated the mechanism of virus entrance, the safety issues of such modified viruses and its efficiency for retargeted gene delivery *in vivo*.

Materials and methods

Cells. The human Jurkat CD3 cell line that was sorted from Jurkat cells for CD3-positivity was grown in RPMI-1640 medium supplemented with 5% inactivated foetal calf serum (FCS), 2 mM L-glutamine, 2% HEPES and 100 U/ml penicillin-100 µg/ml streptomycin. MT-2, a HTLV-1 transformed T cell line, was kindly provided by Dr Masahiko Makino (Department of Microbiology, National Institute of Infectious Disease, Tokyo, Japan). MT-2 cells were propagated in the same RPMI-1640 medium supplemented with 10% FCS. All reagents were purchased from Gibco Life Technologies (Karlsruhe, Germany). Both cell lines were maintained at 37˚C in a humidified atmosphere of 5% CO_2.

Antibodies. Mouse anti-HN monoclonal antibody (HN.B mAb, IgG 2a) and mouse anti-F mAb lcii (IgG1), kindly provided by Dr R.M. Iorio (Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School MA, USA) was used in flow cytometry to detect NDV viral antigens on host cell surfaces. Goat F(ab')$_2$ anti-mouse Ig-RPE was obtained from Southern Biotechnology Associates, Inc. (Birmingham, USA). Biotin labelled anti-HLA-A, B, C mAb (clone: W6/32) was kindly provided by Dr Gerd Moldenhauer (Division of Molecular Immunology, German Cancer Research Center, Heidelberg, Germany). Streptavidin-Alexa Fluor® 647 were from Molecular Probes (Molecular Probes, Leiden, The Netherlands).

Recombinant NDV. The recombinant NDFL-EGFP was derived genetically from non-lytic strain NDV-LaSota by reverse genetics as described previously (18). The parent recombinant NDFL$^+$ virus was generated from cDNA clone of NDV-LaSota (18). The virus was propagated in embryonated chicken eggs, harvested from the allantoic fluid, purified by ultracentrifugation, and cryopreserved in aliquots at -70˚C. The virus was quantified by a hemagglutination assay. One hemagglutination unit (HU) is defined as the smallest virus concentration leading to visible sheep erythrocyte agglutination. NDV was UV inactivated for some experiments as described previously (19).

Modification of NDV with bispecific fusion protein αHN-IL-2. Modification of NDV was performed by incubation of NDV with appropriate amounts of the recombinant protein αHN-IL-2 for 1 h on ice. This protein was constructed by fusion of a single-chain antibody cloned from a neutralizing HN specific hybridoma linked to the human cytokine IL-2 as described previously (17).

NDV binding and infection of tumor cells in vitro. Cell suspensions were washed twice with FCS-free RPMI-1640 medium and 1x10^7 cells were incubated with 100 HU (or 10 HU) of NDV or the same doses of modified NDV/αHN-IL-2 in a final volume of 1 ml for 1 h at 37˚C in a CO_2 incubator. During the incubation, cells were shaken every 15 min. The cells were then washed twice and either stained with antibodies and analyzed by FACS to measure the bound virus or the cells were further cultured for 24 h to allow for viral replication. Viral replication was then evaluated as EGFP fluorescence by flow cytometry.

Flow cytometry. Cells, 5x10^5/sample, were used for analysis by a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). All antibodies were diluted in FACS buffer (PBS containing 5% FCS and 0.1% NaN_3). Cells were washed twice with FACS buffer and then incubated with the first antibody. Subsequently the cells were washed and incubated with goat F(ab')$_2$ anti-mouse Ig-RPE for 30 min on ice in the dark. In some experiments, NDFL-EGFP was used to check for viral replication. Replication of NDFL-EGFP in host cells leads to the expression of EGFP which has a high level of fluorescence that can be measured directly by flow cytometry without antibody staining. All FACS data were analyzed with CELLQuest software (Becton Dickinson, Heidelberg, Germany).

Treatment of cells with MG132. MT-2 cells (1x10^6) were washed twice with FCS-free RPMI-1640 medium and then incubated with MG132 (Calbiochem®, Germany) at a final concentration of 50 µM for 1 h at 37˚C. Without washing, the treated cells were infected by NDV using the standard protocol as described above.

Determination of hemadsorption (HA) and neuraminidase (NA) activities. The HA activity of HN proteins of NDV was determined by testing their ability to adsorb human erythrocytes. Human erythrocytes were isolated from buffy coat by centrifugating at 600 x g and then washed with 3-fold volume of isotonic PBS. Erythrocytes (1x10^6) were co-incubated with NDV for 1 h using the above described protocol and analysed with flow cytometry. NA activity of HN proteins of NDV was also determined by flow cytometry. Briefly, 1x10^6 erythrocytes were co-incubated with NDV for 1 h at 37˚C. After 2 washes in PBS, the erythrocytes were incubated with 3.1 µg/ml peanut agglutinin (PNA)-FITC (Sigma, Munich, Germany) for 30 min at room temperature and then analysed by FACS analysis.

Ex vivo modification in vivo. Pathogen-free DBA/2 mice were obtained from Charles River GmbH (Wiga, Germany) and kept under pathogen-free conditions at the DKFZ animal facilities (Heidelberg, Germany). All procedures involving mice was approved by the Animal Care and Use Committee of German Cancer Research Center. Mice received whole body irradiation at 4.5 Gy 1 day before i.p. injection of 1x10^7 human Jurkat or MT-2 cells which were infected *ex vivo* either with 100 HU NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2 for 1h at 37˚C. For the negative controls, NDV was replaced by PBS. After 24 h, cells in the mouse peritoneal

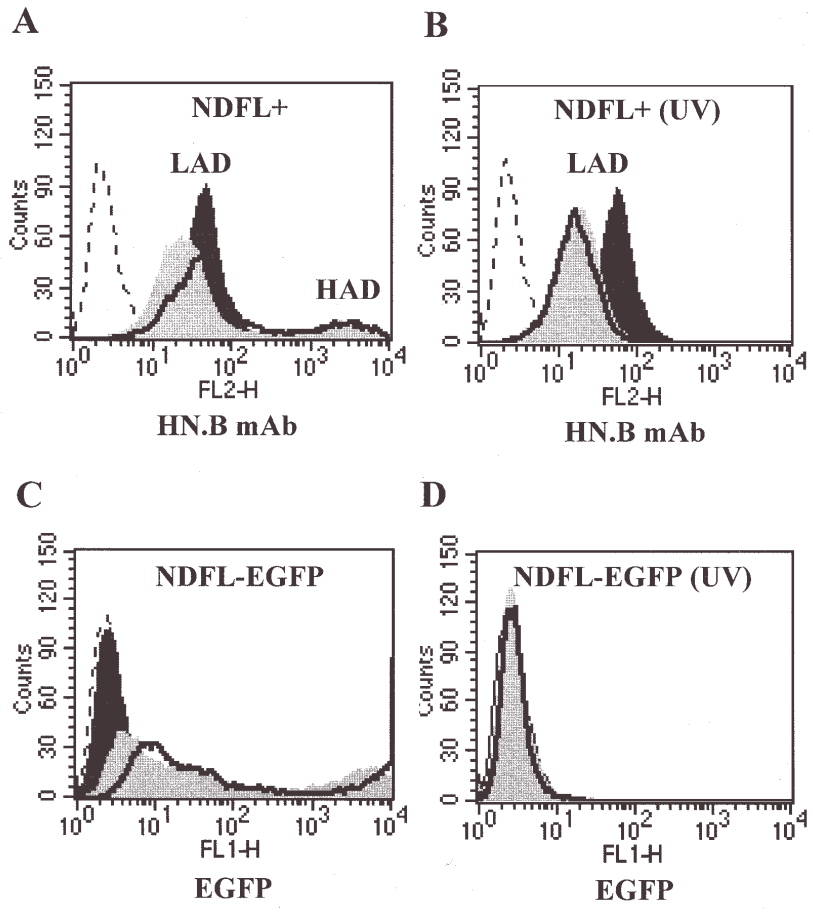

Figure 1. Quantification of NDV binding and replication. MT-2 cells (10^6) were incubated with 10 HU of NDFL$^+$ (A), UV-inactivated NDFL$^+$ (B), NDFL-EGFP (C), or UV-inactivated NDFL-EGFP (D) for 1 h at 37˚C. After washing away unbound virus, cells were examined directly (heavy grey peak), 15 h (light grey peak) or 23 h (bold black line) after incubation. Cells of (A) and (B) were subjected to FACS analysis after staining with HN.B mAb and goat anti-mouse F(ab')$_2$-PE. Cells of (C) and (D) were analyzed for EGFP fluorescence as FL1 by flow cytometry. Dotted lines represent infected cells that were stained with only second Ab (A and B) or cells that were incubated with FCS-free medium (C and D).

cavity were harvested by lavage technique using ice-cold PBS containing 5% FCS. Human cells were differentiated from native mouse cells by two step staining with biotin-anti-HLA-A, B, C mAb and then with streptavidin-Alexa Fluor 647. Viral replication in HLA-I-positive cell population was evaluated by determination of the EGFP fluorescence with FACS analysis.

Results

Correlation between virus replication and transgene expression. Since viral replication is associated with amplification of the two viral surface proteins (HN and F), NDV replication can be evaluated quantitatively by FACS analysis of the infected live cells using anti-HN or anti-F antibodies (Fig. 1A). Twenty-three hours after infection with live virus or after co-incubation with UV-inactivated virus, the human MT-2 leukemia cells were stained with anti-HN mAb. We observed two cell populations: one with high viral antigen density (HAD) and another with low antigen density (LAD). HAD cells were seen only after infection with replication competent live virus and represent cells that had undergone high-copy virus replication. When using UV-inactivated virus, only cell surface bound virus was seen (LAD cells) and no viral replication could be detected (Fig. 1B). To determine whether the fluorescence signal induced by the expression of a transgene (EGFP) inserted into the virus correlates with virus replication, MT-2 cells were also infected with NDV carrying the EGFP transgene (NDFL-EGFP) or the UV-inactivated NDFL-EGFP. Cells with increased EGFP fluorescence intensity were seen only in tumor cells infected with replication-competent live NDFL-EGFP (Fig. 1C). The results demonstrate that high expression of the EGFP gene is correlated with virus replication so that high EGFP fluorescence can serve as surrogate marker for virus replication.

Quantitative and kinetic aspects of NDV modification by αHN-IL-2. To test the effect of the bispecific fusion protein αHN-IL-2 on the binding specificity of NDV, either 10 HU or 100 HU NDFL-EGFP was pre-incubated with different amounts of αHN-IL-2 for 1 h on ice. Then this modified virus (NDFL-EGFP/αHN-IL-2) was co-incubated with IL-2R$^-$ (Jurkat) and IL-2R$^+$ (MT-2) cells for 1 h at 37˚C. At both virus concentrations we saw a retargeted binding of the NDFL-EGFP/αHN-IL-2 to the MT-2 cells (Fig. 2). The binding of NDFL-EGFP/αHN-IL-2 to Jurkat cells was neutralized to ≥99% at concentrations of αHN-IL-2 of 10 μg/ml for 10 HU virus (Fig. 2A, left) and of 20 μg/ml for 100 HU virus (Fig. 2A,

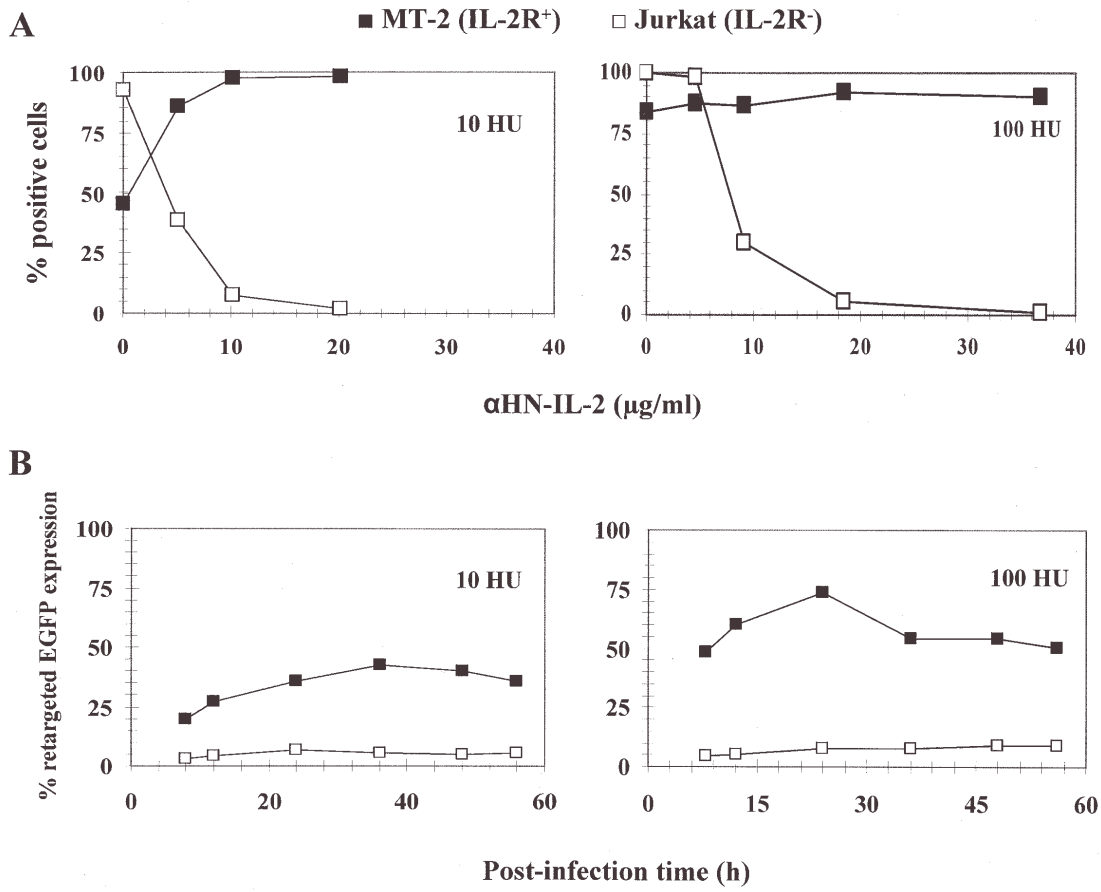

Figure 2. Quantitative and kinetic aspects of NDV modification by αHN-IL-2. (A), 10 HU (left) or 100 HU per 10^7 cells (right) of NDFL-EGFP were pre-incubated with different amounts of αHN-IL-2 for 1 h on ice and then further incubated with 10^6 Jurkat or MT-2 cells for 1 h at 37˚C. After unbound virus was washed away, the cells were stained with anti-F mAb and goat anti-mouse $F(ab')_2$-PE before FACS analysis. (B), 10 HU per 10^7 cells of NDFL-EGFP/αHN-IL-2 were pre-incubated with 9.2 μg/ml of αHN-IL-2 for 1 h on ice (left), or 100 HU per 10^7 cells of NDFL-EGFP were modified with 12 μg/ml αHN-IL-2 (right). The modified viruses were then further incubated with 10^6 Jurkat or MT-2 cells for 1 h at 37˚C. After unbound virus was washed away the cells were further cultured. At different time-points after infection, aliquots of infected cells were analyzed by FACS to detect the EGFP fluorescence. The percentage of retargeted EGFP expression was calculated as the ratio of the percentage of positive cells after infection with the modified virus to the percentage of positive cells after infection with the native virus.

right). A 2.2- and 1.1-fold higher binding of the modified virus was seen to MT-2 cells in comparison to the native virus when using 10 HU or 100 HU, respectively.

We next tested EGFP gene transfer via recombinant NDFL-EGFP/αHN-IL-2. EGFP fluorescence was analysed by flow cytometry at different times after infection of Jurkat and MT-2 cells. Maximal retargeted gene transfer in MT-2 cells was observed at 36 h and 24 h post-infection when using 10 HU (Fig. 2B, left) and 100 HU (Fig. 2B, right) of NDFL-EGFP/αHN-IL-2, respectively. At these two time-points, relative retargeted EGFP expression was observed in 43 and 74% of the MT-2 cells infected with a virus load of 10 HU and 100 HU respectively. In contrast, <10% of the IL-2R negative Jurkat cells expressed EGFP, irrespective of the virus dose used.

Role of the F protein for retargeted virus entry. To study possible mechanisms of cell entry of the modified virus, we first used the proteasome inhibitor MG132. When added at a concentration of 50 μM to the MT-2 cells during infection, we observed no effect on either native NDV infection nor on retargeted virus entry (Fig. 3A). This suggests that the retargeted virus entry to IL-2R+ cells is not mediated through internalization via the IL-2 receptor. We further tested the effect of a mAb specific for the F protein under conditions of retargeted virus entry. As shown in Fig. 3B, retargeted EGFP expression was strongly inhibited by the anti-F mAb in a dose-dependent manner. The data demonstrate: i) that retargeted virus binding to the IL-2R is not sufficient for the process of cellular infection; and ii) that membrane fusion involving the F protein plays a role for retargeted virus entry.

Stability of modified NDFL-EGFP/αHN-IL-2 in vitro. To test for the stability of native virus and of the complex between NDV and αHN-IL-2, aliquots of NDFL-EGFP or NDFL-EGFP/αHN-IL-2 were incubated in serum-free RPMI-1640 medium at 37˚C for different time periods (4-76 h). After each time-point, aliquots were kept at -70˚C until they were added to Jurkat and MT-2 cells. As can be seen from Fig. 4A, more than 50% of virus activity of NDFL-EGFP was lost after 24-h incubation at 37˚C. After 3 days at 37˚C, viral activity of NDFL-EGFP was lost completely. The virus activity

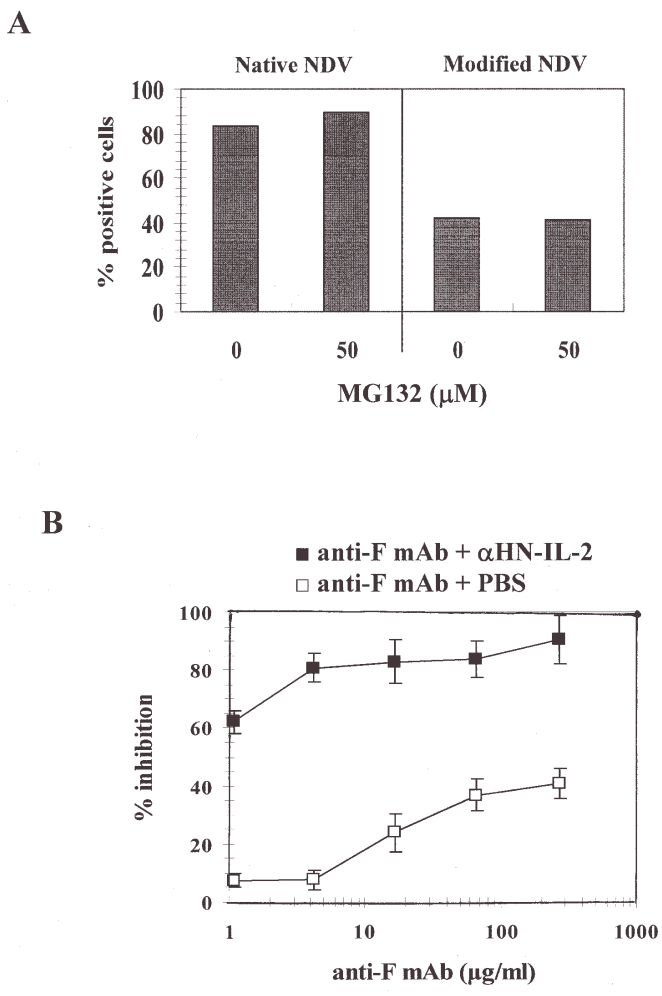

Figure 3. Mechanism of infection by the modified virus NDFL-EGFP/αHN-IL-2. (A), Effect of MG132 on the native NDV infection and the retargeted EGFP expression. MT-2 cells (10^6) in serum-free medium were incubated with MG132 at a final concentration of 50 μM or an equivalent volume of DMSO for 1 h at 37˚C. The treated cells were then infected by NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2 for 24 h at 37˚C. EGFP fluorescence was detected by FACS analysis. (B), Role of the F protein of NDV. To block the F protein of NDV, 10 HU of NDFL-EGFP were pre-incubated with different amounts of anti-F mAb before being further incubated with 12 μg/ml of αHN-IL-2 (■) or PBS (□) for 1 h on ice. This double modified virus was added to 10^6 MT-2 cells for 24 h and the EGFP signal was analyzed by FACS. The percentage of inhibition was calculated as the ratio of the percentage of positive cells after infection with the double modified NDFL-EGFP/anti-F mAb/αHN-IL-2 (or PBS) and the percentage of positive cells after infection with the native virus.

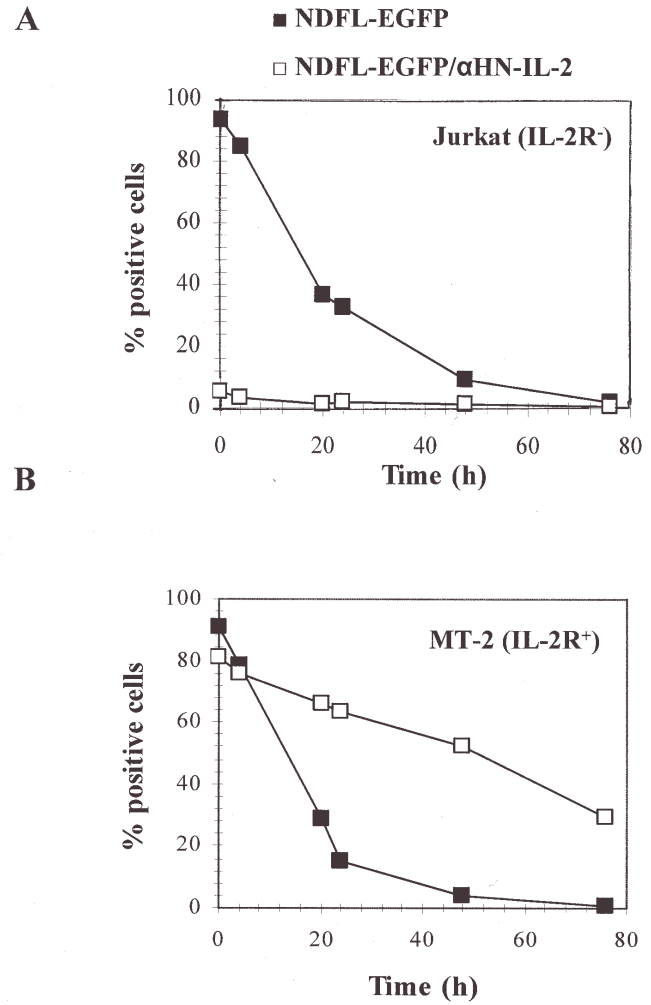

Figure 4. Stability of the modified NDFL-EGFP/αHN-IL-2 *in vitro*. Aliquots of NDFL-EGFP (■) and modified NDFL-EGFP/αHN-IL-2 (□) in the presence of serum-free RPMI-1640 medium were stored at 37˚C for 0, 4, 20, 24, 48 or 76 h. After each time-point, aliquots of virus were kept at -70˚C until they were added to 10^6 Jurkat (A) and 10^6 MT-2 (B) cells for 24 h at 37˚C. The percentage of EGFP-expressing cells was determined by FACS analysis.

of the modified NDFL-EGFP/αHN-IL-2 in IL-2R$^+$ MT-2 cells decreased more slowly compared to native NDFL-EGFP (Fig. 4B). Thus, the bispecific fusion protein αHN-IL-2 did not dissociate from the virus particles, indicating a high stability *in vitro* of the complex between NDV and αHN-IL-2.

Inhibition of HA and NA activities of NDV by αHN-IL-2 in vitro and in vivo. HA activity of native and modified NDV was determined by incubating the viruses with erythrocytes followed by staining with the F-specific mAb and flow cytometry analysis. NA activity was quantified by staining the co-incubated erythrocytes with PNA-FITC which binds to the cleaved sialic acid receptor sites. Fig. 5A shows that αHN-IL-2 inhibited both HA and NA activities in a dose-dependent manner. The 50% inhibitory concentration of αHN-IL-2 was 0.4 μg/ml and 1.5 μg/ml for NA and HA activities, respectively. These results demonstrate that αHN-IL-2 simultaneously blocks HA and NA activities of the HN protein of NDV.

The inhibition of HA activity of NDV by αHN-IL-2 was also tested *in vivo* using the recombinant strain NDFL-EGFP. DBA/2 mice were injected i.v. with 5000 HU of either NDFL-EGFP or NDFL-EGFP/αHN-IL-2. The mice were bled after 1 h or 24 h and the erythrocytes stained with HN-specific mAb. As can be seen in Fig. 5B, 22% of mouse erythrocytes bound the native virus after 1 h. At 24 h, 10% of erythrocytes still had the native virus at their cell surface. Following injection of the modified virus we did not detect any virus bound to the erythrocytes.

Retargeted gene expression in vivo. To evaluate retargeted gene transfer *in vivo*, human MT-2 or Jurkat cells were infected either with native or modified NDV *ex vivo* and then given i.p.

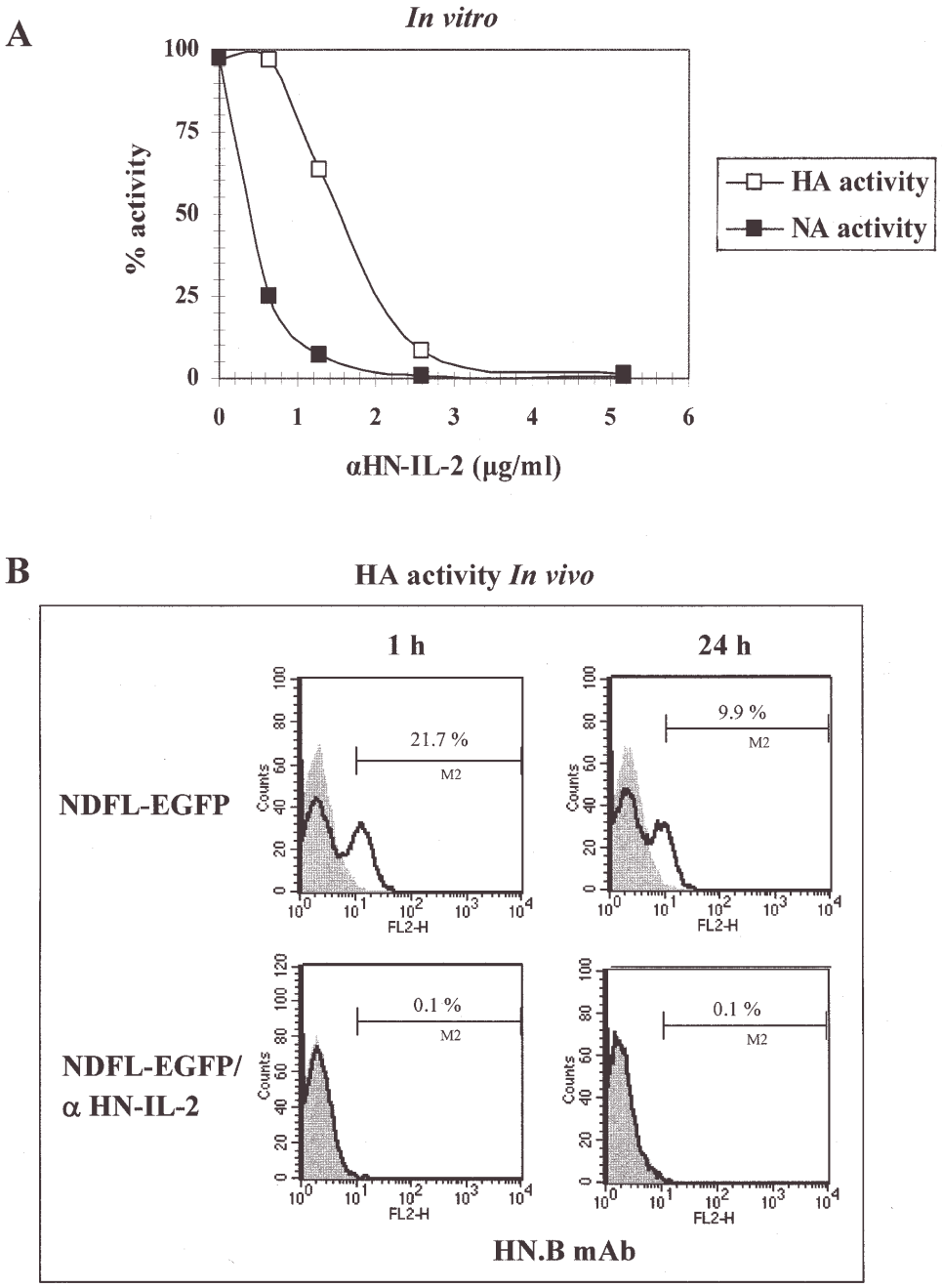

Figure 5. Inhibition of HA and NA activities of NDV by αHN-IL-2. (A), 10^6 human erythrocytes isolated from buffy coat were incubated with 10 HU NDFL-EGFP or same dose of NDFL-EGFP which was modified with different amounts of αHN-IL-2 for 1 h at 37°C. After unbound virus was washed away, the cells were stained with anti-F mAb and goat anti-mouse F(ab')$_2$-PE (□) or with PNA-FITC (■) before FACS analysis. (B), DBA/2 mice were injected i.v. with 5000 HU of either native NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2. At 1 h and 24 h-post injection, the mice were bled and 10^6 isolated erythrocytes were stained with HN.B mAb and goat anti-mouse F(ab')$_2$-PE before FACS analysis.

to DBA/2 mice which were γ-irradiated with 4.5 Gy one day before injection. After 24 h, peritoneal cells were harvested and the human MT-2 and Jurkat cells were stained with anti-HLA-A,B,C mAb to differentiate them from mouse cells. Then EGFP fluorescence was analyzed by FACS analysis. As shown in Fig. 6, 7.0% of MT-2 cells and 0.0% of Jurkat cells were infected by NDFL-EGFP/αHN-IL-2. In contrast, using native NDFL-EGFP there was a similar percentage of cells (31-34%) infected when using the two tumor lines.

Interestingly, MT-2 cells when infected with both native and modified NDV upregulated *in vivo* the expression of HLA class I molecules in comparison to cells treated with PBS instead of virus. As can be seen from the mean fluorescence intensities (Table I), there was a connection between virus replication and HLA class I upregulation. There was no upregulation in Jurkat cells treated with modified virus in which case normal infectivity was blocked.

Discussion

We here demonstrate that NDV, when modified by the bispecific fusion protein αHN-IL-2, is an efficient vector for

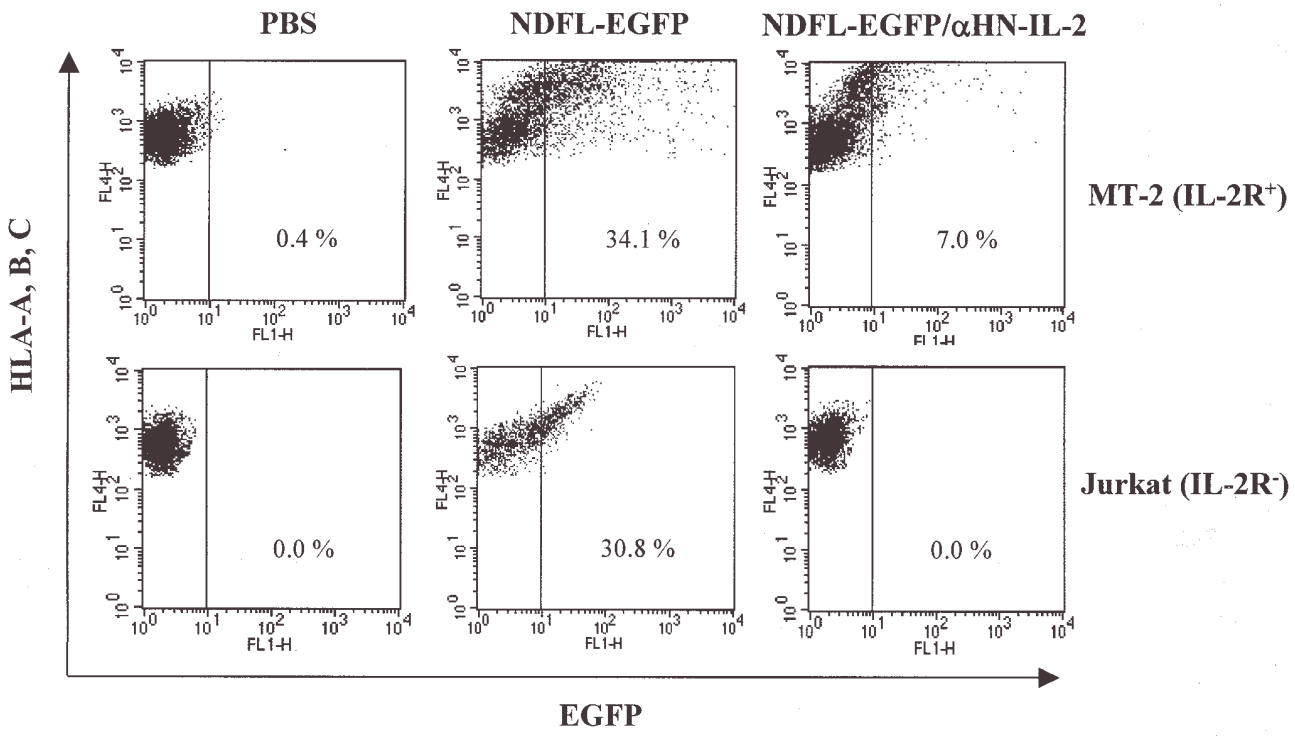

Figure 6. *In vivo* retargeted gene transfer of EGFP via NDFL-EGFP by αHN-IL-2 to tumor cells after *ex vivo* infection. DBA/2 mice were given whole body irradiation with 4.5 Gy 1 day before i.p. injection of 10^7 human MT-2 or Jurkat cells which were infected *ex vivo* either with 100 HU NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2 for 1 h at 37˚C. PBS was used as negative control instead of NDV. After 24 h, cells from the mouse peritoneal cavity were harvested by lavage technique using ice-cold PBS containing 5% FCS. Human cells were differentiated from native mouse cells by staining with biotin-anti-HLA-A, B, C mAb and streptavidin-Alexa Fluor® 647. Viral replication in the HLA class I-positive cell population was evaluated by determination of the EGFP fluorescence by FACS analysis.

Table I. *In situ* HLA class I upregulation on human tumor cells upon NDV infection.

	NDFL-EGFP/αHN-IL-2	NDFL-EGFP	PBS
MT-2 cell	1455	2311	620
Jurkat cells	673	981	658

For experimental details see legend to Fig. 6. The values represent mean fluorescence intensity after staining for expression of HLA-A,B,C molecules at the cell surface followed by FACS analysis.

selective gene transfer into tumor cells. Vector delivery appears to be specific both *in vitro* and *in vivo* for cell lines expressing the defined targeted cell surface molecule.

In this study, we selected the IL-2R as a potential tumor target molecule. Adult T-cell leukemia (ATL) cell lines, which are infected with human T-cell leukemia virus-I (HTLV-I), uniformly express large amounts of IL-2Rα chains (20). The observation that IL-2Rα is not expressed by resting normal cells, but is expressed by a proportion of the abnormal cells in certain forms of lymphoid neoplasia, provides the rationale for the use of the IL-2Rα as a target (21,22). MT-2, a HTLV-1 transformed T cell line, showing high expression of IL-2Rα (20) was used for the retargeting experiments. Jurkat CD3 cells, which do not show any IL-2Rα after staining with human CD25 mAb, were used as negative controls. Using this system, we observed a significant effect of αHN-IL-2 on the tropism of NDV infection. The bispecific molecule abolished the viral native binding capacity, and it provided simultaneously a novel high-affinity binding site.

Several approaches have been reported to improve the targeting capacity of virus vectors. Measles virus (MV), another negative-strand RNA virus of the family Paramyxoviridae, which binds to its native receptor CD46 was genetically modified to target additional novel receptors [e.g. epidermal growth factor (23), CD20 (24), CD38 (25), and human carcinoembryonic antigen (26)] on tumor cells by fusing antigen specific scFv antibody binding sites to the C-terminus of the hemagglutinin of MV. A limitation of this strategy was that the native binding site was not totally abolished. Bispecific reagents have been used in different virus vectors, such as adenovirus (Ad) (27), and adeno-associated virus (AAV) (28) to limit gene transfer to the target cells. By employing a bispecific scFv Ab directed against the renal cell carcinoma (RCC)-associated G250 protein and the Ad fiber knob domain, Jongmans *et al* showed the retargeting of a recombinant Ad to RCC cells with the highly tumor-specific G250 protein as the target (29).

During normal infection of a cell by NDV, interactions between the HN and F proteins in a virus type-specific manner is crucial for efficient membrane fusion and infection.

Binding of the HN protein to its sialic acid-containing cellular receptor is thought to induce a conformational change near the hydrophobic surface of the HN protein thereby triggering the activation of the F protein, which initiates membrane fusion (30,31). Being aware of this, we were curious to find out whether there was also a role for the F protein under our conditions of retargeted virus entry. Alternatively, virus entry could be mediated through internalization of the targeted IL-2R complex. Yu and Malek found that the proteasome inhibitor MG132 at a final concentration of 50 μM impaired the internalization of IL-2-IL-2R and prevented the lysosomal degradation of IL-2 (32). We showed before that retargeted gene delivery by the modified virus in IL-2R$^+$ MT-2 cells is blocked by human recombinant IL-2. This corroborates the specificity of retargeted binding via IL-2R (17). Here we demonstrate that retargeted virus entry could be blocked by anti-F mAb but not by the proteasome inhibitor MG132. This suggests that not only binding of NDV/αHN-IL-2 to the IL-2R is required but also fusion via the F protein to facilitate virus entry.

We further demonstrate that the modification of NDV with bispecific protein could simultaneously block the hemadsorption and neuraminidase activities of the HN protein. The HN protein of NDV is a multifunctional enzyme with three known functions: i) HA activity recognizes sialic acid-containing glycoproteins on host cell surfaces; ii) NA activity cleaves sialic acid residues from newly formed glycoproteins inside the host cell to prevent self-aggregation and ensure proper virus budding and iii) a fusion promoting activity facilitates interaction between HN and F (31,33). Crystal structure analysis confirmed that there is only one site on the HN protein with dual functions, the HA and NA activities (33). By possessing these activities, NDV can cause hemagglutination of red blood cells (35-40). The addition of αHN-IL-2 to NDV is shown here to prevent both HA and NA activities simultaneously.

One of the advantages of the modified NDV is the prevention of aggregation of red blood cells upon systemic administration, suggesting less side-effects. By blocking the HN protein with αHN-IL-2, the HA and NA activities of native NDV were abolished completely with human erythrocytes (data shown) as well as with mouse or sheep erythrocytes (data not shown). αHN-IL-2 also inhibited the hemolytic activity induced by the virulent strain NDV-Italien. The decrease in the hemolytic activity was directly proportional to the αHN-IL-2 concentration (data not shown). Erythrocyte aggregation is one of the main determinants influencing blood circulation at low shear rates by increasing blood viscosity and inducing 'sludging' in the capillary (41). An increase in erythrocyte aggregation was found to be associated with cardiovascular risk factors such as hypertension (42) and hyperlipoproteinemia (43). A phase I trial of intravenous administration of the oncolytic NDV (PV701) showed that hypertension was observed in 5/7 patients. In their study this was suggestive of a vasospasm effect, but no ECG changes were observed. Prophylaxis with antihistamines was found to be ineffective (44). Based on our present study, erythrocyte aggregation by NDV PV701 probably contributes to the hypertension. According to our results, a modified NDV appears to be safer for systemic administration.

In order to extend retargeted gene expression studies to *in vivo* conditions, human MT-2 or Jurkat cells were co-incubated *ex vivo* for 1 h by NDFL-EGFP/αHN-IL-2. These cells were then injected into the peritoneal cavity of mice. Retargeted EGFP fluorescence was observed in MT-2 cells, but not in Jurkat cells. The efficiency of virus infection was, however, reduced compared to the native virus. Previous *in vitro* studies demonstrated that human tumor cell modification by NDV leads to upregulation of HLA class I and cell adhesion molecules, induction of IFN-α, -ß and chemokines and, finally to apoptosis (19). In this study we verified the upregulation of HLA class I molecules upon NDV infection *in vivo*. Hence, by retargeting NDV with a therapeutic gene to the site of a tumor or its metastases, synergistic therapeutic effects can be expected from the therapeutic gene product and from the immunostimulatory properties of this virus.

In conclusion, we demonstrate that modification of recombinant NDV with a bispecific fusion protein can abolish side-effects of the virus on erythrocytes and at the same time redirect the virus to a new tumor target. Significant gene transfer is shown *in vitro* and *in vivo* in retargeted tumors.

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