ORIGINAL ARTICLE

Recombinant Newcastle disease virus (NDV) with inserted gene coding for GM-CSF as a new vector for cancer immunogene therapy

M Janke^{1,4}, B Peeters², O de Leeuw³, R Moorman³, A Arnold¹, P Fournier¹ and V Schirmmacher¹

¹Division of Cellular Immunology, German Cancer Research Center, Im Neuenheimer Feld 280, Heidelberg, Germany; ²Division of Virology, Central Institute for Animal Disease Control Lelystad (CIDC-Lelystad), Lelystad, The Netherlands; ³Division of Infectious Diseases, Animal Sciences Group, Wageningen University and Research Centre, Lelystad, The Netherlands and ⁴Division of Clinical Pharmacology, University Hospital, University of Bonn, Sigmund-Freud-Street 25, Bonn, Germany

This is the first report describing recombinant (rec) Newcastle disease virus (NDV) as vector for gene therapy of cancer. The gene encoding granulocyte/macrophage colony-stimulating factor (GM-CSF) was inserted as an additional transcription unit at two different positions into the NDV genome. The rec virus with the strongest production of the gene product (rec(GM-CSF)) was selected for our study. The insertion of the new foreign gene did neither affect the main features of NDV replication nor its tumor selectivity. The gene product was biologically active and stable. Tumor vaccine cells infected by rec(GM-CSF) stimulated human peripheral blood mononuclear cells (PBMC) to exert antitumor bystander effects in vitro in a tumor neutralization assay. These effects

were significantly increased when compared to vaccine infected by rec(–) virus. Furthermore, rec(GM-CSF) led to a much higher interferon- α (IFN- α) production than rec(–) when added as virus or as virus-modified vaccine to PBMC. Two distinct cell types, monocytes and plasmacytoid dendritic cells were shown to contribute to the augmented IFN- α response of PBMC. In conclusion, the already inherent anti-neoplastic and immunostimulatory properties of NDV could be further augmented by the introduction of a therapeutic gene whose product initiates a broad cascade of immunological effects in the microenvironment of the vaccine.

Gene Therapy (2007) 14, 1639–1649; doi:10.1038/sj.gt.3303026; published online 4 October 2007

Keywords: Newcastle disease virus; granulocyte/macrophage colony-stimulating factor; tumor therapy; vaccine

Introduction

Newcastle disease virus (NDV) is used to treat human cancer either in the form of free virus¹ or in the form of virus-infected tumor cell vaccine.² Three cellular mechanisms have been proposed for its anti-neoplastic activities: (i) oncolysis:³ oncolytic strains may simply kill tumor cells directly. (ii) Provision of danger signals:^{4,5} replication of NDV occurs in the tumor cells' cytoplasm. It is associated with the production of single- and double-stranded viral RNA. NDV infection of tumor cells thus introduces danger signals⁶ that can be recognized by RIG-I⁷ and PKR⁸ in the cytoplasm and by Toll-like receptors⁹ in endosomes. (iii) Immune cell activation: application of NDV may stimulate the host to produce cytokines such as interferons (IFNs) or tumor necrosis factor (TNF), which in turn leads to the activation of natural killer (NK) cells, monocytes, macrophages and sensitized T cells.

In our laboratory, we have established a tumor vaccine, which consists of irradiated autologous tumor cells, infected with the non-lytic NDV strain *Ulster*.

Already in 1986, we first reported on successful post-operative active-specific immunotherapy (ASI) with virus modified—but not with unmodified—murine ESb lymphoma cells, which caused protection from metastases in about 50% of syngenic mice.¹⁰ Protective antitumor immunity in the ESb lymphoma model was highly specific for the autologous tumor line.¹¹

On the basis of these preclinical observations, we subsequently established a similar autologous NDV virus-modified tumor vaccine (ATV-NDV) for human application.^{2,12} Recently, we reported results from two non-randomized phase II studies, which aimed at feasibility, safety and clinical benefit. In both studies (glioblastoma multiforme (GBM)¹³ and head and neck carcinoma (HNSCC)¹⁴), the establishment of primary tumor cell cultures was feasible and successful in about 80–90%. All tumor cells tested could be successfully infected by NDV and showed cell surface expression of MHC I and viral HN molecules. Clinical benefits in GBM included one complete remission and prolongation of median overall-survival (100 weeks versus 49 weeks in controls¹³). Clinical benefits in the HNSCC study included improvement of the 5-year survival rate in stage III and IV (61 versus 38%).¹⁴

In recent years, reverse genetics technology enabled to generate recombinant strains from non-segmented negative strand RNA viruses. Such techniques allowed

Correspondence: Professor V Schirmmacher, Division of Cellular Immunology, German Cancer Research Center (DKFZ), Im Neuenheimer feld 280, Heidelberg D-69120, Germany.

E-mail: V.Schirmmacher@dkfz-heidelberg.de

Received 11 April 2007; revised 29 July 2007; accepted 30 July 2007; published online 4 October 2007

recovering recombinant NDV entirely from cloned cDNA of the non-lytic strain *La Sota*.¹⁵

The aim of this study was to optimize a tumor vaccine by increasing the induced anti-tumor immunity. Recombinant (rec) NDV strains with an inserted therapeutic gene encoding either human or murine GM-CSF were thus produced. We will demonstrate a significant augmentation of anti-tumor activity upon stimulation of human peripheral blood mononuclear cells (PBMCs) from healthy donors with tumor vaccine infected with rec(GM-CSF) in comparison to a vaccine infected with recombinant NDV without a foreign gene (rec(-)).

We observed activation of innate immune cells such as monocytes and dendritic cells (DCs) by tumor vaccine infected with the rec(GM-CSF) when compared to vaccine infected with rec(-). We will show that rec(GM-CSF) vaccine leads to an enhanced IFN- α production by PBMC, which is due to the activity of monocytes and plasmacytoid DCs (PDCs).

In conclusion, our results provide the proof of principle that an incorporated therapeutic gene further augments the immunostimulatory and anti-neoplastic properties of NDV. This opens new possibilities for further optimization of the ATV-NDV tumor vaccine^{13,14,16} and for systemic application of recombinant NDV with incorporated therapeutic genes.

Results

Production of recombinant NDV viruses with the GM-CSF gene

Two new recombinant (rec) NDV strains each containing the human GM-CSF gene incorporated at different positions within the viral genome (in front of the NP gene and between the HN and L gene, see Figure 1a) were generated by reverse genetics. We also generated a rec virus with a murine GM-CSF gene in front of the NP gene. The genes were cloned into the plasmid pNDFL+,¹⁵ which contains the viral genome of NDV. A DNA fragment containing the gene encoding human or murine GM-CSF was obtained by means of PCR using the plasmid pCD-hGM-CSF as template. It was then inserted into plasmid pNDFL+ between the transcription-start box of the NP gene and the NP open reading frame.¹⁵ In order to allow transcription of downstream genes, a synthetic nucleotide sequence corresponding to the consensus transcription-end box and transcription-start box (ATTAAGAAAAAATACGGGTAGAAG) of NDV was inserted behind the therapeutic gene. The resulting plasmid was used to rescue recombinant NDV as described previously.¹⁵ The rescued virus was designated rec(GM-CSF) or rec(mGM-CSF) respectively. The hGM-CSF gene was also inserted between the HN and L gene of NDV by replacing the SEAP gene in plasmid pHZ-SEAP/HN-L¹⁷ and by rescuing the virus referred here as rec(GM-CSF (II)). All rec NDV strains could be grown to high titers in the allantoic fluid of embryonated chicken eggs.

Production of GM-CSF and biological activity

As shown in Figure 1b, human MCF-7 breast carcinoma cells infected with rec(GM-CSF) produced five times more gene product in comparison to rec(GM-CSF (II)). No GM-CSF protein could be detected in the supernatant

Figure 1 Comparison of the recombinant NDV with the GM-CSF gene inserted at two different positions. (a) Insertion sites of the therapeutic GM-CSF gene within the NDV minigenome. In the first construct, the GM-CSF gene was inserted in front of the NP gene (position I). In this case, a GE sequence followed by a GS sequence were added between the inserted gene and the viral NP gene. The position II corresponds to the insertion of the GM-CSF gene between the HN and the L gene. Here, a GE and a GS box were inserted between the HN and the inserted GM-CSF gene. Black boxes indicate conserved gene-end (GE) sequences. Shaded boxes indicate conserved gene-start (GS) sequences. (b) Position effect of the GM-CSF gene within the viral genome on its expression. MCF-7 cells were infected with rec(GM-CSF) or rec(GM-CSF (II)) as described in Materials and Methods (100 HU per 10^7 cells per ml). The cells (1.2×10^5 cells ml^{-1}) were left in culture for up to 4 days and the amount of the GM-CSF protein in the supernatants of these cultures was analyzed by ELISA. GM-CSF, granulocyte/macrophage colony-stimulating factor; NDV, Newcastle disease virus.

of tumor cells infected with rec(-) virus (data not shown). Thus, the integration site of the therapeutic gene within the viral genome has a strong influence on the level of the protein expressed. The supernatants had the capacity to stimulate the growth of the GM-CSF-dependent cell line TF-1 in a linear dose-dependent manner (Figure 2a).

To test for biological activity, we added supernatants from murine ESb tumor cells infected by rec(mGM-CSF) or rec(-) virus to murine bone marrow derived cells and evaluated the generation of CD11c-positive DCs after 10 days of cultivation. As shown in Figure 2b, the supernatant from rec(mGM-CSF)-infected tumor cells caused a similar expansion of bone marrow cell numbers and maturation of DCs as the positive control, a supernatant from Ag8653 tumor cells producing mGM-CSF.

We conclude that the production of rec NDV viruses with incorporated human or murine genes is feasible and that the viruses produce a gene product with biological activity.

Main characteristics of rec(GM-CSF) virus

For tumor vaccine production it is important to know tumor cell-binding properties, virus replication behavior

Figure 2 Biological activity of the produced GM-CSF protein inserted in the recombinant virus. **(a)** Human GM-CSF: TF-1 assays of supernatants of cells infected with rec(GM-CSF). MCF-7 cells were infected with rec(-) or rec(GM-CSF), cultured at 1.4×10^5 cells ml^{-1} and the supernatants of these cultures were taken after 24, 48 and 72 h. They were then added in serial dilutions to GM-CSF-sensitive TF-1 cells and incubated for 72 h. The proliferation of the TF-1 cells was determined by measuring uptake of radioactive ^3H -labeled thymidine during 4 h of pulse. **(b)** Murine GM-CSF: expansion of murine bone marrow-derived cells with supernatants of cells infected with rec(mGM-CSF). A total of 2×10^6 freshly prepared bone marrow cells from DBA/2 (H-2D^d) mice were distributed in four different groups in 10 cm Petri dishes. Then X-Vivo20 medium supplemented with 20% v/v of supernatant from ESb-LCI cells infected with rec(-) for 72 h (5×10^5 cells ml^{-1} ; top), of supernatant from Ag8653 cells that produce mouse GM-CSF (positive control, middle) or of supernatants from ESb-LCI cells infected with rec(mGM-CSF) for 72 h (5×10^5 cells ml^{-1} ; bottom) was added. The fourth group corresponding flow cytometry to X-Vivo20 medium without any supplement showed the same FACS profile as the first flow cytometry group (top). The medium was changed every 3 days. On day 10, the cells were labeled with FITC-labeled anti-mouse CD11c antibody and PE-labeled anti-H2D^d antibody (both diluted 1:100) before being analyzed by flow cytometry. FITC, fluorescein isothiocyanate; GM-CSF, granulocyte/macrophage colony-stimulating factor

and cytotoxic effects. Therefore, we investigated these aspects for the strains rec(-) and rec(GM-CSF) and compared them with NDV strain *Ulster*. As target cells we used the human breast carcinoma line MCF-7 (Figures 3a and b), the human breast hyperplasia-derived epithelial cell line MCF-10A, the colon-carcinoma line HT29 and the T-cell lymphoma line Jurkat. The rec virus strains derived from lentogenic NDV *La Sota* behaved in all cell lines similar to the lentogenic NDV *Ulster*. Viral replication in the cytoplasm was associated with an increase of the density of cell surface-expressed viral HN and F proteins. The rec strains were also similar to NDV *Ulster* in that they were not able to replicate in normal human cells and that supernatants from infected tumor cells were non-infectious due to an uncleaved F-protein in the newly synthesized virion particles (data not shown).

We also compared the rec strains and wild-type NDV *Ulster* with regard to their capacity to induce late apoptotic events in MCF-7 breast carcinoma cells (Figure 3c). The recombinant viruses were about three times more effective in inducing cytotoxicity in the tumor cells than NDV *Ulster*. Only a small difference was seen between rec(-) and rec(GM-CSF) viruses.

Expression and stability of the therapeutic gene product

We infected the above four cell lines and tested their supernatants by enzyme-linked immunosorbent assay (ELISA) for content of GM-CSF after 48 h of *in vitro* culture. Rec(GM-CSF) expressed its gene product in all four tumor cell lines tested. The highest expression was found in MCF-7 cells (Figure 4; 128 ng GM-CSF ml^{-1}). These were followed by MCF-10A and Jurkat cells, while the lowest expression was seen in HT29 cells, which produced only 34 ng GM-CSF ml^{-1} .

We then studied the kinetics of cytokine production and also the effect of γ -irradiation of the infected cells on their capacity to produce cytokine. Figure 4a shows that γ -irradiation had only a small effect on GM-CSF production. Maximum levels of cytokine expression were seen after 48 h of cell culture.

We then evaluated the expression of the therapeutic gene product in dependence on the number of irradiated MCF-7 vaccine cells added per well of a 96-well plate. While 750 cells produced only 12 ng ml^{-1} cytokine, 96 000 cells produced 390 ng ml^{-1} GM-CSF (Figure 4b).

Of further importance was the question of stability. Supernatants taken 1, 2, 3 or 4 days after infection were centrifuged and stored at -20°C , 4°C or incubated at 37°C for 30 days before analyzing the specific protein content by ELISA. The results (Table 1) show that GM-CSF was stable for 30 days at body temperature.

Effect of GM-CSF-producing tumor vaccine in a tumor neutralization assay

This functional assay is basically a mixed lymphocyte-tumor vaccine culture (MLTC) run on top of a tumor cell monolayer. It enables to test human PBMC-mediated bystander antitumor, cytotoxic or cytostatic effects on the tumor monolayer. While we previously performed the tumor neutralization assay (TNA) in 96-well plates with 750 vaccine cells,¹⁸ we had to increase the vaccine cell number to have enough of the therapeutic gene product

Figure 3 Tumor cell binding, virus replication and proapoptotic activity of the recombinant NDV strains. Virus binding (a) and replication (b) 1×10^7 MCF-7 cells were incubated in 1 ml volume with 100 HU of the indicated NDV strains for 1 h at 37 °C and then washed. In (a) the cells were directly stained for expression of viral antigens whereas in (b) they were further incubated for 24 h to allow for virus replication before the cells were stained. Staining was performed with mouse anti-F mAb ($1 \mu\text{g}/5 \times 10^5$ cells) followed by PE-labeled goat anti-mouse Ig secondary antibody (1:50). The dark profiles show staining controls with secondary antibody only. The white histograms show the expression of viral F protein. Notice that without viral replication there is only F-expression at low antigen density whereas after infection and replication the tumor cells show a distinct population of cells with F-expression at high antigen density (HAD). (c) Assessment of apoptosis by Nicoletti staining. Apoptosis induction in 5×10^5 MCF-7 cells per sample was measured by Nicoletti staining 48, 72 and 96 h after infection with the different NDV viruses (wt(Ulster), rec(-) and rec(GM-CSF) (same conditions of infection as in a). The percentage of propidium iodide-positive cells was calculated at each time point. NDV, Newcastle disease virus; GM-CSF, granulocyte/macrophage colony-stimulating factor.

Figure 4 Expression of the therapeutic gene product in tumor cells after infection with rec NDV. (a) Kinetics of the expression of the therapeutic gene product. MCF-7 tumor cells were infected with rec(GM-CSF) and cultured as described for Figure 1b. The experiment was performed with non-irradiated (white bars) or irradiated (black bars) MCF-7 tumor cells as for vaccine production. At different times (24, 48 and 72 h) after NDV infection, the cultured supernatants were tested for content of the therapeutic gene product by ELISA. rec(-)-infected control cultures did not produce GM-CSF (data not shown). (b) Dependence of the number of irradiated and infected MCF-7 vaccine cells on the expression of the therapeutic gene. After infection with rec(GM-CSF), MCF-7 cells were seeded at different numbers per well (750, 12 000 and 96 000 per 200 μl) in a 96-well plate. The expression of the therapeutic gene product was analyzed 48 h later by ELISA. The columns show means and standard deviations from triplicates. NDV, Newcastle disease virus; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte/macrophage colony-stimulating factor.

produced during the assay. An optimized test was thus established in 48-well plates containing 24 000 vaccine cells (V) and 96 000 PBMC as responder cells.

After 5–6 days of culture, the amount of live tumor cells was measured by uptake of the vital dye 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and the produced brown color was quantified in an ELISA reader at 490 nm.

These assays were performed with PBMC from 14 different healthy donors because of individual variability in activity. While in absence of virus infection the vaccine

cells (V) did not induce tumor growth inhibition (TGI) (see Figure 5), we observed about 30% TGI when virus-modified vaccine cells (VN) were added on top of the tumor cell monolayer. Upon addition of PBMC we saw a further increase of TGI up to 80%, while PBMC without vaccine cells had no growth inhibitory effect. The results are summarized in Figure 5. The highest mean of 82.3% TGI was obtained with rec(GM-CSF) in presence of PBMC. This was significantly higher than the effect seen with rec(-) and PBMC and thus provides the proof of principle of an added value of the incorporated gene for the tumor vaccine.

Effect of the recombinant viruses on the IFN- α response of PBMC

To elucidate mechanisms, which could explain the observed higher antitumor activity when using

Table 1 Stability of the expressed GM-CSF protein

MCF-7+rec(GM-CSF)		
Supernatants (hours after infection)	Incubation time: 30 days incubation temperature (°C)	Recovered GM-CSF concentration ng/ml (%)
24	-20	101.8 (100)
	4	109.9 (108)
	37	96.4 (95)
48	-20	117.9 (100)
	4	120.9 (103)
	37	117.2 (99)
72	-20	120.0 (100)
	4	121.9 (102)
	37	117.9 (98)
96	-20	140.0 (100)
	4	128.6 (92)
	37	127.1 (91)

GM-CSF, granulocyte/macrophage colony-stimulating factor; ELISA, enzyme-linked immunosorbent assay.

MCF-7 cells were infected with rec(GM-CSF) (100 HU per 10^7 cells per ml) and cultured as described for Figure 1b. After culture for 24, 48, 72 and 96 h, the supernatants were collected and stored at three different temperatures (-20, 4 and 37 °C) for 30 days. All samples were simultaneously analyzed for GM-CSF by ELISA. Numbers in brackets represent the percentage of GM-CSF concentration determined after storage at 4 or 37 °C taking as reference the sample stored at -20 °C.

rec(GM-CSF) instead of rec(-)-infected tumor vaccine, we analyzed the supernatants of the TNA for the expression of a panel of cytokines. These tests revealed that the amount of IFN- α was significantly higher in the TNA assays performed with rec(GM-CSF) than those with rec(-). When the TNA was performed with human purified T cells instead of PBMC as effector cells, no increase of IFN- α was seen (data not shown).

We next tested the IFN- α response of PBMC after co-incubation with increasing amounts of either rec(-) or rec(GM-CSF). The results obtained with PBMC from 10 different healthy donors are shown in Figure 6a. rec(GM-CSF) induced a significantly higher IFN- α response in comparison to rec(-). Also, rec(GM-CSF)-infected MCF-7 vaccine cells induced an IFN- α response in PBMC, which was significantly higher than that induced by rec(-)-infected vaccine cells (Figure 6b). PBMC control cultures or those stimulated with uninfected vaccine cells (data not shown) were negative. True infection of tumor cells by the rec viruses was important since UV inactivated virus was much less efficient (data not shown).

The increased IFN- α response of PBMC to rec(GM-CSF) virus could theoretically be due to a combined effect of GM-CSF and infection by NDV. The results from experiments testing this possibility are shown in Figure 6c. When recombinant GM-CSF protein (recGM-CSF) was added to rec(-) virus or V(rec(-)) vaccine to stimulate PBMC, we did not observe an increased IFN- α response. Even higher amounts of GM-CSF did not work while rec(GM-CSF) virus was effective over a broad dose range (data not shown). Thus, rec(GM-CSF) is a new vector with an added value in comparison to rec(-) virus and also in comparison to GM-CSF protein.

To find out which cell types in PBMC are responsible for the increased IFN- α response seen with rec(GM-CSF), we tested purified monocytes, which were positively

Figure 5 Effect of GM-CSF producing tumor vaccine in a tumor neutralization assay. To produce an MCF-7 monolayer, 48-well plates were seeded with 14 000 live MCF-7 cells and incubated for 4 h to enable the adherence to the bottom. Then wells of different experimental groups received 24 000 irradiated MCF-7 vaccine cells (V) or vaccine cells infected by rec(-) (VN[rec(-)]) or by rec(GM-CSF) (VN[rec(GM-CSF)]) and 96 000 fresh human PBMC as effector cells (400 μ l volume total). Since the effector activity of PBMC is variable depending on the donor, we performed separate experiments with PBMC from 14 different healthy donors. Data represent the mean of TGI of all donors, which was determined by triplicate measurements. In addition, the mean is shown under the respective group. A Student's *t*-test revealed the significance of the differences between certain groups. * $P < 10^{-5}$, ** $P < 0.004$. TGI, tumor growth inhibition.

Figure 6 IFN- α response of PBMC from normal donors. Stimulation was performed (a) with the recombinant viruses rec(-) and rec(GM-CSF); (b) with tumor vaccine cells infected with these two recombinant viruses or (c) with recombinant GM-CSF protein in addition to rec(-) or to the tumor vaccine infected with rec(-). (a) In a 24-well plate, 5×10^4 PBMCs from eight healthy donors were co-incubated or not with the two recombinant viruses rec(-) and rec(GM-CSF) at a concentration of 1 HU per 10^7 PBMC and in a final volume of 500 μ l. Results show the IFN- α expression in the supernatant 24 h later as tested by ELISA (* $P=0.0096$). (b) The same PBMCs as in (a) (5×10^5 per well of a 24-well plate) were stimulated with 4.8×10^4 vaccine cells pre-infected with rec(-) or rec(GM-CSF) (10 HU per 10^7 vaccine cells) in a total volume of 500 μ l. After 24 h, the supernatants of these co-cultures were collected and tested for IFN- α by ELISA. The addition of uninfected MCF-7 vaccine cells to the different PBMCs did not lead to induction of IFN- α (data not shown) (** $P=0.014$). (c) Recombinant GM-CSF protein was given at a final concentration of 20 ng ml $^{-1}$ onto PBMCs (5×10^5 cells per well) of the different donors in addition to rec(-) present at a concentration of 10 HU per 10^7 PBMC or to MCF-7 vaccine cells pre-infected with rec(-) as in (b) in a final volume of 500 μ l. After 24 h, the supernatants were tested for IFN- α by ELISA. NS, not significant. IFN- α , interferon- α ; GM-CSF, granulocyte/macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cells.

selected with CD14 magnetic beads and PBMC depleted of CD14-positive monocytes. As shown in Figures 7a and b both cell fractions responded to rec(GM-CSF) stimulation. We further fractionated CD14 $^{-}$ PBMC with BDCA-4 and BDCA-2 monoclonal antibody (mAb) staining and cell sorting and found that only the BDCA-4 $^{+}$ BDCA-2 $^{+}$ double positive fraction of PDCs responded to stimulation with NDV. This response could be blocked by BDCA-2-specific mAb, an antibody that is known to block the IFN- α response of PDCs,¹⁹ while a respective isotype control did not interfere with the response to

rec(GM-CSF)-purified PDC (data not shown). Similar results were obtained with CD14 $^{-}$ PBMC (Figure 7b–d), suggesting that PDCs are responsible for the response of this fraction. We thus conclude that rec(GM-CSF) stimulates two sub-populations of PBMC for IFN- α production, namely CD14 $^{+}$ monocytes and CD14 $^{-}$ BDCA-2 $^{+}$ PDCs.

Discussion

The aim of the study was to further improve the tumor vaccine ATV-NDV, which is elaborated by infection of irradiated tumor cells with the lentogenic strain Ulster of NDV and showed promising results in clinical studies.^{13,14,16} To this end, we inserted an additional gene coding for GM-CSF within the viral genome in such a way that viral infection of tumor cells leads to secretion of GM-CSF. We show here that the insertion of this additional therapeutic gene significantly increases the immune stimulatory properties of the virus and the antitumor effects of a respective tumor vaccine in a tumor neutralization assay *in vitro*.

Using NDV as vector for tumor gene therapy offers several advantages over other viral expression systems: (1) high safety profile. This is due to the fact that NDV is an avian RNA virus. Viral infection and replication occur exclusively in the cytoplasm. No viral DNA is generated and no genetic recombination can occur with host cell DNA; (2) tumor cell infection efficiency. All the tumor cell lines tested in our laboratory were easy to infect and showed a high permissivity with 80% of the cells being infected. Viral replication is independent of cell proliferation and proceeds efficiently in tumor cells inactivated by γ -irradiation.²⁰ Already after 24 h, the cells are fully infected by the virus. No selection for tumor cell transfectants is necessary as is the case with retroviral vectors for which technical hurdles have been described.²¹ In fact the efficiency of the retroviral vector approach was so low that from 116 malignant gliomas only three transfectants were successfully established and could be applied to the patients.²¹ For comparison, in our glioblastoma study,¹³ the efficiency of ATV-NDV tumor vaccine production (autologous cell cultures plus virus infection) was 90%.

Since NDV is a negative strand RNA virus, reverse genetics technology had to be employed for the elaboration of recombinant NDV. The rescue system has been established with the strain *La Sota*.¹⁵ Cloned *La Sota* virus NDV was found to have similar properties as *Ulster*. The *La Sota* strain has already been shown to be safe in a clinical trial with stage I–IV malignant tumors of the digestive tract.²² Other data obtained by us (not shown here) revealed that NDV (i) shows tumor selective replication as observed for NDV *Ulster*,²³ (ii) has a monocyclic replication cycle in tumor cells and (iii) produces non-infectious progeny virus similar to NDV *Ulster*.²¹

The only difference between the two virus strains was that NDV (rec(-)) had about three times higher cytotoxic effects on tumor cells as revealed by the Nicoletti assay. Such increased tumor cytotoxic effect may lead to an improvement of the immune response through uptake of apoptotic bodies and cross-presentation of tumor-associated antigens via host DCs.²⁴ Secretion of GM-CSF by infected tumor cells should activate

Figure 7 IFN- α response to rec(GM-CSF) virus by monocytes and plasmacytoid DCs. Monocytes were purified or depleted from PBMC of a healthy donor using anti-CD14-specific mAb and magnetic beads. Staining and flow cytometry analysis confirmed a high purity of the obtained monocytes fraction. A 5 HU of rec(-) or rec(GM-CSF) (or no virus as control) was added to 5×10^5 cells in 24-well plates (500 μ l volume per well) to the monocytes (a) or to the remaining CD14 depleted PBMC (b). c and d additionally contained anti-BDCA-2 mAb or isotype control mAb, respectively (3 μ g ml⁻¹). After 1 day of incubation, the level of IFN- α was determined by ELISA. IFN- α , interferon- α ; GM-CSF, granulocyte/macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.

and attract antigen-presenting cells, thereby inducing a tumor-specific cellular immune response. The GM-CSF of the vaccine produced with rec(GM-CSF) could further stimulate this process by recruiting more DCs to the site of vaccination and by improving cross-presentation via IFN- α and by upregulation of costimulatory molecules on DCs. This is a critical first step in the induction of an optimal immune response to any immunotherapy product at the injection site.^{25,26}

GM-CSF appears to be a very attractive therapeutic gene because it was the most potent among multiple cytokines and growth factors transduced into a syngeneic, non-immunogenic, B16 melanoma tumor cell vaccine.^{26,27} GM-CSF was shown to recruit and mature DCs, macrophages and neutrophils.²⁶ It showed already

interesting effects in vaccination studies^{25,27–29} and also some objective clinical regression of metastatic breast cancer.³⁰

In this study, two positions of insertion for the GM-CSF therapeutic gene were tested. The position close to the 3' proximal end showed a higher recombinant protein expression than the other one. The NDV RNA-dependent RNA polymerase initiates its binding to the RNA template at the leader sequence close to the 3' end of the genomic RNA. It performs the transcription of the six different genes within the viral genome in a discontinuous manner by starting the transcription of each gene at the Gene Start (GS) sequence and ending it at the Gene End (GE) sequence. This leads to the generation of a gradient of mRNAs from the 3' end to

the 5' end and a corresponding gradient in the amounts of the different proteins. This explains the faster (within the first 24 h) and stronger expression of the therapeutic gene product when testing the virus with the GM-CSF gene inserted in front of the NP gene. Furthermore, irradiation of the tumor cells before infection with rec(GM-CSF) did not interfere with the level of protein produced.

GM-CSF expressed by rec(GM-CSF)-infected tumor cells was stable up to 30 days and it was biologically active. The cytokine served as a growth factor for GM-CSF-dependent TF-1 cells and murine bone marrow cells and caused maturation of DCs. GM-CSF was not only produced by tumor cells but also by PBMC upon infection with rec(GM-CSF) (data not shown). We reported before that NDV can infect PBMC and produce positive strand viral RNA²³ but the antiviral response of PBMC²³ prevents further viral replication and associated cell death.

To investigate the antitumoral activity of PBMC upon stimulation with tumor vaccine cells infected by rec viruses, we used a previously established TNA, which allows testing for bystander antitumor activity. We demonstrate that bystander antitumor activity can be increased significantly when using rec(GM-CSF) virus in comparison to rec(–) virus for the elaboration of the tumor vaccine. These TNA experiments were performed with 14 different PBMC donors because of the great variability in the activity of PBMC from different donors. The findings corroborate results from other tumor vaccine systems in which either vaccinia virus,³¹ retrovirus³⁰ or HSV-1 amplicons³² were used as vectors to transfer the GM-CSF gene into tumor cells.

An unexpected but encouraging finding was that PBMC responded to addition of rec(GM-CSF) with a strong production of IFN- α . This response was significantly higher than the IFN- α response to either rec(–) virus or to recombinant GM-CSF. There seems to be a synergistic effect in this response between virus infection and production of GM-CSF thus providing rec(GM-CSF) with an added value in comparison to the recombinant protein. Addition of irradiated MCF-7 vaccine cells infected with rec(GM-CSF) to PBMC also showed a significantly enhanced IFN- α response. IFN- α has a central importance not only as an antiviral agent but also for innate and adaptive immune responses.^{19,33} It may thus also be involved in the observed increased bystander antitumor effects.

Recent preclinical studies have demonstrated further enhancement of antitumor immunity when a tumor vaccine secreting GM-CSF is combined with (i) blockade of CTLA-4,³⁴ (ii) blockade of vascular endothelial growth factor³⁵ or (iii) immunomodulatory doses and schedules of certain chemotherapeutic agents.³⁶ Also a combination of IFN- α and GM-CSF secreting tumor vaccine was shown to significantly enhance the potency of the immunotherapeutic effect.³⁷ The expression of the HN protein of NDV at the cell surface of the ATV-NDV tumor vaccine⁵ as well as the replication of NDV within these cells may be of central importance for the induction of IFN- α at the vaccination site and consequently for the observed antitumor response in clinical studies based on NDV tumor vaccine.¹⁶

Here, we show that a tumor vaccine obtained by irradiation and infection with rec(GM-CSF) induces a

stronger IFN- α response in human monocytes and PDCs than a vaccine infected with the virus without incorporated foreign gene. Interestingly, rec(GM-CSF) virus was found to exert immunostimulatory effects; which were superior to a mixture of rec(–) virus and GM-CSF protein. Earlier experiments had revealed that the type I IFN response leads to upregulation of TRAIL on monocytes and to monocyte mediated antitumor cytotoxicity.³⁸ Soluble TRAIL-Fc molecules were able to inhibit such cytotoxicity³⁸ and they also inhibited partially (about 50%) the effects measured by the TNA test (data not shown). Preliminary tests also revealed that in a TNA with rec(GM-CSF)-infected vaccine, monocytes show increased TRAIL expression, DCs show increased expression of CD80 and CD86 costimulatory molecules and tumor cells from the monolayer show increased expression of Apo 2.7, a marker for early apoptotic events (M Janke, unpublished results). Through the stronger activation of innate immune effects, we expect that a tumor vaccine infected with rec(GM-CSF) will also induce a stronger adaptive immune response because IFN- α provides a link between both immunity systems.^{39,40}

In summary, this study shows that the immune stimulatory and antitumoral properties of NDV can be further augmented by incorporation of a foreign gene. More specifically our data suggest that rec(GM-CSF), a recombinant NDV that produces GM-CSF and induces a strong IFN- α response represents a promising new viral vector for human tumor immunogene therapy.

Materials and methods

Antibodies and chemicals

For flow cytometry analysis, the following mAbs against mouse antigens were used at 1:100 dilution: CD11c-FITC, H2D^d-PE (Pharmingen, Hamburg, Germany). Mouse anti-HN mAb (HN.B mAb, IgG2a) and mouse anti-F mAb lci (IgG1) were kindly provided by Dr Iorio (Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School, Worcester, MA, USA) and used at $1 \mu\text{g}/5 \times 10^5$ cells. These two antibodies were used to detect NDV viral antigens on host cell surfaces. To detect the unconjugated antiviral antibodies, cells were incubated with secondary mAb goat F(ab)₂ anti-mouse Ig-RPE (1:50) obtained from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). Propidium iodide was purchased from Sigma (Deisenhofen, Germany).

For blocking experiments, we used unlabeled anti-human BDCA-2 (IgG1) mAb at $3 \mu\text{g ml}^{-1}$ (Miltenyi, Bergisch Gladbach, Germany). The isotype control HD20 (IgG1) was a gift from Dr Gerd Moldenauer (DKFZ, Heidelberg, Germany) and was used at identical concentrations. ELISA experiments for the detection of human GM-CSF (Pharmingen, Hamburg, Germany) and human IFN- α (Bender Medsystems, San Bruno, CA, USA) were performed in accordance to the manufacturer's guidelines. Recombinant GM-CSF protein (commercial trade name: Leukine Sargramostin) was purchased from Berlex Laboratories Inc. (Richmond, VA, USA).

MTS Cell Titer 96 Aqueous solution was purchased from Promega (Mannheim, Germany).

Cell lines and culture

All tumor cells were obtained from the Tumorbank DKFZ (Heidelberg, Germany) except the MCF-10A human breast hyperplasia-derived cell line, which was purchased from ATCC (Manassas, VA, USA).

The human Jurkat cell line was grown in RPMI-1640 medium supplemented with 10% inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. The human breast carcinoma cell line MCF-7 and the human colon-adenocarcinoma cell line HT29 were maintained in Dulbecco's modified Eagle's medium containing 10% inactivated FCS, 2 mM L-glutamine, 10 mM HEPES, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin.

The non-tumorigenic but immortalized human breast epithelial cell line MCF-10A was cultured in Dulbecco's modified Eagle's medium with 5% horse serum, 10 mM HEPES, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 500 ng ml⁻¹ hydrocortisone, 0.01 mg ml⁻¹ insulin, 20 ng ml⁻¹ epidermal growth factor and 100 ng ml⁻¹ cholera toxin. TF-1 is a human premyeloid cell line, which was originally established by Dr Kitamura from a patient with erythroleukemia. This cell line, whose growth is dependent on GM-CSF was purchased from ATCC (cat. no. CRL-2003). The myeloma cell line Ag8653 expressing mouse recombinant GM-CSF was cultured to produce mouse GM-CSF. All these cells were grown in RPMI-1640 medium with 10% FCS, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 2.5 mg ml⁻¹ glucose and 25 µg ml⁻¹ GM-CSF.

All reagents for cell culture were purchased from Gibco Life Technologies (Karlsruhe, Germany) except FCS, which was from Biochrom (Krefeld, Germany) and the horse serum, hydrocortisone, insulin, epidermal growth factor and cholera toxin were purchased from Sigma (Deisenhofen, Germany). GM-CSF used as standard was obtained from Promocell (Heidelberg, Germany). All cell lines were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

Plasmids and virus

The plasmid called pNDFL, which contains the six genes of the negative-stranded RNA genome of NDV strain *La Sota* in the order 3'-NP-P-M-F-HN-L-5' has been described by Peeters *et al.*¹⁵ The plasmid pCD-hGM-CSF encoding the human GM-CSF gene was purchased from ATCC (cat. no. 57594).

NDV-Ulster 2C was obtained in 1984 from Dr PH Russel (University London, UK).

All the viruses (recombinant or not) were propagated in embryonated chicken eggs, harvested from the allantoic fluid, purified by ultracentrifugation and quantified by hemagglutination as described previously.² In some experiments, the virus was inactivated with UV light for 5 min (254 nm, 2 mW cm⁻², 7 cm distance).

Purification of blood-derived cells

Human PBMCs were isolated from buffy coat of healthy donors by Ficoll-Paque (Amersham Pharmacia Biotech, Freiburg, Germany) density gradient centrifugation.

In some assays, PBMCs were depleted from monocytes by using magnetic beads coated with CD14-specific mAbs and MACS columns (both Miltenyi, Bergisch

Gladbach, Germany). We also tested the positively selected CD14⁺ monocytes.

Production of virus-infected tumor vaccine stimulator cells

As stimulator cells for mixed lymphocyte-tumor cell cultures (MLTC) and the TNA, we used MCF-7 breast carcinoma cells that were inactivated with 200 Gy irradiation via a ¹³⁷Cs source (Gammacell 1000, Atomic Energy of Canada, Chalk River, Canada) and then infected with the respective NDV strain (100 HU per 10⁷ cells per ml) as described.²

Flow cytometry

A total of 5 × 10⁵ cells were used per sample. All antibodies were diluted in flow cytometry buffer (PBS containing 5% FCS and 0.1% NaN₃) as described in the antibody section. Cells were incubated with a primary (un-) conjugated antibody for 20 min on ice in the dark. Subsequently cells were washed two times with flow cytometry buffer and, if necessary incubated with a conjugated secondary antibody for 20 min on ice in the dark. Then the cells were washed two times, adjusted to a volume of 100 µl and analyzed in a FACS Calibur flow cytometer (Becton & Dickinson, Heidelberg, Germany). All flow cytometry data were analyzed with CELLQuest Pro software (Becton & Dickinson, Heidelberg, Germany).

Apoptosis assays

For determination of late apoptotic cells a Nicoletti staining was performed. Briefly, 5 × 10⁵ cells were washed in PBS, incubated with 250 µl Nicoletti solution (0.05 mg ml⁻¹ propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100 in PBS) for at least 48 h at 4 °C in the dark and then analyzed by flow cytometry.

Tumor neutralization assay

The assay was performed in 48-well round-bottom plates. A total of 1.4 × 10⁴ live MCF-7 tumor cells in Dulbecco's modified Eagle's medium with 5% FCS were added to each well and incubated for 4 h at 37 °C to adhere.

Subsequently, 9.6 × 10⁴ freshly isolated non-activated PBMC from normal healthy donors were added to the respective wells. A total of 2.4 × 10⁴ stimulator cells per well were prepared by irradiation and virus infection as indicated. The total volume per well was 400 µl. The microtiter plates were then wrapped in cling film to prevent evaporation of medium and incubated in a CO₂ incubator at 37 °C. The time of incubation depended on the time for confluence of the tumor cell monolayer in control wells without stimulator or effector cells. All experimental wells were performed in triplicates. At the end of the assay, supernatants were removed by suction and the plates were carefully washed twice with RPMI containing 5% FCS to remove effector and stimulator cells. Then 200 µl of RPMI with 5% FCS and 40 µl MTS solution (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, MTS) were added per well and incubated for 20–40 min at 37 °C in the dark. After color switch, the reaction was stopped by transfer of each well content to a 96-well plate containing 20 µl 10% SDS per well. The plates were then evaluated

in an ELISA reader at 490 nm. The background was taken from control wells containing only medium and MTS solution. The result was displayed as percent tumor growth inhibition related to an untreated monolayer (positive control) calculated according to:

$$1 - \left(\frac{A_{490} \text{ experimental well} - A_{490} \text{ background}}{A_{490} \text{ positive control} - A_{490} \text{ background}} \right) \times 100$$

= % growth inhibition

Cytokine-dependent proliferation assay

We used GM-CSF-dependent TF-1 cells to analyze the biological activity of the expressed integrated cytokine genes released during viral tumor cell infection.

One day prior to the assay, the indicator cells were transferred to cytokine-free medium. For the assay, the cells were then harvested, washed three times with cytokine free medium and adjusted to a cell number of $2 \times 10^5 \text{ ml}^{-1}$. A 50 μl of cell suspension was added to a 96-well plate, which contained serial 1:4 dilutions of the supernatants of infected tumor cells. The assay was performed in duplicates for each dilution step. After 48–72 h at 37 °C 1 μCi ^3H -thymidine in 50 μl RPMI with 5% FCS was added to each well of the plate, which then was incubated for additional 4 h at 37 °C. By using a micro cell harvester the radioactive DNA was transferred onto a fiberglass mat, which was analyzed in a liquid scintillation counter (Perkin Elmer Wallac, Freiburg, Germany) for thymidine incorporation.

References

- Lorence RM, Pecora AL, Major PP, Hotte SJ, Laurie SA, Roberts MS *et al.* Overview of phase I studies of intravenous administration of PV701, an oncolytic virus. *Curr Opin Mol Ther* 2003; **5**: 618–624.
- Schirmacher V, Haas C, Bonifer R, Ahlert T, Gerhards R, Ertel C. Human tumor cell modification by virus infection: an efficient and safe way to produce cancer vaccine with pleiotropic immune stimulatory properties when using Newcastle Disease Virus. *Gene Therapy* 1999; **6**: 63–73.
- Sinkovics JG, Horvath JC. Newcastle Disease Virus (NDV): brief history of its oncolytic strains. *J Clin Virol* 2000; **16**: 1–15.
- Gallucci S, Matzinger P. Danger signals: SOS to the immune system. *Curr Opin Immunol* 2001; **13**: 114–119.
- Zeng J, Fournier P, Schirmacher V. Induction of interferon α and tumor necrosis factor-related apoptosis-inducing blood mononuclear cells by hemagglutinin-neuraminidase but not F protein of Newcastle Disease Virus. *Virology* 2002; **297**: 19–30.
- Fournier P, Zeng J, Schirmacher V. Two ways to induce innate immune responses in human PBMCs: paracrine stimulation of IFN- α responses by viral protein or dsRNA. *Int J Oncol* 2003; **23**: 673–680.
- Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Akira S *et al.* Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 2005; **23**: 19–28.
- Clemens MJ, Elia A. The double-stranded RNA-dependent protein kinase PKR: structure and function. *J Interferon Cytokine Res* 1997; **17**: 503–524.
- Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; **4**: 499–511.
- Heicappell R, Schirmacher V, von Hoegen P, Ahlert T, Appelhaus B. Prevention of metastatic spread by postoperative immunotherapy with virally modified autologous tumor cells. I: parameters for optimal therapeutic effects. *Int J Cancer* 1986; **37**: 569.
- Schirmacher V, Heicappell R. Prevention of metastatic spread by postoperative immunotherapy with virally modified autologous tumor cells. II: Establishment of specific systemic anti tumor immunity. *Clin Exptl Met* 1987; **5**: 147–156.
- Washburn B, Schirmacher V. Human tumor cell infection by Newcastle Disease Virus leads to upregulation of HLA and cell adhesion molecules and to induction of interferons, chemokines and finally apoptosis. *Int J Oncol* 2002; **21**: 85–93.
- Steiner HH, Bonsanto MM, Beckhove P, Brysch M, Geletneky K, Ahmadi R *et al.* Anti-tumor vaccination of patients with glioblastoma multiforme in a case-control study: feasibility, safety and clinical benefit. *J Clin Oncol* 2004; **22**: 4272–4281.
- Karcher J, Dyckhoff G, Beckhove P, Reisser C, Brysch M, Zionta Y *et al.* Anti-tumor vaccination with HNSCC with autologous virus-modified tumor cells. *Cancer res* 2004; **64**: 8057–8061.
- Peeters BP, de Leeuw OS, Koch G, Gielkens AL. Rescue of Newcastle Disease Virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. *J Virol* 1999; **73**: 5001–5009.
- Schirmacher V. Clinical trials of antitumor vaccination with an autologous tumor cell vaccine modified by virus infection: Improvement of patient survival based on improved anti-tumor immune memory. *Cancer Immunol Immunother* 2005; **54**: 587–598.
- Zhao H, Peeters BPH. Recombinant Newcastle disease virus as a viral vector: effect of genomic location of foreign gene on gene expression and virus replication. *J Gen Virol* 2003; **84**: 781–788.
- Haas C, Lulei M, Fournier P, Arnold A, Schirmacher V. A tumor vaccine containing anti-CD3 and anti-CD28 bispecific antibodies triggers strong and durable anti-tumor activity in human lymphocytes. *Int J Cancer* 2005; **118**: 658–667.
- Dzionek A, Sohma Y, Nagafune J, Cella M, Colonna M, Facchetti F *et al.* BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J Exp Med* 2001; **194**: 1823–1834.
- Schirmacher V, Haas C, Bonifer R, Ertel C. Virus potentiation of tumor vaccine T-cell stimulatory capacity requires cell surface binding but not infection. *Clin Cancer Res* 1997; **3**: 1135–1148.
- Parney IF, Chang LJ, Farr-Jones MA, Hao C, Smylie M, Petruk KC. Technical hurdles in a pilot clinical trial of combined B7-2 and GM-CSF immunogene therapy for glioblastomas and melanomas. *J Neurooncol* 2006; **78**: 71–80.
- Liang W, Wang H, Sun TM, Yao WQ, Chen LL, Jin Y *et al.* Application of autologous tumor cell vaccine and NDV vaccine in treatment of tumors of digestive tract. *World J Gastroenterol* 2003; **9**: 495–498.
- Fiola C, Peeters B, Fournier P, Arnold A, Bucur M, Schirmacher V. Tumor selective replication of Newcastle Disease Virus: Association with defects of tumor cells in anti-viral defence. *Int J Cancer* 2006; **119**: 328–338.
- Scheffer SR, Nave H, Korangy F, Schlote K, Pabst R, Jaffee EM *et al.* Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response *in vivo*. *Int J Cancer* 2003; **103**: 205–211.
- Soiffer R, Hodi FS, Haluska F, Jung K, Gillesen S, Singer S *et al.* Vaccination with irradiated, autologous melanoma cells engineered to secrete granulocyte-macrophage colony-stimulating factor by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma. *J Clin Oncol* 2003; **21**: 3343–3350.
- Jaffee EM, Lazenby A, Meurer J, Marshall F, Hauda KM, Counts C *et al.* Use of murine models of cytokine-secreting tumor vaccines to study feasibility and toxicity issues critical to designing clinical trials. *J Immunother Emphasis Tumor Immunol* 1995; **18**: 1–9.
- Dranoff G, Jaffee E, Lazenby A, Golumbek P, Pardoll D, Mulligan RC *et al.* Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage

- colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci USA* 1993; **90**: 3539–3543.
- 28 Driessens G, Hamdane M, Cool V, Velu T, Bruyns C. Highly successful therapeutic vaccination combining dendritic cells and tumor cells secreting granulocyte macrophage colony-stimulating factor. *Cancer Res* 2004; **64**: 8435–8442.
- 29 Luiten RM, Kueter EW, Mooi W, Gallee MP, Rankin EM, Gerritsen WR *et al*. Immunogenicity, including vitiligo, and feasibility of vaccination with autologous GM-CSF-transduced tumor cells in metastatic melanoma patients. *J Clin Oncol* 2005; **23**: 8978–8991.
- 30 Wiseman CL, Kharazi A. Objective clinical regression of metastatic breast cancer in disparate sites after use of whole-cell vaccine genetically modified to release sargramostim. *Breast J* 2006; **12**: 475–480.
- 31 Qin HX, Chatterjee SK. Construction of recombinant vaccinia virus expressing GM-CSF and its use as tumor vaccine. *Gene Therapy* 1996; **3**: 59–66.
- 32 Santos K, Duke CM, Dewhurst S. Amplicons as vaccine vectors. *Curr Gene Ther* 2006; **6**: 383–392.
- 33 Stetson DB, Medzhitov R. Type I interferons in host defense. *Immunity* 2006; **25**: 373–381.
- 34 Hurwitz AA, Yu TF, Leach DR, Allison JP. CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. *Proc Natl Acad Sci USA* 1998; **95**: 10067–10071.
- 35 Li B, Lalani AS, Harding TC, Luan B, Koprivnikar K, Huan Tu G *et al*. Vascular endothelial growth factor blockade reduces intratumoral regulatory t cells and enhances the efficacy of a GM-CSF-secreting cancer immunotherapy. *Clin Cancer Res* 2006; **12**: 6808–6816.
- 36 Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D *et al*. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res* 2001; **61**: 3689–3697.
- 37 Prell RA, Li B, Lin JM, VanRoey M, Jooss K. Administration of IFN- α enhances the efficacy of a granulocyte macrophage colony stimulating factor-secreting tumor cell vaccine. *Cancer Res* 2005; **65**: 2449–2456.
- 38 Washburn B, Weigand MA, Grosse-Wilde A, Janke M, Stahl H, Rieser E *et al*. TNF-related apoptosis-inducing ligand mediates tumoricidal activity of human monocytes stimulated by Newcastle disease virus. *J Immunol* 2003; **170**: 1814–1821.
- 39 LeBon A, Tough DF. Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol* 2002; **14**: 432–436.
- 40 Tough DF. Type I interferon as a link between innate and adaptive immunity through dendritic cell stimulation. *Leuk Lymphoma* 2004; **45**: 257–264.