# Tumor-targeted gene transfer *in vivo* via recombinant Newcastle disease virus modified by a bispecific fusion protein

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**Abstract.** Previously we have demonstrated that a recombinant Newcastle disease virus (NDV) carrying the transgene EGFP can be retargeted to IL-2 receptor positive tumor cells by a bispecific fusion protein αHN-IL-2 *in vitro*. The purpose of the present study was to investigate the specificity and efficiency of gene delivery to tumor cells *in vivo* via this modified RNA virus. Prior *ex vivo* infection of murine lymphoma cells by the modified virus resulted in selective EGFP expression in IL-2R$^+$ target tumor cells *in vivo*. Direct fluorescence microscopy and immunohistology showed viral replication in target positive tumor tissue resulting in much more EGFP expression than in target negative tumor tissue, 24 h after intratumoral injection of the αHN-IL-2 modified NDV. A quantitative real-time RT-PCR for EGFP mRNA. confirmed the selective gene expression in IL-2R$^+$ tumor cells. Biodistribution studies showed that EGFP transgene delivery was reduced by 35-100% in liver, spleen, kidney, lung and thymus by the modified virus, while 98% of the transgene was delivered to IL-2R$^+$ tumors. In conclusion, the modification of NDV by the bispecific protein does not compromise severely the efficiency of gene delivery into IL-2R-positive tumors, but greatly reduces viral gene expression in IL-2R-negative tumors and in normal tissues.

## Introduction

Newcastle Disease Virus (NDV), an avian negative-strand RNA virus of the family Paramyxoviridae, has characteristics which are very attractive for cancer therapy (1-3). First, it shows tumor selective replication. Integral to the life cycle of all RNA viruses is the formation of double-strand RNA (dsRNA). dsRNA activates Toll-like receptor 3 and a spectrum of cellular defense mechanisms including the activation of RNA binding protein kinase (PKR) and the release of interferon α/ß (4-6). Since tumor cells are frequently defective in their PKR signaling and interferon response pathways, they are relatively permissive for infection by RNA viruses. Second, its replication occurs in the cytoplasm of host cells and thus avoids potentially deleterious interactions with the genomic DNA. Third, reverse genetics techniques allow the modification of the viral RNA genome. Several recombinant NDV carrying transgenes, such as enhanced green fluorescent protein (EGFP) or human secreted alkaline phosphatase were generated (7-9). Fourth, clinical applications of this virus are numerous. They are mainly based on two strategies. One is exploiting the immune stimulatory properties of NDV in the form of autologous tumor cell vaccine (ATV-NDV) to treat solid tumors. Clinical studies based on this strategy revealed significant improvement of patient survival (10-13). Another clinical application makes use of the oncolytic properties of some NDV strains (14,15).

Two main drawbacks limit the use of NDV *in vivo*. First, NDV binds to every cell, whether it is a normal or a tumor cell since the sialic acid-containing receptors for NDV are ubiquitously expressed on cell surfaces. Although NDV shows a preferential replication in tumor cells, its binding to normal cells might compromise its therapeutic effect and cause side effects when administered systemically *in vivo*. Second, *in vivo* administration of very high doses of NDV could produce toxic side effects.

To avoid the binding of NDV to normal cells and to selectively target it to tumor cells, we suggest to modify its properties by adding a linker molecule to its surface. We have previously shown *in vitro* that, by doing so, a recombinant NDV could be retargeted selectively to marker-defined tumor cells (16,17). For this, we pre-incubate the virus with a specially designed recombinant bispecific fusion protein (αHN-IL-2). As described before, αHN-IL-2 binds to the HN molecule of the virus, thereby neutralising it, and to the IL-2 receptor (IL-2R), which is used as a tumor marker. Thus the protein assures the selective binding of the virus to the targeted tumor cells.

In this study, we investigated *ex vivo* and *in vivo* the delivery of genes to tumor and normal tissues via such a modified NDV vector.

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## Materials and methods

*Cells, antibodies and bispecific fusion protein*. The metastatic mouse tumor cell line p11-R-Eb was obtained after i.p. passages of the non-metastatic Eb lymphoma cells into DBA/2 mice (18). The cells were propagated in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) at 37˚C in a humidified atmosphere of 5% $CO_2$.

Polyclonal rabbit anti-NDV serum was prepared by immunizing rabbit four times s.c. with 40000 HU/ml NDV-Ulster mixed 1:1 with incomplete Freund's adjuvant. Pre-immune serum and the antiserum were heat-inactivated (for 45 min at 56˚C), aliquoted and stored at -20˚C.

The bispecific fusion protein αHN-IL-2 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 (16).

*Recombinant NDFL-EGFP*. The recombinant NDFL-EGFP was derived genetically from non-lytic strain NDV-LaSota by reverse genetics as described previously (7,16,19). 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.

*In vitro infection of tumor cells with NDV*. Cell suspensions were washed twice with FCS-free RPMI-1640 medium and $1x10^6$ cells were incubated with 10 HU of NDV or the same doses of modified NDV/αHN-IL-2 in a final volume of 100 µl 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 further cultured for 24 h to allow for viral replication. Viral replication was then evaluated as EGFP fluorescence by flow cytometry and FACS data were analysed with CellQuest software (Becton-Dickinson, Heidelberg, Germany).

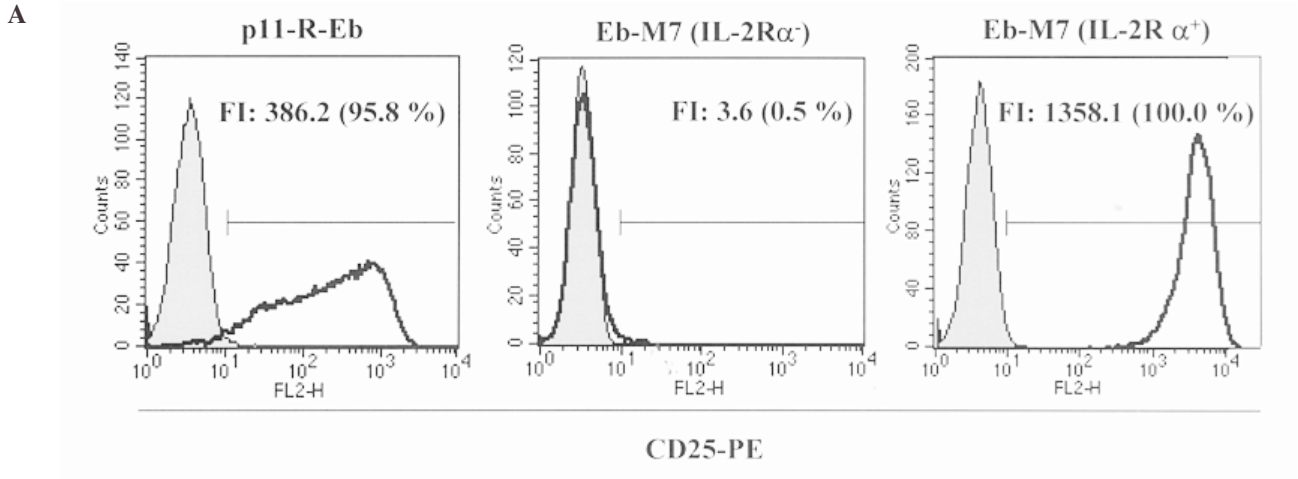
*1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) labeling*. To differentiate the Eb-M7 cells from the mouse peritoneal cavity cells, Eb-M7 cells were labelled with DiR (Molecular Probes, Leiden, The Netherlands) which has absorption and emission maxima in the near infrared region, where non-labelled cells are optically transparent. After 2 washes, the Eb-M7 cells were suspended at a density of $1x10^6$/ml in serum-free medium. Two µl of a 50 mg/ml stock solution of DiR was then added to the cells. After incubation for 15 min at 37˚C, the cells were washed twice with pre-warmed serum-free medium.

*Ex vivo infection of tumor cells with NDV*. 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 the German Cancer Research Center. DiR-labeled Eb-M7 cells ($1x10^7$) which were incubated either with 100 HU NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2 for 1 h at 37˚C *in vitro* were then injected into the peritoneal cavity of mice that received whole body irradiation at 4.5 Gy 1 day before (the procedure here is named *ex vivo* modification). For the negative controls, NDV was replaced by PBS. After 24 h, cells in mouse peritoneal cavity were harvested by lavage technique using ice-cold PBS containing 5% FCS. The EGFP fluorescence of DiR-positive cells from the mouse peritoneal cavity was analysed by flow cytometry.

*Intratumoral EGFP expression and immunohistochemistry (IHC) with NDV specific antibody*. DBA/2 mice received s.c. implants of $5x10^6$ syngeneic Eb-M7 (IL-2Rα$^+$) or Eb-M7 (IL-2Rα$^-$) cells/100 µl PBS. One week after tumor cell implantation, NDFL-EGFP (100 HU/100 µl) or modified NDFL-EGFP/αHN-IL-2 (100 HU/100 µl) or an equivalent volume of PBS was injected intratumorally. To assess EGFP expression and virus replication, mice were euthanized at 1 or 24 h after virus injection. Tumors were harvested and fixed overnight in PBS containing 1.5% paraformaldehyde and 20% sucrose. Section (6-µm) were prepared from frozen tissues previously embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe B.V., distributed by Vogel GmbH, Giessen, Germany). EGFP was visualized by fluorescence microscopy using an EGFP filter. Sections on slides coated with 3-triethoxysilylpropylamine (Sigma, Taufkirchen, Germany) were subjected to IHC using rabbit polyclonal anti-NDV serum (1:800 dilution), followed by chicken anti-rabbit Ig (H+L) Alexa Fluor® 594 (1:400 dilution) (Molecular Probes). IHC sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

*Quantification of the in vivo EGFP expression by quantitative real-time reverse transcriptase-polymerase chain reaction (RRT-PCR)*. DBA/2 mice bearing either Eb-M7 (IL-2Rα$^+$) or Eb-M7 (IL-2Rα$^-$) tumors were injected intratumorally either with 100 HU/100 µl of NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2. Other DBA/2 mice bearing both Eb-M7 (IL-2Rα$^+$) and Eb-M7 (IL-2Rα$^-$) tumors on their right and left flanks respectively were injected i.v. with 5000 HU of NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2 into the tail vein. Twenty-four hours after injection, i.v. injected-mice were perfused transcardially through the aorta with DEPC-treated PBS until lung, liver and kidney turned pale. Tumors (IL-2Rα$^+$ and IL-2Rα$^-$), liver, spleen, kidney, lung and thymus from i.v. injected-mice and tumors from intratumoral-injected mice were removed and immediately submerged in RNAlater RNA Stabilization solution (Qiagen GmbH, Hilden, Germany). RNA was extracted following the Qiagen RNeasy procedure for animal tissue (Qiagen). Then, first-strand cDNA was synthesized from it using the SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen).

RRT-PCR was carried out using a Gene Amp® 5700 Sequence Detection System (Applied Biosystems GmbH, Weiterstadt, Germany). Amplifications were run in separate tubes to illustrate relative quantitation of EGFP and normalized with an endogenous control, mouse ß-actin. Primers for EGFP (sense, 5'-ATC ATG GCC GAC AAG CAG AAG AAC-3'; antisense, 5'-GTA CAG CTC GTC CAT GCC GAG AGT-3') (20) and mouse ß-actin (sense, 5'-ACG GCC AGG TCA TCA CTA TTG-3'; antisense, 5'-AGG ATT CCA TAC CCA AGA AGG AA-3') were synthesized by the German



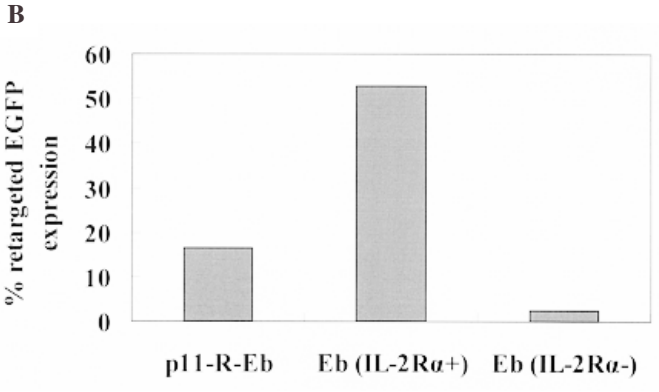




Figure 1. (A), IL-2Rα expression on parent p11-R-Eb and cloned Eb-M7 cell lines. $10^6$ p11-R-Eb, Eb-M7 (IL-2Rα$^+$) or Eb-M7 (IL-2Rα$^-$) cells were washed twice with FACS buffer (PBS containing 5% FCS and 0.1% $NaN_3$) and then incubated with mouse mAb CD25-PE for 30 min on ice in the dark. After being washed twice with FACS buffer, cells were analyzed by flow cytometry with CellQuest software. Cells without staining (grey peak) were used as negative controls. (B), Enhanced-retargeted EGFP expression in Eb-M7 (IL-2Rα$^+$) cells *in vitro*. NDFL-EGFP (10 HU) was pre-incubated with 12 μg/ml of αHN-IL-2 for 1 h on ice, then further incubated with $10^6$ p11-R-Eb, Eb-M7 (IL-2Rα$^+$) or Eb-M7 (IL-2Rα$^-$) cells for 1 h at 37˚C. After being washed twice with medium, cells were further cultured for 24 h. Parallel experiments were done with same dose of native NDFL-EGFP. Cells were analyzed for EGFP fluorescence by flow cytometry. The percentage of retargeted EGFP expression was calculated as the ratio of the percentage of EGFP-positive cells infected with the modified NDFL-EGFP/αHN-IL-2 to the percentage of EGFP-positive cells infected with the native NDFL-EGFP.

Cancer Research Center. Each RRT-PCR was performed in a 25 μl reaction mixture containing 17.5 μl Master Mix (qPCR™ Core Kit for SYBR® Green I, Eurogentec, Köln, Germany), 300 nM of each primer for EGFP or 300 nM (sense) and 900 nM (antisense) for ß-actin. cDNA from PBS-treated mice was used as negative controls and was included in each set of experiments. A common threshold was set in the exponential phase of the PCR reactions to determine a specific threshold cycle ($C_T$) number for each sample. Comparative $C_T$ method was used to relatively quantify the EGFP expression.

## Results

*Cloning of Eb-M7 (IL-2Rα$^+$) and Eb-M7 (IL-2Rα$^-$) cells*. To prove the principle of retargeted virus infection of tumors we decided to first select target positive and target negative tumor cell lines from the mouse lymphoma p11-R-Eb. This cell line contains IL-2Rα positive and negative cells as revealed by staining with mouse mAb CD25-PE and FACS analysis (Fig. 1A). After three rounds of sorting we were able to select a high IL-2Rα expressing cell population (here called Eb-M7 IL-2Rα$^+$) and a very low IL-2Rα-expression cell population (here called Eb-M7 IL-2Rα$^-$). One hundred percent of Eb-M7 (IL-2Rα$^+$) cells were positive for IL-2Rα and the mean fluorescence intensity (FI) for IL-2Rα was enhanced 3.5-fold in comparison to the parental p11-R-Eb cells. In contrast, the Eb-M7 (IL-2Rα$^-$) cells were completely negative for IL-2Rα expression. Both cell populations were stable with regard to their expression of IL-2R over many culture passages.

*Enhanced-retargeted gene transfer via NDFL-EGFP by αHN-IL-2*. We first tested the effect of the bispecific fusion protein αHN-IL-2 on the tropism of NDV. For that, 10 HU of NDFL-EGFP was pre-incubated with 12 μg/ml of αHN-IL-2 for 1 h on ice. This modified virus was then used to infect p11-R-Eb, Eb-M7 (IL-2Rα$^+$) and Eb-M7 (IL-2Rα$^-$) cells for 24 h at 37˚C. FACS analysis (Fig. 1B) showed that the relative EGFP expression was enhanced by a factor 3.2 in the Eb-M7 (IL-2Rα$^+$) cell population in comparison to the parental p11-R-Eb cells. The relative EGFP expression in the Eb-M7 (IL-2Rα$^-$) cell population was only 2.6%. We conclude that virus binding and replication was improved in IL-2Rα$^+$ cells and reduced considerably in the IL-2Rα$^-$ cells.

*Retargeted gene expression in vivo after ex vivo infection*. To evaluate *in vivo* the retargeted gene transfer, DiR-labeled Eb-M7 (IL-2Rα$^+$) or Eb-M7 (IL-2Rα$^-$) cells were infected *ex vivo* either with native or modified NDFL-EGFP. They were then given i.p. to DBA/2 mice which were γ-irradiated with 4.5 Gy one day before injection. After 24 h, peritoneal cells were harvested and DiR-positive cells were gated and analyzed by flow cytometry for EGFP expression. As shown

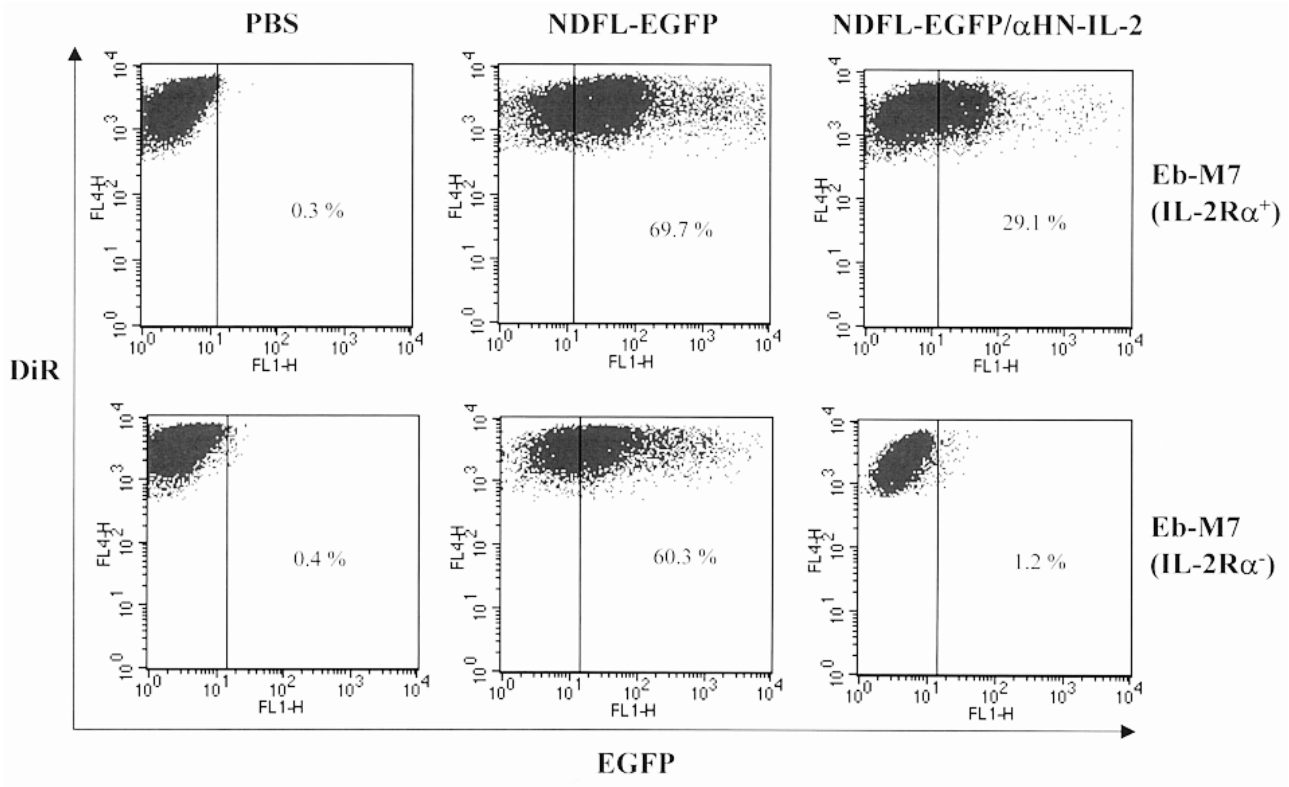



Figure 2. *In vivo* retargeted EGFP transgene expression via NDFL-EGFP by αHN-IL-2 to tumor cells after *ex vivo* modification. DBA/2 mice were given whole body irradiation at 4.5 Gy 1 day before i.p. injection of $10^7$ DiR-labeled Eb-M7 (IL-2Rα$^+$) or Eb-M7 (IL-2Rα$^-$) cells which were incubated *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 virus. After 24 h, cells in mouse peritoneal cavity were harvested by lavage technique using ice-cold PBS containing 5% FCS. DiR-positive cell population was evaluated by determination of the EGFP fluorescence by FACS analysis.

in Fig. 2, 29.1% of Eb-M7 (IL-2Rα$^+$) cells and only 1.2% of Eb-M7 (IL-2Rα$^-$) cells were infected by NDFL-EGFP/αHN-IL-2. In contrast, when using native NDFL-EGFP there was almost a similar percentage of Eb-M7 (IL-2Rα$^+$) and Eb-M7 (IL-2Rα$^-$) cells (69.7-60.3%) infected. We conclude that *ex vivo* infection of tumor cells by the modified virus leads to selective replication of the virus in the IL-2Rα$^+$ target positive tumor cells.

*Intratumoral NDV infection and EGFP expression in situ*. In order to test viral infection of tumor cells *in vivo*, DBA/2 mice bearing tumors of 0.5 cm diameter were injected intratumorally with 100 HU of native NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2. Tumors were dissected at 1 or 24 h after virus application and subjected to immunohistochemistry (IHC) or to direct fluorescence microscopy. IHC revealed that tumor tissues from 1 h had virus bound to cell membranes (Fig. 3b), while tumor tissues from 24 h had virus located in the cytoplasm (Fig. 3c). After 24 h, viral replication in the tumor tissue caused in some areas tumor necrosis (high expression of viral antigens overlapping with an area of reduced cell nuclei, Fig. 3d).

When analyzing the tumor sections by fluorescence microscopy, no EGFP fluorescence was observed in tumors harvested 1 h after virus inoculation (data not shown). In contrast, a strong EGFP fluorescence could be observed 24 h later. When using modified virus (NDFL-EGFP/αHN-IL-2) instead of NDFL-EGFP, target positive tumors expressed much more EGFP than target negative tumors (Fig. 4, right panels).

*Establishment of quantitative RRT-PCR for EGFP detection*. To achieve a high degree of sensitivity for the quantification of the EGFP signal, we established a real-time RT-PCR (RRT-PCR) assay. Mouse ß-actin served as endogenous control. Fig. 5a shows kinetics of RRT-PCR reactions obtained for the EGFP and ß-actin genes from Eb-M7 (IL-2Rα$^+$) cells that were infected *in vitro* with NDFL-EGFP. Melting temperatures of dissociation curves (Fig. 5b) indicated that the amplicons were specific for EGFP and ß-actin, respectively. To determine whether the two amplicons have the same amplification efficiency, we analyzed how the difference in threshold cycles ($C_T$) obtained with EGFP and ß-actin ($\Delta C_T$) varied when the template was diluted. Theoretically, if the efficiencies of the two genes are approximately equal, the plot of log input amount versus $\Delta C_T$ has a very low slope (<0.1). As shown in Fig. 5c, regression analysis of $C_T$ data for EGFP and ß-actin showed a horizontal line with a negligible absolute slope (0.09). This demonstrates that ß-actin can serve as a reliable reference to normalize the EGFP measurements.

*In vivo determination of EGFP-mRNA by quantitative RRT-PCR after intratumoral virus inoculation*. To quantify transgene expression delivered by the modified recombinant NDFL-EGFP/αHN-IL-2 in comparison to the native NDFL-

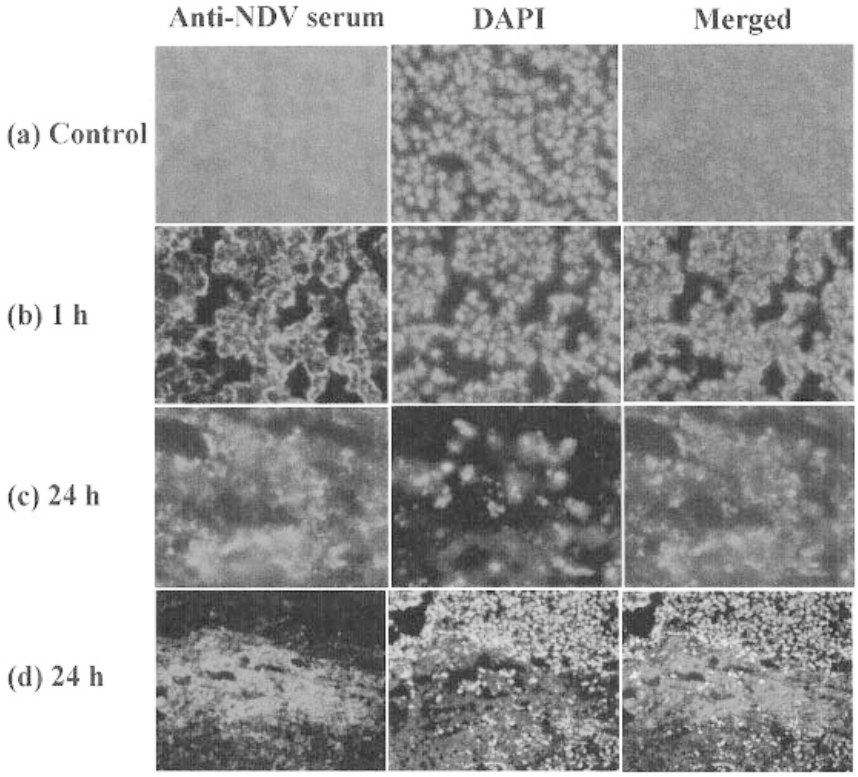



Figure 3. *In situ* intratumoral virus infection with modified NDFL-EGFP/αHN-IL-2 in Eb-M7 (IL-2Rα+). DBA/2 mice bearing Eb-M7 (IL-2Rα+) tumors (0.5 cm) on their flanks received intra-tumorally NDFL-EGFP/αHN-IL-2 (100 HU/100 µl). Mice were sacrificed at 1 or 24 h after virus injection. Tumor sections were stained with rabbit polyclonal anti-NDV serum, followed by Alexa 594-conjugated chicken anti-rabbit secondary antibodies and DAPI for nuclear staining before being analyzed by fluorescence microscopy. Tumor treated with PBS was used as negative control (a). Virus binding on the cell membrane was observed at 1 h (b). After 24 h, virus was visualized in the cytoplasm and interstitium (c). Necrosis in part of the tumor was induced by virus replication (d).

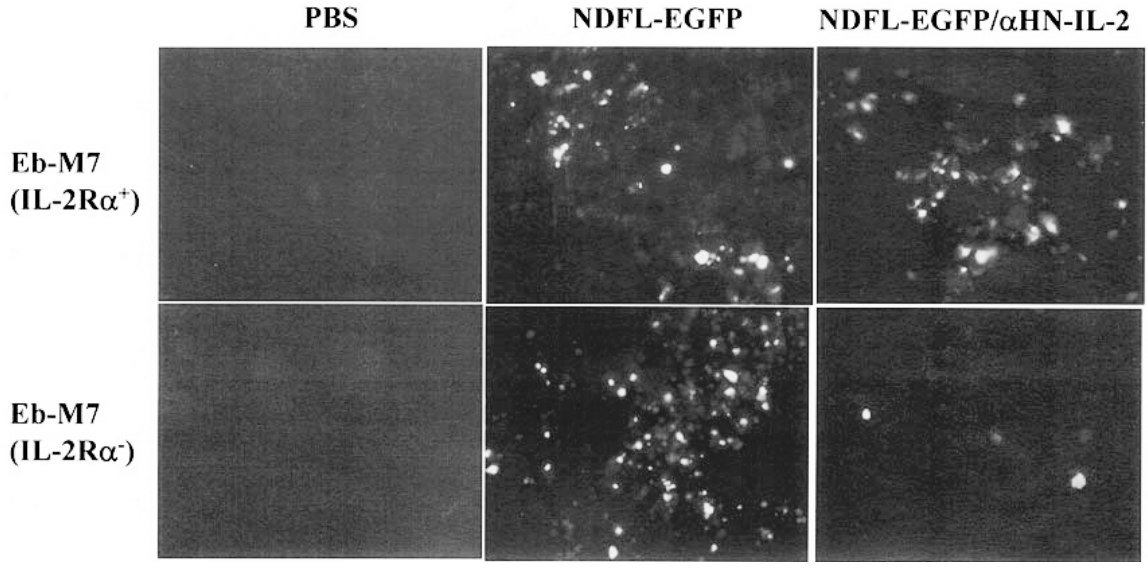



Figure 4. Visualization of EGFP expression by fluorescence microscopy. DBA/2 mice bearing Eb-M7 tumors (0.5 cm) on their flanks received intratumorally either NDFL-EGFP or NDFL-EGFP/αHN-IL-2 (100 HU/100 µl). Mice were sacrificed at 24 h after virus injection. Tumor sections were observed directly under the fluorescence microscope. Tumor treated with PBS was used as negative control.

EGFP in tumor tissues, cDNA from whole Eb-M7 (IL-2Rα+) and Eb-M7 (IL-2Rα-) tumors was synthesized by reverse transcription of RNA extracted from the tumors. EGFP expression in virus-treated tumors relative to PBS-treated tumors was calculated using the comparative $C_T$ method (Table I). A prominent retargeted EGFP transgene expression was detected in Eb-M7 (IL-2Rα+) cells infected with modified NDFL-EGFP/αHN-IL-2. In comparison to EGFP expression in Eb-M7 (IL-2Rα+) tumors infected with native NDFL-EGFP, a 6.3-fold higher gene delivery occurred in such tumors when infected

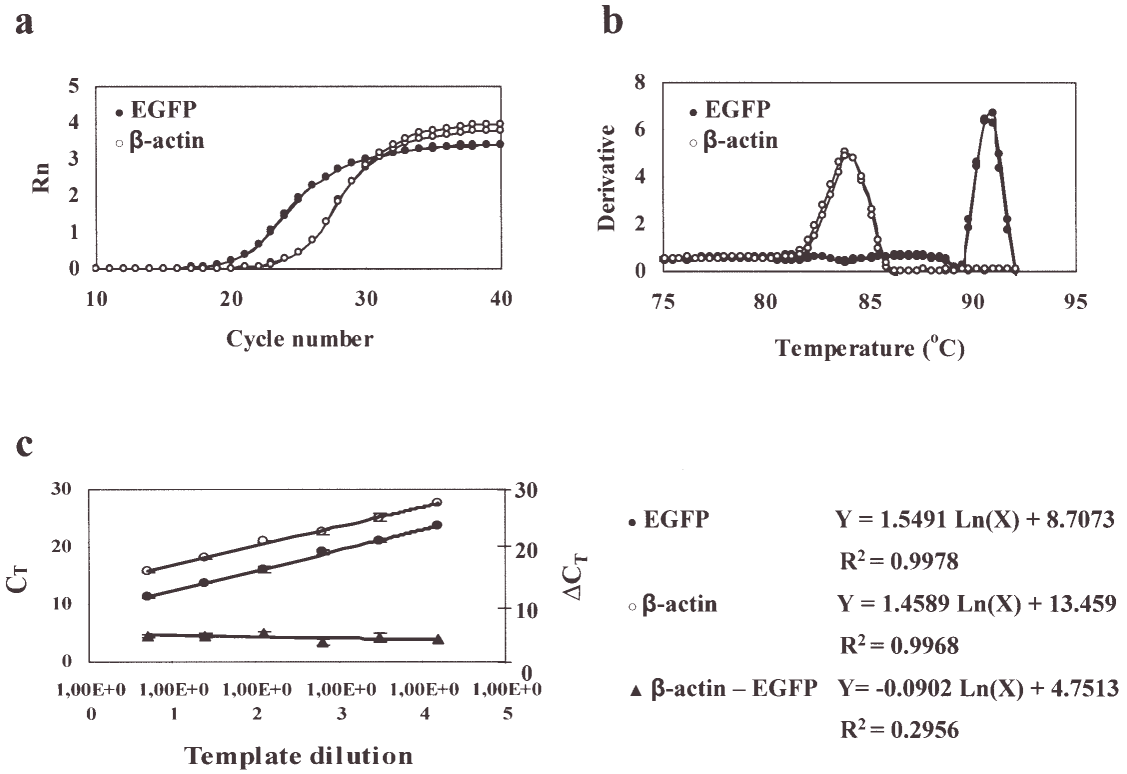



Figure 5. Establishment of a quantification test of EGFP transgene by RRT-PCR. Amplification plots (a) and dissociation curves (b) for EGFP and mouse ß-actin were derived from Eb-M7 (IL-2Rα⁺) cells infected with NDFL-EGFP. (c), Comparison of amplification efficiencies of EGFP and ß-actin was done in separate tubes, which were derived from a series of six 1:5 dilutions of the template similar as (a) and (b).

Table I. Quantification of the EGFP expression in tumors by RRT-PCR after intratumoral injection of native or modified NDFL-EGFP.

| Tumor | EGFP copies | % retargeted EGFP expression |
|---|---|---|
| (A) native virus | | |
| IL-2Rα⁺ | 44762.4 | |
| IL-2Rα⁻ | 5345894.7 | |
| (B) modified virus | | |
| IL-2Rα⁺ | 282913.2 | 632.0 |
| IL-2Rα⁻ | 65536.0 | 1.2 |

DBA/2 mice bearing either Eb-M7 (IL-2Rα⁺) or Eb-M7 (IL-2Rα⁻) tumors (0.5 cm) on their flanks received intratumorally native NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2 (100 HU/100 µl). Mice were sacrificed at 24 h after virus injection and tumors were analyzed for transgene EGFP presence by RRT-PCR. The percentages listed in the column represent the ratio of gene copies for modified virus to the gene copies for native virus. (A), Native NDFL-EGFP injected; (B), Modified NDFL-EGFP/αHN-IL-2 injected.

by NDFL-EGFP/αHN-IL-2. Gene delivery via modified virus to Eb-M7 (IL-2Rα⁻) cells was only 1.2% compared to target positive cells.

*Biodistribution of the EGFP transgene after intravenous application of NDV*. DBA/2 mice bearing Eb-M7 (IL-2Rα⁺) and Eb-M7 (IL-2Rα⁻) tumors on their right and left flanks respectively received i.v. either 5000 HU of NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2. Twenty-four hours later, the organ distribution of the EGFP mRNA was determined by RRT-PCR (Table II). We observed relatively high levels of EGFP mRNA in the lung, thymus, liver and spleen. Some EGFP mRNA could also be detected in tumors although at lower amounts. Compared to the native NDFL-EGFP, 98% of transgene was delivered to Eb-M7 (IL-2Rα⁺) tumors by modified NDFL-EGFP/αHN-IL-2, while 28% of transgene was found in Eb-M7 (IL-2Rα⁻) tumors. Importantly, the EGFP-mRNA expression decreased significantly (by 35-100%) in normal organs when the virus NDFL-EGFP was modified with αHN-IL-2.

## Discussion

In this study we demonstrate that recombinant NDV, when modified by the bispecific fusion protein αHN-IL-2, is an efficient vector for selective gene transfer into IL-2R-positive tumor cells both *ex vivo* and *in vivo*. The modification of NDV by the bispecific protein does not compromise severely the efficiency of gene delivery into IL-2R-positive tumors, but greatly reduces viral gene expression in IL-2R-negative tumors and in normal tissues.

Much attention has recently been focused on RNA viruses as vectors for tumor therapy, including measles virus, vesicular stomatitis virus (VSV), reovirus, and NDV. These are

Table II. Biodistribution of the transgene EGFP by RRT-PCR after i.v. injection of native or modified NDFL-EGFP.

| Tissue | EGFP copies | % retargeted EGFP expression |
|---|---|---|
| (A) native virus | | |
| Tumor (IL-2Rα$^+$) | 44453.2 | |
| Tumor (IL-2Rα$^-$) | 24322.4 | |
| Liver | 2511294.9 | |
| Spleen | 1070608.8 | |
| Kidney | 237900.7 | |
| Lung | 30876374.0 | |
| Thymus | 4372418.3 | |
| (B) modified virus | | |
| Tumor (IL-2Rα$^+$) | 43538.4 | 97.9 |
| Tumor (IL-2Rα$^-$) | 6841.0 | 28.1 |
| Liver | 189258.7 | 7.5 |
| Spleen | 696614.0 | 65.1 |
| Kidney | 9674.7 | 4.1 |
| Lung | 299044.4 | 0.0 |
| Thymus | 172950.5 | 4.0 |

DBA/2 mice bearing both Eb-M7 (IL-2Rα$^+$) and Eb-M7 (IL-2Rα$^-$) tumors (0.3-0.4 cm) on their right and left flanks respectively received i.v. 5000 HU of native NDFL-EGFP or modified NDFL-EGFP/αHN-IL-2. After 24 h, mice were perfused transcardially through the aorta with DEPC-treated PBS until lung, liver and kidney turned pale. Tumors and organs of interest were analyzed for the presence of the EGFP transgene by RRT-PCR. The percentages listed in the column represent the ratio of the number of gene copies for modified virus to the number of gene copies for native virus. (A), Native NDFL-EGFP injected; (B), Modified NDFL-EGFP/αHN-IL-2 injected.

currently being developed as a novel class of anti-tumor agents either as a viral vector or as an oncolytic agent (21-23). Our previous *in vitro* studies showed that lentogenic recombinant NDV with monocyclic replication properties can be retargeted to the IL-2R$^+$ human leukemia-like cell line, MT-2, by αHN-IL-2, while the native tropism via the interaction between HN proteins and sialic-acid containing receptors was abolished (16). Furthermore, we demonstrated that EGFP transgene expression was retargeted to MT-2 by NDFL-EGFP via αHN-IL-2 *ex vivo* and that virus hemadsorption was inhibited by αHN-IL-2 *in vivo* (17). To prove the principle of virus retargeting *in vivo*, we first had to establish a model consisting of target-positive and target-negative tumor cell lines. For this purpose, we selected two cell populations, Eb-M7 (IL-2Rα$^+$) and Eb-M7 (IL-2Rα$^-$) out of the parental p11-R-Eb cells. DBA/2 mice inoculated s.c. with $5x10^6$ Eb-M7 (IL-2Rα$^+$) and $1x10^7$ Eb-M7 (IL-2Rα$^-$) cells developed similar tumors of 0.5-cm diameter on their flanks at 1 week. IHC staining demonstrated a similar difference of IL-2Rα expression *in vitro* (data not shown).

Despite the fact that our bispecific fusion protein αHN-IL-2 was constructed from the human IL-2 sequence, FACS analysis showed specific binding of αHN-IL-2 to the murine IL-2R$^+$ Eb cell line (data not shown). Human and murine IL-2 show a high sequence homology (62%). We demonstrated that 16.5% of mouse IL-2R$^+$ p11-R-Eb cells were retargeted by modified NDFL-EGFP/αHN-IL-2 to express the EGFP transgene in comparison to native NDFL-EGFP. The value was improved to 52.7% when using the IL-2Rα-high expression cell line, Eb-M7 (IL-2Rα$^+$). This indicates that the percentage of retargeted EGFP expression was proportional to the amount of the IL-2R molecules on the cell surface. We did not observe retargeted EGFP expression in Eb-M7 (IL-2Rα$^-$) cells by modified NDFL-EGFP/αHN-IL-2, thus corroborating the previous results obtained with the human IL-2R$^-$ Jurkat cell line (16,17).

*Ex vivo* infection of Eb-M7 (IL-2Rα$^+$) and Eb-M7 (IL-2Rα$^-$) cells either with NDFL-EGFP or with NDFL-EGFP/αHN-IL-2 in DBA/2 mice showed 41.8% of retargeted EGFP delivered by NDFL-EGFP/αHN-IL-2 in Eb-M7 (IL-2Rα$^+$) cells in comparison to native NDFL-EGFP, and only 2.0% in Eb-M7 (IL-2Rα$^-$) cells. The percentage of retargeted EGFP delivery to Eb-M7 (IL-2R$^+$) cells was 2.0-fold higher when compared with human IL-2R$^+$ MT-2 cells (17).

After intratumoral injection of the modified virus into Eb-M7 (IL-2Rα$^+$) tumors, the retargeted binding *in vivo* of NDFL-EGFP/αHN-IL-2 to the cell membrane and virus replication in the cytoplasm were visualized by IHC at 1 and 24 h of post-injection, respectively. Necrotic areas were seen at 24 h in tumor sections, demonstrating the efficiency of this approach of tumor treatment. Ebert *et al* reported on GFP expression in tumors injected with recombinant VSV-GFP as a proof for viral replication (24). We saw no EGFP fluorescence in tumors dissected at 1 h (data not shown). The results are consistent with IHC staining which showed the binding of virus to the cell membranes. The EGFP fluorescence was observed in tumor sections dissected at 24 h, in which retargeted EGFP expression was evident impressively in Eb-M7 (IL-2Rα$^+$) tumors, but not in Eb-M7 (IL-2Rα$^-$) tumors. These results manifested that recombinant NDV can be retargeted to defined tumors by bispecific fusion proteins *in vivo*, causing tumor destruction via virus replication.

Several studies (20,25,26) employed real-time PCR to quantify EGFP or GFP transgene expression. To quantify EGFP expression *in vivo*, we also applied RRT-PCR in this study. Relative to native NDFL-EGFP, high amounts of the EGFP gene was transferred by modified NDFL-EGFP/αHN-IL-2 upon intratumoral inoculation of Eb-M7 (IL-2Rα$^+$) tumors. RRT-PCR confirmed that only very low amounts of the EGFP gene was delivered by NDFL-EGFP/αHN-IL-2 to Eb-M7 (IL-2Rα$^-$) tumors.

We showed before that NDFL-EGFP viral replication accompanied by EGFP expression can serve as a surrogate maker for virus replication (17). Here, it is the first time that we evaluated the biodistribution of lentogenic NDV in tumor-bearing mice by RRT-PCR. By quantifying EGFP mRNA levels, we found that NDV can replicate in all the tested tissues (tumor, liver, spleen, lung, spleen and thymus). Among them, lung had the highest amounts of virus accumulation which may not be surprising after i.v. inoculation of the virus. The tissue tropism of the same strain NDFL-EGFP has been investigated in 14-day-old embryo-

nated chicken eggs by Al-Garib *et al*, who observed that lung and the chorioallantoic membrane were the initial target organs as determined by fluorescence microscopy (7). It was also reviewed by Morrison that avirulent NDV results largely in respiratory tract infections (27). We have shown previously no replication of NDV in normal resting human T cells or in IL-2 pre-activated T cells, but replication in a broad spectrum of human and mouse tumor cells *in vitro* by flow cytometry (11). Up to now no data exist about NDV replication in tissues of mammals. We show here that NDV to some degree can replicate in the digestive and respiratory tract of mice. Whether this is accompanied by cell death or tissue destruction is not known.

After intravenous injection of the modified NDFL-EGFP/αHN-IL-2 into tumor-bearing mice, Eb-M7 (IL-2Rα$^+$) tumors were the most prominent retargeted tissue. All normal organs were relatively refractory to this modified virus when compared to native NDFL-EGFP. These results demonstrate that, upon systemic delivery, recombinant NDV, when modified by a bispecific molecule i) can be retargeted to target positive tumors followed by retargeted transgene expression and ii) shows decreased side effects on normal tissues.

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