Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by
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born in Głogów, Poland
Oral examination:

Evaluation of IP-10 and TNFα-transducing parvoviral vectors as antitumoral agents in animal glioblastoma models

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To my husband Bernhard

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Summary

This work evaluated the efficacy of parvoviral vectors expressing human IP-10 or mouse TNFα as tools against subcutaneous glioblastoma tumors in two animal models.

First, new recombinant MVMp- and H1- based vectors expressing human IP-10 or mouse TNFα were constructed. It was shown that parvoviral vectors could effectively infect both human and murine glioblastoma cells. High amounts of the transgene proteins were produced upon infection with particular vectors. All tested cell lines were sensitive to wild type parvoviruses.

Two animal models were established: murine GI261 glioma cells were used for inducing subcutaneous tumors in C57/Bl6 mice and human U87 glioblastoma cells produced subcutaneous tumors in cd1 swiss nude mice. The antitumoral effects mediated *in vivo* by recombinant and wild type parvoviruses (MVMp and H1) were investigated in these animal models.

High efficacy of IP-10 and TNFα-encoding parvoviral vectors could be demonstrated in both models.

Infecting tumor cells with recombinant parvoviruses encoding IP-10 or TNFα as well as treating established tumors with these vectors provided conditions to observe antitumor effect. In nude mice combined IP-10/TNFα expression resulted with significant tumor growth delay, reduced tumor volume and prolongation of animal survival. This effect was not dependent on angiogenesis inhibition. It is possible that NK cells participate in observed antitumoral effects.

The best therapeutic effect – complete tumor eradication – could be demonstrated in immunocompetent animals. This effect was reached when both types of virus (IP-10 and TNFα-expressing) were administered simultaneously. Histological analysis and MRI study showed that antitumoral effects in this system (tumor growth delay, reduced tumor volume and prolongation of animal survival) were not dependent on the inhibition of angiogenesis.

We were able to show that intact immune system is necessary to obtain a strong antitumor effect. Rechallenged animals are protected from tumor growth. GI261 glioma cells can be specifically recognized by host spleenocytes. The data from the literature suggest that the main effectors in the antitumoral response could be $CD8^+$ T cells. TNFα - expressing vector demonstrated the ability to support dendritic cell maturation.

In the systems investigated here the effectiveness of wild type H1 and MVMp viruses could not be demonstrated.

Taken together, the data obtained in this work are promising and suggest that recombinant parvoviruses are good candidates for gene therapy of glioma.

In the future, antitumoral effects of these vectors should be investigated in the intracranial system like well-described GI261 model.

Zusammenfassung

Diese Dissertation wertet die Wirksamkeit parvoviraler Vektoren, die menschliches IP-10 oder TNFα der Maus exprimieren, als Hilfsmittel gegen subkutane Glioma in zwei Tiermodellen aus. Zuerst wurden neue rekombinante MVMp- und H1- basierte Vektoren, die menschliches IP-10 oder TNFα der Maus exprimieren, konstruiert. Es wurde gezeigt, dass parvovirale Vektoren sowohl die menschlichen, wie auch die Glioma der Maus wirkungsvoll infizieren können. Nach der Infektion mit bestimmten Vektoren wurden hohe Mengen von transgenen Proteinen erzeugt. Alle getesteten Zelllinien waren zum Wild-Typ-Virus sensitiv.

Es wurden zwei Tiermodelle eingerichtet: Mit Gl261 Gliomazellen der Maus wurden subkutane Tumore bei C57/Bl6 Mäusen induziert und menschliche U87 Glioblastomazellen erzeugten subkutane Tumore in cd1 swiss Nacktmäusen. Die antitumoralen Effekte, die *in vivo* durch rekombinante und Wildtypparvoviren (MVMp und H1) vermittelt wurden, wurden in diesen Tiermodellen untersucht.

Die hohe Wirksamkeit von IP-10 und TNFα-transduzierenden Parvoviren konnte in beiden Modellen gezeigt werden.

Das Infizieren der Tumorzellen sowohl mit IP-10 und TNFα-transduzierenden Parvoviren, als auch die Behandlung erzeugter Tumore mit diesen Tumoren schufen die Bedingungen, den antitumoralen Effekt zu beobachten. In Nacktmäusen erzeugte die kombinierte IP-10/TNFα- Exprimierung eine signifikante Tumorwachstumsverzögerung, verringertes Tumorvolumen und ein verlängertes Überleben des Tieres. Dieser Effekt war nicht abhängig von der angiogenetischen Inhibition. Es ist möglich, dass NK-Zellen einen Anteil an den beobachteten antitumoralen Effekten haben.

Den besten therapeutischen Effekt – die vollständige Vernichtung des Tumors – konnte an immunokompetenten Tieren gezeigt werden. Dieser Effekt wurde erreicht, als man beide Typen des Virus (IP-10 und TNFα- exprimiert) gleichzeitig verabreichte. Die histologische Analyse und die MRI-Studie zeigten, dass der antitumorale Effekt in diesem System (Tumorwachstumsverzögerung, verringertes Tumorvolumen und ein verlängertes Überleben des Tieres) nicht abhängig sind von der Inhibition der Angiogenese.

Wir konnten zeigen, dass ein intaktes Immunsystem notwendig ist, um eine starke antitumorale Wirkung zu erhalten. Tiere, die zum zweiten Mal mit Tumorzellen infiziert wurden, waren vor Tumorwachstum geschützt. Gl261 Gliomazellen können besonders durch Wirtsmilzzellen erkannt werden. Angaben aus der Literatur lassen vermuten, dass der Hauptverursacher der antitumoralen Antwort $CD8^+$ T-Zellen sein könnten. Der TNFα-exprimierte Vektor demonstrierte die Fähigkeit, die Reifung dendritischer Zellen zu unterstützen. In dem hier untersuchten System konnte die Wirksamkeit des Wildtyps H1 und des MVMp-Viruses nicht nachgewiesen werden.

Zusammenfassend gesagt sind die Daten, die in dieser Arbeit erzielt wurden, viel versprechend und legen nahe, dass rekombinante Parvoviren gute Kandidaten für die Gentherapie bei Gehirntumoren sind.

In der Zukunft sollte der antitumorale Effekt dieser Vektoren im intrakranialen System, wie in dem gut beschriebenen Gl261-Modell, untersucht werden.

1 Introduction

1.1 Gliomas

Gliomas are primary central nervous system tumors that arise from astrocytes, oligodendrocytes or their precursors. Gliomas can be classified into several groups according to their histological characteristics; the most malignant of the gliomas is glioblastoma multiform (GBM). Malignant gliomas are characterised by biological features that make them intractable diseases. These include uncontrolled tumor cell proliferation, invasion into normal brain parenchyma, induction of tumor angiogenesis, inhibition of apoptosis and suppression of the immune system (Okada H. et al. 2001). Gliomas are refractory to most standard therapies, including surgical resection, radiation therapy and chemotherapy. Nearly all glioma patients die of their disease with a mean survival of one year. Despite of established treatments these tumors will recur and cause neurological deterioration and death (Dai C. et al. 2001).

The new WHO Classification of Tumors affecting the Central Nervous System:
In 1993 the WHO ratified a new comprehensive classification of neoplasms affecting the central nervous system. The classification of brain tumors is based on the premise that each type of tumor results from the abnormal growth of a specific cell type. To the extent that the behaviour of a tumor correlates with basic cell type, tumor classification dictates the choice of therapy and predicts prognosis. A shortened classification is provided below (Kleihues P. et al. 1993).

Neuroepithelial Tumors of the CNS	**Other CNS Neoplasms**
Astrocytic tumors	Tumors of the Sellar Region
Oligodendroglial tumors	Hematopoietic tumors
Ependymal cell tumors	Germ Cell Tumors
Mixed gliomas	Tumors of the Meninges
Neuroepithelial tumors of uncertain origin	Non-menigothelial tumors of the meninges
Tumors of the choroid plexus	Tumors of Cranial and Spinal Nerves
Neuronal and mixed neuronal-glial tumors	Local Extensions from Regional Tumors
Pineal Parenchyma Tumors	Metastatic tumors
Tumors with neuroblastic or glioblastic elements (embryonal tumors)	Unclassified Tumors
	Cysts and Tumor-like Lesions

1.1.1 Molecular mechanisms involved in glioma development

Molecular studies have identified some of the genetic changes that underlie the pathologic differences among astrocytic tumors; progression in tumor grade is associated with an ordered accumulation of mutations. Approximately 33% of low grade infiltrating astrocytomas have mutations detected in the p53 gene on chromosome 17p. Overexpression of PDGF (platelet-derived growth factor) and its receptors occurs in all grades of gliomas. Low-grade astrocytomas (WHO grade 2) are characterised by overexpression of VEGF (vascular endothelial growth factor), TGFβ (transforming growth factor β) and chromosomal loss (parts) of 13q what results in RB (retinoblastoma) gene disorders. Amplification of cell cycle regulators (CDK4, MDM2) as well as protease overexpression occurs (Mentlein R. et al. 2003). Anaplastic astrocytomas (WHO grade 3) - whether found in preexistent low grade astrocytomas or detected *de novo* - have a similar incidence of p53 mutations but, in addition, show a loss of heterozygosity on chromosome 19q in more than 40% of cases. Progression from astrocytoma to anaplastic astrocytoma also involves mutations in other tumor suppressor genes including the retinoblastoma gene on chromosome 13q. Finally, glioblastomas have the same incidence of these genetic aberrations and in addition 70 percent have lost heterozygosity for chromosome 10 and one third have amplification of the epidermal growth factor receptor (EGF-R) gene. High-grade gliomas show often overexpression of platelet-derived growth factor (PDGF) gene and expression of angiogenic factors such as vascular endothelial growth factor (VEGF) (Castro M.G. et al. 2003, Kyritsis A.P. et al. 1993). Glioma patients are often immunosuppressed. Molecules like transforming growth factor-β (TGF-β), IL-10 and prostaglandin E_2 are reported to be involved in down-regulation of the cellular immune response in glioma patients. It can be manifested by induction of growth arrest and apoptosis in immune cells, suppression of MHCII expression and by inhibition of the development cytotoxic T lymphocytes (Castro M.G. et al. 2003).

1.1.2 Glioma-directed therapy (standard therapies)

Currently, the standard of care for the treatment of patients with high-grade malignant glioma is resection followed by focal radiotherapy (RT). Even when treated with aggressive combined surgery, chemotherapy and radiotherapy, in patients with gliomas recurrence occurs between 6 and 12 months.

1.1.2.1 Surgery

For patients in whom surgical resection is possible, total resection should be the goal; the extent of tumor resection may be a decisive prognostic factor. However, complete resection is not possible in the majority of patients due to the lack of a defined tumor edge or localization in the critical areas of the brain. An aggressive removal of macroscopic tumor could influence early re-growth from tumor remnants and possibly influence survival. (Giese A. et al. 2001). Even partial resections improve body functions, relieve pressure in the brain and disrupt the brain-blood barrier. It also provides the space for tumor growth and growing cells are more sensitive to radiation and chemotherapy. The biopsies of inoperable tumors provide information for possible implementation of other therapies (Castro M.G. et al. 2003).^

1.1.2.2 Adjuvant therapy

1.1.2.2.1 postoperative radiation therapy:

Adjuvant therapy increases survival in patients with high-grade malignant glioma. It has become the standard adjuvant treatment for these patients. (Brandes A.A. 2003). Radiotherapy is necessary because extensive tumor infiltration into normal brain structures makes resection of the entire primary tumor impossible (DeAngelis L.M. 2003). Stereotatcic surgery allows delivering high radiation doses directly to tumor mass, minimizing the exposure of normal brain tissue. Interstitial radiation therapy uses radioactive pellets implanted within the tumor. Hyperthermia is used to enhance the response of the tumor to the radiation and to the chemotherapy. Radiosensitizers and radioprotectors are drugs used either to sensitize tumor mass or to protect normal tissue (Castro M.G. et al. 2003).

1.1.2.2.2 adjuvant chemotherapy:

Patients treated with RT plus adjuvant chemotherapy had a survival advantage. BCNU (bischloroethyl nitrosourea; carmustine), PCV (the combination of procarbazine, carmustine and vincristine) and temozolomide are the most commonly used chemotherapy regimens in patients with recurrent glioma. BCNU and temozolomide are lipid-soluble agents that cross the blood-brain barrier. Systemic delivery of commonly used medicaments may cause haematological toxicity, skin reactions, hepatic toxicity and pulmonary toxicity. Meta-analyses have suggested that adjuvant chemotherapy,

specifically with nitrosurea-based regimens, is associated with improved survival. (Giese A. et al. 2001). Nevertheless, chemotherapy of brain tumors is not curative and the goals of the treatment are mainly to control the growth of the tumor and to maintain good performance and quality of life for the patient for as long as possible (Castro M.G. et al. 2003).

Several strategies of local treatment were established.

a) local chemotherapy

- supraselective intra-arterial delivery. This method is based on the placement of microcatheters into branches of the midcerebral artery.
- intralesional deposition of biodegradable polymers. Implanting biodegradable polymers, which show slow-release of encapsulated drug (for example BCNU) increases its concentration within the tumor. This strategy spares systemic toxicity.
- convection-enhanced delivery. Convection-enhanced drug delivery is obtained by placing catheters into non-resectable tumors followed by a positive pressure infusion.

b) radiation therapy

Local radiation has very strict limitations regarding the size of treatable lesion. As radionuclides, iridium as well as iodine-125 has been used, with I-125 as temporary high-energy implants or permanent low-activity implants. Radiation point source can be also inserted stereotactically and left in place for a short period of a few minutes and removed after application of the total dose (Giese A. et al. 2001).

1.2 Gene Therapy

New approaches are developing very dynamically; for the moment they are used as adjuvant strategies complementing current strategies (surgery, radiation, chemotherapy). The resistance of brain tumors to the attempts at conventional therapy have made them particularly attractive for gene therapy trials.

“Gene therapy” can be defined as the transfer of genetic material into a patient’s cells for therapeutic purposes. Such a definition includes a variety of therapeutic approaches. Over the last 10 years, more than 300 protocols for cancer and genetic

diseases have been initiated worldwide (Bansal K. et al. 2000). Genetic material can be introduced through direct delivery into the target organ (*in vivo* technique). This is the most common case for patients with brain tumors. Therapeutic agent is applied at the time of tumor resection or by means of stereotactic injections. Another method of genetic material delivery is *ex vivo* technique, where the cells are initially outside of the host and then implanted into affected area. Commonly used methods of delivering genes into cells are:

- plasmid DNA injections
- calcium phosphate transfection
- electroporation
- cationic liposomes
- viral vectors (Bansal K. et al. 2000).

Using the broad definition, gene therapy approaches fall into several categories:

- gene transfer-based immunotherapy
- enzyme/prodrug therapy
- transfer of the therapeutic transgenes into cells
- antisense strategies
- viral vectors

1.2.1 Gene transfer-based immunotherapy

Attempts at gene therapy for boosting the activity of the immune system against cancer cells have often focused on activating cell-mediated immunity. It includes:

- induction of increased immunogenicity of glial tumor cells by enhancement of the immune response using cytokines, for example by inducing the production of IL-2, IL-4 or GM-CSF within the tumor (Okada H. et al. 1999)
- reversal of tumor derived immunosuppression by enhancement of T cell activation (by upregulation MHC I on the tumor cell surface, upregulation of tumor antigenes, upregulation of B7 costimulatory antigens) (Parney I.F. et al. 1997)
- *ex vivo* manipulation of effector cells, for example manipulation of dendritic cells as tool for brain cancer immunotherapy by stimulating them with specific antigens or promoting their maturation (Aoki H. et al. 2001)
- antibodies-coupled compounds delivery, for example anti-CD44 directed antibodies conjugated with isotopes or immunotoxines (Breyer R. et al. 2000)

- local application of activated tumor-infiltrating lymphocytes or NK cells. The interactions between activated lymphocytes and glial cells are still poorly understood, what makes such approaches unclear and risky (Ishikawa E. et al. 2004)

1.2.2 Enzyme/prodrug therapy

In GDEPT (Gene-Directed Enzyme Prodrug Therapy) a gene encoding an enzyme that converts a nontoxic product into a toxic drug is delivered and expressed in tumor cells. The patient is then treated with a prodrug, and when it reaches the tumor cell, it is converted into toxic drug by the newly introduced enzyme. For malignant glioma, most interest has focused on inserting the herpes simplex virus thymidine kinase gene, followed by treatment with the prodrug ganciclovir. Thymidine kinase phosphorylates the ganciclovir, creating a toxic nucleotide analogue, which blocks the function of DNA polymerase, leading to the death of target cell. A variety of vectors have now been used to transfer the thymidine kinase gene, including retrovirus, adenovirus, herpes simplex virus, and direct injection into the tumor (Bansal K. et al. 2000). Herpes simplex thymidine kinase gene (HSV-tk) transfer followed by ganciclovir treatment as adjuvant gene therapy is reasonable and appears to be satisfactorily safe as an adjuvant to the surgical resection of recurrent glioblastoma, but any benefit appears to be marginal (Shand N. et al. 1999).

1.2.3 Transfer of the therapeutic transgenes into cells

Such strategy involves replacement gene therapy and/or induction of beneficial proteins. Replacement gene strategy intends to incorporate a functional gene, such as tumor suppressor gene into the cells of the patient because the gene of interest is defective or absent. For gliomas it could be a restoration of normal p53 function. This protein normally causes growth arrest in the G_1 phase of the cell cycle in case of DNA injury. This gives the time for DNA repair. If DNA injury exceeds a critical repair threshold, p53 induces apoptosis. Another example is RB protein that inhibits progression of the cell cycle. Adenoviral vectors to deliver genes coding for RB protein have demonstrated antitumor effects in animal models (Riley D.J. et al. 1996).

Tumor angiogenesis can be targeted by gene transfer-mediated expression of anti-angiogenic agents. In this approach an expression of beneficial protein is induced,

even if that protein is not actually deficient. An example is viral-mediated transfer of anti-angiogenic peptide platelet factor 4 to inhibit endothelial proliferation (Tanaka T et al. 1997).

1.2.4 Antisense strategies

The target of antisense therapy is to block the expression of cancer genes. Antisense constructs hybridize in an anti-parallel orientation to mRNA of intrest. During triple-helix formation process antisense constructs bind to double stranded DNA in the nucleus. Two main antisense strategies have been employed:

- transfection of cells with antisense cDNA;
- treatment of cells with the shorter antisense oligodeoxynucleotides (ODNs).

For brain tumors therapy antisense constructs against the following genes were used: basic fibroblastic growth factor (bFGF), protein kinase C, isotype α (PKCα), insulin-like growth factor 1 (IGF-1), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF) and many others. Antisense ODNs have been used successfully to block glioblastoma gene expression in vitro and expression of multiple genes within the CNS of experimental animals (Engelhard H.H. 1998).

1.2.5 Viral vectors

They can be used as effective vectors for transferring foreign genes into the cells in the approaches boosting the activity of the immune system, in the enzyme/prodrug therapy and in the transfer of therapeutic transgenes. Very often they are also used as direct oncolytic factors.

Gene therapy using viral vectors for the treatment of brain tumors has proven to be a promising novel treatment modality. One of the first available reports instituting a relationship between viral infection and cancer regression appeared in 1912 when a woman suffering from cervical cancer was bitten by a dog, injected with the attenuated Pasteur's viral vaccine and subsequently showed tumor regression. The properties of an ideal viral vector should include:

- selectivity to neoplastic cells
- minimal toxicity to normal tissue
- systematic killing of tumor tissue
- high efficacy.

The quantity of replication-competent viruses could potentially increase after inoculation; this benefit is not offered by any existing cancer treatments. Any viral-based gene therapy vectors to be useful in patients must be safe, potent against tumors and complement or ever synergize with existing standard therapies (Shah A.C.et al.2003).
To the most studied viruses proposed for the use in the brain tumor gene therapy belong:

1.2.5.1 Herpes simplex virus-1 (HSV-1)

HSV-1 is a nuclear replicating, icosahedral, enveloped DNA virus. The HSV-1 genome is a linear, double stranded DNA duplex 152 kbp in length. It is non-integrating, neurotropic virus whose essential and non-esential genes have been identified. Genes involved in neurovirulence of HSV-1 are separate and distinct from those that confer oncolytic properties, such that deletion of the neurovirulence genes allows selective targeting to glioma cells without elimination of its oncolytic abilities. Furthermore, up to 30 kbp of the HSV genome can be replaced with foreign DNA while still retaining the virus' ability to replicate. The first engineered mutant of *HSV-1 was a tk (thymidine kinase) deletion mutant*. This mutation restricted viral replication to mitotic cells, which supply tk necessary for the replication. Another mutant of HSV-1, *G207*, retains its susceptibility to standard ani-HSV therapies like acyclovir, since the tk gene is intact. G207 passed some studies that confirmed its safety for the use in patients. *HSV1716* is a single-mutant replication-selective virus with attenuated ability to replicate in neurons of the CNS (Shah A.C.et al.2003).

In the clinical studies the safety of G207 and HSV1716 vectors was evaluated. G207 was moved into Phase I clinical trials in 2000. This dose-escalation study was intended to determine the maximally tolerated dose (MTD) and any dose-limiting toxicities of G207. The results showed the safety of vector administration. Injecting up to $3x10^9$ PFU did not cause any toxicity; there was no evidence of encephalitis or any major inflammatory reactions observed (Markert J.M. et al. 2000).

HSV1716 underwent clinical trials to evaluate its toxicity in patients with recurrent malignant glioma. The study showed that at least up to $1x10^5$ PFU of the vector could be administered without signs of side effects. Again, no MTD was established because the highest dose administered in the study was well tolerated (Rampling R. 2000). In 2002 the Glasgow group reported a second clinical trial appraising the efficacy of

HSV1716 virus and suggesting that replication occurs in at least some of the high-grade gliomas treated with the intratumoral injections (Papanastassiou V. et al 2002).

HSV-1 mutants serve not only to kill tumor cells vie infecting, replicating and lysing them, but also by functioning as vectors to deliver antitumor agents. Engineered HSV-1 that express IL-4 and IL-2 can increase antitumor efficacy in syngenic murine models of brain tumors over treatment with oncolytic HSV-1 that does not express these cytokines (Andreansky S. et al. 1998).

1.2.5.2 ONYX-015

Adenoviruses are non-enveloped viruses with a single, linear, double-stranded DNA genome of approximately 36-38 kbp in size. *ONYX-015* is an adenovirus that selectively replicates in and lyses neoplastic cells with defects in p53 or the p53 pathway. A deletion in the 55 kD protein E1B resulted in its selective phenotype (Bischoff J.R. et al. 1996). ONYX-015 has joined G207 and HSV1716 as the third selective, oncolytic and replication-competent virus to be investigated in a clinical trial for the treatment of malignant glioma. A Phase I study in patients with a malignant glioma demonstrated that injection of ONYX-015 into glioma cavities is well tolerated at doses up to 10^{10} PFU (Chiocca E.A. et al. 2004). Prior to this, the safety and potential efficacy of ONYX-015 was evaluated in the treatment of head and neck cancers both in Phases I and II trials. In patients no dose-limiting toxicity was observed (the highest administered dose was $1x10^{11}$ virus particles) and the adverse symptoms included mostly low-grade flu-like symptoms. In Phase II combining ONYX-015 with chemotherapy showed much greater efficacy and potential (Khuri F.R. et al. 2000), so a Phase III has been undertaken for head and neck cancer.

01/PEME is another adenovirus engineered to replicate in a p53-dependent fashion. When infecting cells with intact p53, the virus prevents its own replication. *CN706* has been genetically modified from the parent strain by the insertion of a prostate-specific enhancer, targeting the virus to prostate cancers. *Ad5lucRGD* adenovirus expresses a recombinant RGD fiber (Arg-Gly-Asp) sequence that interacts with a_V integrins, which are abnormally expressed in many cancers (Suzuki K. et al 2001).

In human trials, adenoviral vectors caused inflammatory reactions, formation of antibodies to adenoviruses and transient fever, but they have not been linked to any human malignancies (Ylä-Herttuala S. et al. 2003). However, the first gene therapy-

associated death occurred when a high dose of adenovirus vector was given intraportally into a patient who suffered from a genetic defect causing ornithine transcarbamylase deficiency. In retrospect, the death seems to have been a result of toxicity caused by the adenoviral vector in conjunction with the underlaying disease (Lehrman S. 1999).

1.2.5.3 Newcastle disease virus (NDV)

It belongs to avian paramyxoviruses (PMV). Nine members of this family have a single stranded, linear RNA. The total genome is roughly 16kbp. Replication of the virus takes place in the cytoplasm of the host cell. NDV was found to have increased replication efficiency in cancer cells compared to non-neoplastic human cells. Because it replicates in the host cell cytoplasm with strong cell-binding properties and exhibits great properties as an adjuvant, NDV was shown to be very potential as a cancer vaccine therapy. NDV-infected cells exhibit enhanced recruitment and activation of NK cells and T_C cells. The hemagglutin-neuraminidase molecule expressed on the surface of infected cells has great costimulatory function. NDV facilities recognition of tumor cells via its strong induction of the cytokines TNFα and IFNγ (Zorn U. et al. 1994).

A Phase I trial in the US looked at the safety of utilizing PV701, an oncolytic NDV, for 79 patients with various advanced solid cancers. The most common adverse event in this trial was fever and other flu-like symptoms. However, some serious adverse events were noted, with one death that may have been associated with virus administration (Pecora A.L. et al. 2002). A progressive shrinking of glioblastoma tumor was demonstrated in a patient treated with NDV vaccine (Csatary L.K. et al. 1999).

NDV-infected cells have been investigated as cancer vaccines through treatment with both oncolysates and whole-cell vaccine. Oncolysate immunotherapies have been used in four clinical trials (one Phase I and three Phase II) in the US against advanced melanoma and in two Phase II clinical trials in Germany against advanced renal cell carcinoma. For the melanoma trials, two of the Phase II trials showed benefit of oncolysate vaccine use whereas the last showed no significant benefit (Shah A.C. et al. 2003). Several clinical trials (Phase I and Phase II) have been conducted in the treatment of breast, ovarian, renal cell and colorectal carcinomas. Clinical trials of antitumor vaccination with an autologous tumor cell vaccine modified by virus infection demonstrated improvement of patient survival based on improved antitumor immune memory (Schirrmacher V. 2004).

1.2.5.4 Reovirus

Reoviruses are non-enveloped viruses with a segmented, double-stranded RNA genome. Total genome is 18-30 kbp long. When reoviruses infect the cell, double-stranded RNA can activate the host protein kinase (PKR), which shuts down protein synthesis to protect the cell from viral infection. Upregulation of Ras pathway interferes with PKR signalling. Therefore tumors with Ras mutation permit successful infection of reoviruses. In malignant gliomas Ras mutations can be rare. However, EGFR and PDGFR are commonly overexpressed in gliomas, what leads to overexpresion of Ras and up-regulation of the Ras signalling pathway (Wilcox M.E. et al. 2001). This property makes reoviruses interesting tool against tumors with functional p53 expression, which are resistant to adenoviruses like ONYX-015. Preclinical studies demonstrate that reovirus is a promising oncolytic agent for primary brain tumors in experiments *in vitro, in vivo* and *ex vivo*.

The company Oncolytics reported about completed Phase I clinical study with reovirus (REOLYSIN ®). The study examined the administration of escalating dosages of REOLYSIN® directly into subcutaneous tumors. No serious adverse events were attributed to the administration of the virus and no dose-limiting toxicity was reached (March 21, 2002 - Oncolytics Biotech Inc. Announces REOLYSIN® Phase I Clinical Trial Results -- Study indicates potential anti-cancer agent is safe for human use).

In spring, 2002, Oncolytics initiated a clinical trial examining the use of REOLYSIN® in cancer confined to the prostate gland. Patients were treated with a single injection of REOLSYIN® directly into the prostate gland. Final results showed evidence of apoptotic tumour cell death in four of six patients, with no safety concerns (March 31, 2003 - Oncolytics Biotech Inc. Reports Presentation of Results from Interim Assessment of T2 Prostate Cancer Trial).

A Phase I/II Clinical Trial to Evaluate Dose Limiting Toxicity and Efficacy of Intralesional Administration of REOLYSIN® for the Treatment of Patients with Histologically Confirmed Recurrent Malignant Gliomas was announced in 2002. Positive safety results of the Phase I study could be demonstrated (December 23, 2002 - Oncolytics Biotech Announces Positive Interim Safety Results from REOLYSIN® Phase I Malignant Glioma Study).

In May 2004, the Company announced that the first patient had been enrolled in its systemic administration trial in the United Kingdom. The primary objective of Oncolytics' first systemic administration study is to determine the safety of REOLYSIN®

when administered intravenously. The secondary objective is to observe tumour and immune system response to intravenous infusion of REOLSYIN®, which will help to determine dosage levels in subsequent clinical studies (May 26, 2004 - Oncolytics Biotech Starts Patient Treatment in UK Phase I Cancer Trial Investigating Systemic Delivery of REOLYSIN®)

1.2.5.5 Poliovirus-derived viruses

An *intergeneric poliovirus PV1* is an attenuated version of poliovirus. Polioviruses are non-enveloped RNA viruses that cause illnesses from minor infection to paralytic poliomyelitis as a consequence of acute neurovirulence. Intergeneric poliovirus PV1 showed promising effects as anti-glioma factor in animal experiments. *Poliovirus replicons* are alternative candidates derived from poliovirus for the use in therapy of malignant glioma. They are replication-incompetent particles; due to deletion of capsid gene (P1) they cannot form progeny virions. The capsid gene can be substituted with a gene of interest such as the carcinoembrionic antigen, TNFα, IL-1 (Bledsoe A.W. et al. 2000).

Animal studies demonstrated that live-attenuated poliovirus has potent oncolytic activity against human neuroblastomas in vitro and in vivo and it may be useful for the treatment of advanced and refractory neuroblastomas, however, further studies are necessary to evaluate the safety of the method (Toyoda H. et al. 2004).

Poliovirus replicons have been administrated to non-human primates with no consequent deleterious effects according to Replicon Neurothechnologies, Inc. (URL: http://www.replicontechnologies.com/Technology.htm). Replicon Technologies Inc. (RTI) announced that the National Cancer Institute (NCI) and Small Business Innovation Research Program (SBIR) have awarded RTI a Phase II grant of more than $750,000 to develop replicons (modified viruses that seek out and destroy cancer cells) as brain tumor therapies (Business Wire, Nov 6, 2001).

1.2.5.6 Vaccinia virus

Vaccinia virus is a member of the *Poxviridae* family of viruses, and has also been studied as a possible virus for the use as an anti-tumor agent. The recombinant vaccinia virus expressing p53 was tested in combination with radiation therapy as an anti-tumor vector for the C6 rat glioma model, a p53-deficient tumor cell line. Antitumor effect can

be also obtained by the administration of vaccinia viruses expressing IL-2 and IL-12. Deletion of tk gene or vaccinia growth factor genes allows targeting the vector to neoplastic tissue (Shah A. et al. 2003)

Vaccinia virus vectors have been applied for a few cancer therapy trials. The prostate-specific antigen expressed from vaccinia virus vectors in a phase I study on patients with advanced prostate cancer (Eder J. et al. 2000). Stable antigen levels and disease inhibition were obtained in some patients. In another study on mesothelioma tumors replication-restricted vaccinia virus expressing IL-2 was used for the intratumoral treatment. The study showed minimal toxicity and the expression of IL-2 lasted 2 up to 3 weeks (Mukherjee S. et al. 2000). In a study on metastatic breast cancer the patients received repeated intramuscular injections of an attenuated vaccinia virus expressing the human MUC1 gene and the IL-2 gene. Partial tumor regression (>50%) was observed in 2 patients and stable disease in 15 patients (Scholl S. et al. 2003).

Among current and terminated trials, retroviruses are the most frequently used (28%), closely followed by adenoviruses (26%). Poxviruses represent 3,4%, herpes simplex viruses 2,8% and AAV 2,1% of the trials (Lundstrom K. 2004).

Once the safety and efficacy of these nonintegrating viruses through simple oncolysis has been proven, along with their utility in conjunction with standard therapies, these agents can be considered as potential gene delivery vectors. Many of these viruses can be genetically engineered to express therapeutic genes to further enhance efficacy profiles. Collectively, the clinical trials conducted thus far, as well as the results of ongoing preclinical studies involving the use of novel ‘virotherapy’ agents, have served to invigorate and expand the enthusiasm for cancer biotherapy, justify further clinical studies, and will hopefully soon succeed in extending the time and quality of the lives of terminally ill patients with malignant gliomas. The ultimate proof of the significance of oncolytic viral therapy in the treatment of glioma, either alone or in combination with other therapies, will require the successful performance of Phases II and III trials.

1.3 Parvoviruses

The first parvoviral isolates were derived from tumors and tumor cell lines (Kilham L. et al 1959). These facts initially brought parvoviruses into the class of oncogenic viruses, but this was not confirmed since parvovirus infections did not correlate with higher tumor incidence. On the contrary, later investigations revealed that parvoviruses could

prevent the formation of tumors in animal models (Dupressoir T. et al. 1989; Rommelaere J. et al. 1991).

The Parvovirus Family subdivides into two subfamilies. The insect parvoviruses are classified in the *Densoviridae* subfamily and the vertebrate parvoviruses - in the *Parvovirinae* subfamily (see Table 1).

Dependoviruses or adeno-associated viruses (AAV) require a coinfection with a helper virus for efficient productive infection. In the absence of such, they integrate into the host chromosome in a site-specific manner. When a latently- infected cell is superinfected with a helper virus (adeno-, herpes simplex-or human papilloma- virus), the AAV genome rescued, and replicates, leading to a productive infection. AAV capsid can package minus or plus single-strand genomes with equal efficiency. Humans often carry an asymptomatic AAV infection (Cheung A.K. et al. 1980).

Family	Subfamily	Genus	Characteristic Representatives
Parvoviridae	*Parvovirinae*	Autonomous PV	
		Parvovirus	MPV, MVMp, MVMi H-1 KRV, FPV, CPV, PPV, LuIII, GPV, ADV, BPV
		Erythrovirus	B19, V9, SPV
		Helper-dependentPV	
		Dependovirus	AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6
	Densovirinae	*Densovirus*	JcDNV
		Iteravirus	BmDNV
		Contravirus	AaDNV

MPV – mouse parvovirus, **MVM –** minute virus of mice (prototype (p) or immunosupressive (i) strain), **H-1 –** H-1 parvovirus, **KRV –** Kilham's rat virus, **FPV –** feline panleukopenia virus, **CPV –** canine parvovirus, **LuIII –** LuIII virus, **GPV –** goose parvovirus, **ADV –** aleuthian mink disease virus, **BPV –** bovine parvovirus, **SPV –** simian parvovirus, **B19 –** parvovirus B19, **V9 –** human erythrovirus V9, **AAV –** adeno-associated virus, **JcDNV –** Junonia coenia desendovirus, **BmDNV –** Bombyx mori desendovirus, **AaDNV –** Aedes aegypti desendovirus

Table 1 Taxonomy of parvoviruses

Only one member of the Erythrovirus Genus - B19 is known to be pathogenic to humans. Acute infection causes fifth disease in children, polyarthropathy syndromes in adults or chronic anemia due to persistent infection in immunocompromised patients. Infection in pregnancy can lead to hydrops fetalis with possible fetal loss or congenital

infection (Cassinotti P. et al. 1994). Autonomous parvoviruses cause infectious pathology in some animal species but none of them has been associated with human diseases (Rommelaere J. et al. 2001).

1.3.1 Structure and properties of the parvoviral particle

Parvoviruses are small (18 to 26 nm), nonenveloped, nuclear-replicating viruses. The parvoviral capsid packages a single-stranded DNA genome of about 5000 nucleotides. Full parvoviral particles have a density of 1.39-1.4 g/cm^3 in CsCl gradients. They are stable in the pH range of 3 to 9, for 60 min at 56°C and can survive storage for a very long period of time without significant loss of infectivity. MVM is the best-studied member of autonomous parvoviruses up to date. Its particle has an icosaedrical shape.

The capsids consist of three structural proteins VP1 (83-86 kDa), VP2 (64-66 kDa) and VP3 (60-62kDa) with VP2 being the most prominent (80%). VP3, a cleavage product of VP2, is present in small, varying amounts in DNA-containing virions (Agbandje-McKenna M. et al. 1998).

1.3.2 Gene expression

The two promoters upstream of each of the two large ORFs at map units 4 and 38 are named P4 and P38, respectively. Two P4 transcripts, R1 (4.8kb) and R2 (3.3kb), are generated upon splicing and encode the two nonstructural proteins NS1 and NS2 (L, Y, R isoforms). The P38 transcript R3 (3.0kb) produces the VP-1 and VP-2 proteins after differential splicing (Morgan W.R. et al. 1986).

Transcription from the P4-directed promoter is regulated by TATA and GC boxes localized between nucleotides 180 and 150 from the left end. The transcription factors TFIID and Sp1 bind to the viral TATA and GC boxes, respectively. The activity of the P4 promoter is regulated in a cell cycle-dependent manner at the G1/S-phase transition, through E2F. P4 is repressed in confluent noncycling cells (contact inhibition), while transformation of cells with several oncogenes including ras and following uncontrolled proliferation, correlates with an increase in the activity of this promoter (Deleu L. et al. 1998).

P38 is a weak promoter directing transcription from localised nt 2005. It comprises a TATA and GC boxes, as well as a transactivation response region (TAR)

localised at nt –145 to –115. P38 has a low basal activity but can be transactivated by the viral NS1 protein. NS1 is binding sequence-specifically to an ACCA motif, which occurs at multiple positions through the parvoviral genome (Cotmore F. et al. 1995).

1.3.3 Parvoviral proteins

NS1 is an 83 kDa multifunctional protein. It is relatively stable with a half-life of approximately 6 hours (Miller C.L. et al. 1995). Some of its functions include NTP-binding, helicase activity, nuclear targeting, homo- and hetero-oligomerization, DNA binding, site-specific DNA nicking. NS1 is the only viral DNA protein being essential for DNA replication in all cell types tested. It has also a transcription-activating domain in its C-terminal region (Krady K.J. et al. 1995).

NS1 can also inhibit DNA replication from heterologous origins. For instance, SV40 ori-driven replication of hybrid plasmids was reduced in the presence of NS1 when sequences from SV40 and either B19 (Beard C. et al. 1989) or MVMp (Tenenbaum L. et al. 1993) parvovirus were combined. NS1 expression was found to disregulate transcription driven by various nonparvoviral (including cellular) promoters like SV40, the Harvey-ras promoter and HIV I LTR promoter (Rhode S.L.III et al. 1987). A productive parvoviral infection may be associated with cell death, for which NS1 is considered to play a major role (Vanacker J.M. et al. 1995). H-1 parvovirus has been reported to induce apoptotic cell death in rat glioblastoma cells (Ohshima T. et al. 1998) and human leukemic U-937 cells. In the last system induction of apoptosis can be assigned to the cytotoxic non-structural proteins (Rayet B. et al. 1998).

Minute virus of mice NS1 protein is a multifunctional phosphoprotein endowed with a variety of enzymatic and regulatory activities necessary for progeny virus particle production. To regulate all of its different functions in the course of a viral infection, NS1 has been proposed to be modulated by posttranslational modifications, in particular, phosphorylation. NS1 is a target for PKClambda phosphorylation in vivo and that this modification is essential for the helicase activity (Nuesch J.P. et al. 2003). PKCeta phosphorylates NS1 and in consequence is able to activate the viral polypeptide in concert with PKClambda for rolling circle replication (Lachmann S. et al. 2003).

NS2 is a small protein with a molecular weight of 23-25kDa. In contrast with NS1, NS2 has a predominantly cytoplasmic localisation. *In vitro* and *in vivo* experiments point to the fact that NS2 of MVMp is essential for a productive viral infection in mouse (the

natural host) cells (Naeger L.K. et al. 1993). NS2 from MVMp interacts with 14-3-3 protein family members (Brockhaus K. et al. 1996) and nuclear export factor CRM1 (Bodendorf U. et al. 1999). It was also shown that the NS2 proteins of MVMp are required for efficient nuclear egress of progeny virions in mouse cells (Eichwald V. et al. 2002).

VP proteins are derived from differentialy spliced R3 transcripts. The VP1/VP2 ratio is about 1:5, and this is the proportion in which they appear in the capsid. However, empty capsids can be assembled from VP2 alone. The role of the VP proteins is first to assemble into empty capsids. The interaction between VP proteins and the 3‘ terminal hairpin end of RF (replicative form) DNA has been suggested to be involved in the subsequent displacement and packaging of single-stranded progeny DNA (Willwand K. et al. 1991). VP2 is necessary for the accumulation and encapsidation of virus progeny single-stranded DNA. VP1 is dispensable for these functions but is required to produce an infectious virion. Virus that lacks VP1 binds to cells as efficiently as wild-type minute virus of mice but fails to initiate a productive infection (Tullis G.E. et al. 1993).

The virion of CPV (canine parvovirua) contains a potential nuclear localization signal as well as a phospholipase A(2) like domain in N-terminus of VP1. Permeability of endosomal membranes apparently changes during CPV infection, probably due to the PLA(2) activity of the virus. These results suggest that parvoviral PLA(2) activity is essential for productive infection and presumably utilized in membrane penetration process of the virus (Suikkanen S. et al. 2003).

Parvovirus DNA replication and packaging occur in the nucleus. The process involves three different steps: the synthesis of the viral complementary (positive) strand (conversion) leading to the generation of a monomer length replicative form (mRF), DNA amplification through the formation of multimeric RFs, the subsequent packaging of single-stranded progeny DNA into preformed capsids and release (Cotmore S.F. et al. 1995).

1.3.4 Oncosuppressive effects of parvoviruses

Persistent or latent infections with parvoviruses may significantly protect against spontaneous tumorigenesis. Epidemiological studies showed that hamsters that have survived parvovirus H-1 infections at birth, without a syndrome, had a fivefold lower cancer incidence than their uninfected siblings. When parvoviruses were used to infect tumor cells, which were subsequently implanted into rodents or dogs, inhibition of tumor development was observed in the recipient animals (Toolan H.W et al. 1967).

Many human and murine cell lines of in vitro transformed cells of fibroblastic and epithelial origin were shown to be much more sensitive to viral infection than the normal cells from which they derive. Transformation by physical and chemical carcinogenes, as well as viral oncogenes (such as large T antigen of SV40, middle T antigen of polyomavirus, v-src, Ha-ras), correlates with an increased cytopathic effect of parvoviruses (Rommelaere J. et al. 1991). Conversely, the functional inactivation of the tumor suppressor gene product p53 was shown to correlate with a sensitisation of rat cells to H-1 virus infection (Telerman A. et al. 1993). It seems that the cell transformation coincided with the stimulation of an intracellular step(s) in the parvoviral life cycle, in particular viral DNA amplification and/or viral gene expression.

Similarly to the *in vitro* transformed cells, many human cell lines established from various tumors (fibrosarcoma, epidermoid and mammary carcinomas, gastric cancer, hepatoma and lymphoma) are more susceptible to killing by MVMp or H-1 virus than the corresponding normal cells (for a review see Cornelis J.J. et al. 2001). Humans are hosts for a number of parvoviruses like the autonomous B19 as well as AAV-2, AAV-3 and AAV-5. This gives hope of including some of these viruses or their components, in the arsenal of anticancer therapeutic agents. It should be stated however that tumors might arise in autonomous parvovirus-infected organisms (as shown, for instance by the isolation of parvoviruses from human tumor material implanted in animals). The antitumor response could be limited by the initial quantity of virus present at the site, by inefficient local production of new particles or by the host's immune response. Hence, there is still need for optimizing the oncosuppressive activity of parvoviruses. Our approach to this end involves the development of parvoviral vectors combining the intrinsic parvoviral anticancer properties with the additional therapeutic effect of a transgene.

1.3.5 Vectors derived from autonomous parvoviruses

Vectors have been derived from the autonomous parvoviruses MVMp, H-1 and LuIII. The development of vectors derived from MVMp and H-1 viruses has been hampered by low titres and contamination with replication-competent virus (RCV) that ist generated through homologous recombination with helper plasmids. Several approaches have been used to avoid recombination between vectors and helpers (for a review see Brandenburger A. et al. 2004).

Kestler et al. demonstrated that the production of H1 viral vectors by cotransfection of recombinant clones and helper plasmids providing the structural proteins (VPs) in trans, drastically decreased when more than 800 bp was removed from the VP transcription unit (Kestler J. et al. 1999). To minimise recombination between the vector and helper genomes a cell line in which the MVM helper functions are inducibly expressed from a modified MVM genome that is stably integrated into the host cell chromosome can be used. Using this MVM packaging cell line, MVM vector stocks that contained no detectable helper virus could be reproducibly generated (Brandenburger A. et al. 1996).

Dupont et al. have developed a second-generation MVMp-based vector system specifically designed to reduce the probability of RCV generation by homologous recombination. They constructed a new MVMp-based vector and a new helper genome with minimal sequence overlap. The generation of contaminating viruses in medium-scale rMVMp preparations was substantially reduced (approximately 200 x), but not completely eliminated. The contaminating viruses arising from the new packaging system cannot initiate secondary infection rounds (so they are not RCVs) (Dupont F. et al. 2001). A new vector/helper system has been presented that takes advantage of the similarity between MVMp and H-1 (Wrzesinski et al. 2003). Viral sequences downstream of the transgenes were exchanged between MVMp and H-1 vectors. RCV production was greatly reduced in the chimeric vector stocks but it was not altogether abolished at least for the MVMp-based vector. Packaging an MVMp vector into an H-1 capsid, and *vice versa*, abolished the generation of RCV after cotransfection. (Wrzesinski et al. 2003).

1.3.6 Autonomous parvovirus-mediated tumor therapy

The anti-cancer effect of H-1 or MVM vector-mediated transduction of various cytokines/chemokines was evaluated by monitoring the formation of tumors following the implantation of human or mouse neoplastic cells in recipient mice (for a review see Cornelis J.J. et al. 2004).

Tumor cells	Parvovirus backbone	Therapeutic product	References
HeLa (human cervical carcinoma)	H-1	human IL-2	Haag A. et al. 2000
	H-1	human MCP-3	Wetzel K. et al. 2001
	H-1	human MCP-1	Haag A. et al. 2000
K1735 (mouse melanoma)	MVMp	human IL-2	El Bakkouri K. et al. 2000
B78/H1 (mouse melanoma)	MVMp	human MCP-3	Wetzel K. et al. 2000
H5V (mouse endothelioma)	MVMp	mouse IP-10	Giese N. A. et al. 2002

Table 2 Anti-tumor effect of parvoviral vectors transducing immunomodulating products in animal experiments (according to Cornelis J.J. et al. 2004)

First experiments were carried out using interleukin 2 (IL-2). The formation of tumors from HeLa cells infected *in vitro* with H-1/IL-2 vector prior to implantation in nude mice was reduced by 90% compared with mock-infected cells (Haag A. et al. 2000). IL-2 – transducing MVMp was also found to be endowed with a strong antitumor activity in immunocompetent animals (El Bakkouri K. et al. 2000).

An H-1 virus - based vector expressing the human monocyte chemotactic protein 3 (MCP-3) was also evaluated for its capacity to suppress HeLa tumors in nude mice. The MCP-3 - transducing vector had only a modest antitumor effect which was shown to be mediated by macrophages and NK cells (Wetzel K. et al. 2001). In contrast, a complete tumor suppression was observed after the subcutaneous implantation of *in vitro* MVMp/MCP-3 – infected B78/H1 mouse melanoma cells in immunocompetent mice (Wetzel K. et al. 2000).

A mouse chemokine, interferon γ - inducible protein 10 (IP-10) was also expressed from an MVMp – based vector. MVMp/IP-10 proved to have potent anticancer effects in the mouse hemangiosarcoma H5V (Giese N.A. et al. 2002).

A full cure from established thymomas was achieved by the injection of recombinant MVMi transducing the co-stimulatory molecule B7-1 (Palmer G.A. et al. 2000).

Altogether, the pilot pre-clinical studies described above are very encouraging, since low doses of parvoviruses were given per animal and no deleterious effects could be detected even after repeated virus injections.

1.4 Angiogenesis

Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100 to 200 µm of blood vessels – the diffusion limit for oxygen. Vessels in an embryo are assembled from endothelial precursors (vasculogenesis). Subsequently, this primitive network expands by sprouting or intussusception (angiogenesis) (Jain R.K. 2003). These processes are regulated by a balance between pro- and antiangiogenic molecules and are derailed in various diseases, especially cancer. Tumor vessels develop by sprouting or intussusception from pre-existing vessels. Circulating endothelial precursors shed from the vessel wall or mobilized fro the bone marrow, can also contribute to tumor angiogenesis. In addition, tumor cells can co-opt existing vessels (Carmeliet P. et al. 2000).

Various signals that trigger the “angiogenic switch” have been discovered. These include metabolic stress (low pO_2, low pH or hypoglycaemia), mechanical stress (pressure generated by proliferating cells), immune/inflammatory response (the cells that have infiltrated the tissue), and genetic mutations (activation of oncogenes or deletion of tumor-suppressor genes that control production of angiogenesis regulators). Pro- and antiangiogenic molecules can emanate from cancer cells, endothelial cells, blood and the extracellular matrix.

Several molecules have been implicated in these processes. During sprouting angiogenesis, vessels initially dilate and become leaky in response to VEGF. Ang1 and the junctional molecules VE-cadherin and platelet-endothelial cell-adhesion molecule (PECAM) tighten vessels and their action needs to be overcome during angiogenesis. Ang2 and proteinases mediate dissolution of the existing basement membrane and the interstitial matrix. Numerous molecules stimulate endothelial proliferation, migration and assembly, including VEGF, Ang1 and bFGF (Ferrara N. et al 2003). Cell-matrix receptors such as the $\alpha_v\beta_3$ and α_5 integrins mediate cell spreading and migration. Maturation of nascent vessels involves formation of a new basement membrane and investment of new vessels with pericytes and smooth muscle cells. PDGF-BB recruits smooth muscle cells, whereas signalling by TGF-β1 and Ang1/Tie2 stabilizes the

interaction between endothelial and smooth muscle cells. Proteinase inhibitors (for example, PAI-1) prevent degradation of the provisional extracellular matrix around nascent vessels. VEGF, bFGF, granulocyte macrophage-colony stimulating factor (GM-CSF), IGF-1 and angiopoietins have been implicated in the mobilization of endothelial precursors, angiopoietins are also important in vessel co-option (Carmeliet P. et al. 2000).

The structure and function of tumor blood vessels is different then normal vessels.

They have chaotic architecture and blood flow. Vascular permeability is high. Vascular endothelium expresses non-uniform surface markers. Very often a functional lymphatic is missing.

Gliomas are particularly highly vascularized and, therefore, serve as a model to elucidate the process of tumor angiogenesis and to investigate new antiangiogenic therapies. The microvessels in brain tumors characteristically lose their normal blood-brain properties and leak fluid into the brain. A cerebral oedema is a consequence. The proteins building tight junctions are downregulated in these tumors what results in the opening of the blood-brain barrier. VEGF seems to be the most important angiogenic factor in gliomas. Its mRNA and also the protein are abundant. High-grade gliomas (WHO grade 4) produce more VEGF than low-grade astrocytomas (Ferrara N. et al. 2003). VEGF targets predominantly endothelial cells. It induces their migration and the expression of several genes involved in extracellular matrix degradation. VEGF increases also vascular permeability. Expression of VEGF is regulated by hypoxia and growth factors/cytokines. This protein plays a key role in the transformation of normal glial cells to malignant glioma cells (Mentlein R. et al. 2003).

To the most important angiogenic pleiotropic factors produced by glioma cells belong PDGF, bFGF, PTN and TGFβ. PDGF and bFGF are growth factors that act like VEGF – on tyrosine kinase receptors. This results in a mitogenic response as well as the transcription of responsive genes (e.g. VEGF). Both factors act also on perivascular cells. PTN (pleiotrophin) is another growth factor. It is produced by glioma cells, but not by normal glial cells. It is not only a mitogen and a chemoattractant for endothelial cells, but also for microglial cells/monocytes that invade tumors. TGFβs belongs to the family of proteins that regulate cell growth, differentiation, morphogenesis, immunosuppression and apoptosis. TGFβs contribute to tumor angiogenesis by their chemotactic effect on endothelial cells, stimulation of extracellular matrix protein

formation, on which endothelial cells adhere and spread as well as by increasing VGFE and VEGFR expression (Breier G. et al. 2002)

Several endogenous angiostatic factors are known. These include proteins and peptides, e.g. angiostatin, endostatin, pigment epithelium-derived factor (PEDF), somatostatin, interferons, chemokines, and lipids, e.g. retinoids and some steroids, also other substances such as nitric oxide. Antiangionetic approaches find use in the treatment of vascular malformations, heart and lung diseases, obesity and cancer. These trials are based on strategies that:

- interfere with angiogenic ligands, their receptors or downstream signalling (inhibiting VEGF expression by antisense constructs or protein neutralization by the antibodies)
- upregulate or deliver endogenous inhibitors (angiostatin and endostatin delivered by gene transfer)
- directly target tumor vasculature (thalidomide-mediated matrix breakdown) (Mentlein R. et al. 2003).

1.4.1 IP-10 as an antiangiogenic factor

There are at least four families of chemokines, but only two have been extensively characterized. CXC chemokines attract neutrophils and lymphocytes, whereas chemokines belonging to the CC family act primarily on monocytes, but they can also attract lymphocytes, basophils, eosinophils, dendritic and NK cells. The CXC chemokine family of cytokines appear to have proinflammatory and reparative activities. These cytokines are basic heparin-binding proteins less than 10 kDa, and have four highly conserved cysteine amino acid residues with the first two cysteines separated by one non-conserved amino acid residue. These chemokines are all clustered on human chromosome 4 and exhibit between 20% to 50% homology on the amino acid level. To CXC family belong: IL-8, GRO-α, β, γ (growth-related oncogene, PF4, PBP (platelet basic protein), βTG (beta-thromboglobulin), NAP-2 (neutrophil-activating peptide) and epithelial-derived NAP (ENA)-78, IP-10 (interferon-inducible protein 10), Mig (monokine induced by gamma-interferon) and ITAC (interferon inducible T cell α chemoattractant).

Human interferon-inducible protein 10 (IP-10), a member of the α chemokine family (CXC chemokines), inhibits bone marrow colony formation, has antitumor activity *in vivo*, is chemoattractant for human T cells, and promotes T cell adhesion to

endothelial cells. IP-10 inhibits neovascularization *in vivo*. Addition of IP-10 to Matrixgel impregnated with bFGF resulted in marked reduction in the number of endothelial cells invading the plug and the absence of blood vessels. In the presence of IP-10, the endothelial cells formed small aggregate structures, but they were not differentiating into tubelike structures (Angiolillo A. et al. 1995). IP-10 can effectively inhibit IL-8 or bFGF-induced angiogenesis (by inhibiting endothelial cells proliferation) (Strieter R.M et al. 1995).

In all members of the CXC chemokine family that activate neutrophils a sequence Glu-Leu-Arg (the ELR motif) is highly conserved. It plays a role in ligand/receptor on neutrophiles. The members of CXC family that lack ERL motif (PF4, IP-10, Mig), in contrast to members that contain these three amino acids (IL-8) are angiogenesis inhibitors (Strieter R.M. et al. 1995b).

CXCR3 (CD183) receptor binds IP-10, Mig and interferon-inducibleT cell α chemoattractant (I-TAC) with high affinity. It is highly expressed in IL-2 activated T lymphocytes, but not detectable in resting T lymphocytes, as well as on B lymphocytes, monocytes, granulocytes, NK cells and vascular pericytes. It mediates Ca^{2+} mobilization (transient rise of the cytosolic free Ca^{2+} concentration) and chemotaxis in response to IP-10 and Mig (Loetscher M. et al. 1996).

CXCR3 ligands activate Ras/ERK, Src, and the PI3K/Akt pathway, thereby regulating critical cellular functions such as cell proliferation and migration. CXCR3 activates all the components of the ERK cascade, including Ras, Raf-1, and MEK. Activation of both ERK and PI3K contributes to the cell chemotaxis and proliferation mediated by CXCR3 (Bonacchi A. et al. 2001).

Endothelial cell receptors for IP-10 and Mig have not yet been identified and the mechanisms responsible for the effect of these chemokines on angiogenesis are still unclear. CXCR3 is expressed by a small percenrage of microvascular endothelial cells in several human normal (thymus, liver, kidney, thyroid, gut) and pathological tissues. IP-10 can inhibit DNA synthesis and proliferation of endothelial cells. Anti-CXCR3 monoclonal antibody effectively inhibited antiproliferative effect of IP-10 (Romagnani P. et al. 2001).

Examination of CXCR3 and its ligands also demonstrated an important role for these molecules in acute encephalitis. IP-10 and its receptor are expressed by the CNS and by CNS infiltrating lymphocytes, only in patients with ongoing CNS inflammation, suggesting an important role for these molecules in the pathogenic process. IP-10 is

essential for the recruitment of T-lymphocytes into the CNS during development of encephalitis. However, IP-10 expression alone is not sufficient to induce pathology (Klein R. 2004).

1.4.2 Cytokines and antitumor immunity

The cytokine milieu plays an important role in tumor progression. It can be manipulated in order to upregulate antitumor immune responses. Despite recent progress in the understanding of the cytokine network there is still no complete picture of how cytokines work under different *in vivo* conditions and in combinations with other synergistic/antagonistic cytokines. However, a variety of potential cytokine-related strategies will have wide applications in the future.

It is well known that cell-mediated immunity is suppressed in patients with neoplastic diseases. One critical determinant of host immunity is the cytokine/chemokine milieu in the tumor microenvironment. A critical component thereof is likely to be the cytokine interleukin (IL) 10, which has been shown to hinder a number of immune functions, i.e. T lymphocyte proliferation, Th1 type cytokine production, antigen presentation, and lymphokine-activated killer cell cytotoxicity. High plasma level of IL-10 correlates with bad prognosis for patients with melanomas, haematological malignancies, nasopharyngeal carcinoma and other solid tumors (Fortis C. et al. 1996). TGFβ is also well established as a multifunctional immunosuppressive cytokine. Produced by tumors, significantly reduces the potency of DC/tumor fusion vaccines. It stimulates on autocrine and paracrine way the production of VEGF at the transcriptional level, contributing to the malignant phenotype. Circulating IL-6 is associated with worse survival in patients with metastatic breast cancer and is correlated with the extent of the disease (Müller L. et al. 2003).

Since the cytokines play a critical role in regulating antitumor immune response, modulation of the cytokine network should be therapeutically exploitable. Tumor cells express tumor-associated antigens, which can be recognized by the immune system, but mostly of them, represent self-proteins and as a result of tolerance are poorly immunogenic. Immunomodulation with cytokines may break tolerance and allow tumor-reactive T cells to eradicate tumor cells. Many cytokines, including IL-12 and IL-2 have demonstrated their immunomodulatory activities. IL-12 is known to be a crucial cytokine for inducing a T cell-mediated immune response. As reported for breast cancer,

melanoma, renal cancer and neuroblastoma, a combination of IL-2 and IL-12 *in vitro* may strongly enhance the development of tumor specific CTLs. Synergistic effects of IL-4 with IL-12 on IFNγ production by DCs have also been shown (Fukao T. et al. 2000). Dendritic cells are the most potent of the antigen presenting cells, capable of acitivating both $CD4^+$ and $CD8^+$ cells. DCs can be generated from peripheral blood by culture in GM-CSF and IL-4, then maturation by TNFα, and employed as vaccines well able to present a range of naturally processed epitopes as well as synthetic peptides. Cytokine-induced DCs can be effectively used for the peptide presentation *in vivo* (Allavena P. et al. 2000).

In humans, immunotherapy with cytokines is an established but still largerly poor-effective method for treatment of cancer. IL-2 and IFNα are the main immunobiological agents used in the therapy of melanoma, renal cell carcinoma and haematological malignancies (Müller L. et al. 2003). GM-CSF and IL-4 may provide a mechanism for increasing the number and function of antigen presenting cells in patients with cancer. Combination cytokine immunotherapy and chemotherapy may offer some advantages over either alone. The immunotherapy with cytokine infusions requires large amounts of material to be injected over extended periods of time. It is necessary due to the very short plasma half-life of most cytokines. Systemic administration may cause serious side effects. To avoid this, it has been attempted to complex cytokine with antibodies or to use implantation of genetically engineered cytokine-secreting cells. There may be significant advantages associated with a gene therapy approaches using cytokines. Cytokine gene transfer to tumor cells has been demonstrated to induce tumor rejection in different murine models suggesting that vaccination with tumor cells producing cytokines is an attractive strategy to enhance antitumor responses also in patients (Müller L. et al. 2003). In multiple murine models, GM-CSF proved to be the most potent immunostimulatory product. It enhances recruitment of DCs and macrophages, stimulates T cells and antibodies mediated immunity. Transfection of murine cells with IL-12 significantly reduced their tumorogenicity and metastatic potential and generated protective CTL response. Intraperitoneal vaccination with irradiated IL-1β - secreting melanoma cells results in protection against subsequent subcutaneous challenge. Use of IL-15 – secreting cells resulted in no metastatic tumor growth (Meazza R. et al. 2000).

The mechanism by which anti-tumor activity is produced varies with the transduced cytokine and the haematopoietic and immune effector cells recruited. These

mechanisms include generation of CTLs, which specifically recognize tumor cells, enhancement of antigen presentation, and recruitment of non-specific cytotoxic cells such as eosinophils and neutrophils. It was demonstrated that tumor cells transfected to mediate overexpression of a cytokine gene activate immunologic effector cells for an improved proliferation rate and significantly higher antitumoral cytotoxic activity. The strategies strategies include gene delivery into tumor cells and into cellular components of the immune system, including cytotoxic T cells, NK, macrophages and dendritic cells. Transducing cytokine genes into tumor cells enhances haematopoietic and immune system defence against tumor.

1.4.3 TNFα (tumor necrosis factor α)

The cytokines are proteins regulating proliferation and differentiation of the cells by binding to the specific receptors on the cell surface. TNFα (tumor necrosis factor α) is a pleiotropic cytokine produced mainly by activated monocytes and macrophages. However, at certain conditions, almost all the cells, with the exception of erythrocytes, are able to synthesise TNFα (Nagata S.1997).

TNFα mediates both physiological and pathological effects. It plays a role in normal physiological processes like: embryogenesis, haematopoiesis, inflammation, and protection against infections. TNFα influences the hormonal regulation – for example insulin activity, it plays a role in cachexia, anorexia and obesity. Pathological effects mediated by TNFα appear upon stress, which generally increases TNFα production. Well known is TNFα contribution to the induction of sepsis, arthritis, pancreas inflammation and ischemia (Reimold A. M. 2003).

TNFα is both a stimulator and an inhibitor of cell growth. It acts on autocrine and paracrine manner on the growth of different tumors and tumor cell lines (leukaemia, neuroblastoma, and ovarian cancer) as well as normal cells (B lymphocytes and macrophages differentiation, growth of the fibroblasts, astrocytes and thymocytes) (Aggarwal B.B et al. 1996).

TNFα gene is located in the MHC region of the human chromosome 6 and murine chromosome 17. Its expression is regulated on the transcriptional, translational and posttranslational level. Mature human protein contains 157 amino acids (mouse, rat and rabbit 156 amino acids). In the active form TNFα is a trimeric protein of molecular weight 45 kDa. Trimer formation is necessary for the effective receptor binding and

signal transduction. TNFα shows biological activity as a transmembrane protein as well as a soluble one. Production of the soluble form is mediated by membrane metalloproteinase (Grell M. 1995). TNFα production can be induced by gram-positive and gram-negative bacteria, yeast, mycoplasma, viruses, tumor cells, cytokines and mitogens. Many drugs, UV, fever and stress induce TNFα production as well. TNFα gene expression can be downregulated by such stimuli as steroids, prostaglandin inhibitors, immunosuppressive drugs, some viruses and cytokines. Some cellular signalling pathways playing a role in the stimulation of TNFα production may participate in its inhibition.

TNFα is cytotoxic for many transformed cell lines and may cause hemorrhagic necrosis of the tumors *in vivo* (Lans T.E. et al. 2004). Depending on the target cell and metabolic inhibitors TNFα can induce apoptotic or necrotic call death. Due to the potential use in the cancer therapy, antitumor properties (tumor growth suppression) of TNFα are being intensively studied now. TNFα-mediated antitumor effect is often depending on the dose, however, high doses of this cytokine result with systemic toxicity. For many cell lines TNFα is only cytostatic, but not cytolytic and many lines are totally resistant to its cytotoxic activity. Some tumors undergo regression upon combined treatment (TNFα applied with IFNγ or with cytostatic drugs). Up to now there is no known mutation in TNFα gene that would result on oncogenic transformation.

Cellular receptors belonging to the TNF receptor family are key mediators of the immune response both in the normal or pathological conditions. Trimeric TNFα is binding to the specific receptor and inducing its trimerization. TNFα receptors (TNF-R) are present on all the cell types and their number varies from 100 to 10000 per cell. Two TNF-Rs were identified: TNF-R55 (TNF-R1/CD120a/p55/p60) and TNF-R75 (TNF-R2/CD120b/p75/p80). The present model of the events leading to the apoptosis is described below:

- ligand binding to the transmembrane receptor, aggregation and internalization of the receptor
- interaction of cytoplasmatic receptor domain with adaptor proteins
- signal transduction to the effector proteins (Zhang S.Q. et al. 2000).

TNFα is widely studied in different antitumor approaches: as direct antitumor agent or as immunostimulatory molecule (enhancer of DCs maturation or TIL (tumor infiltrating lymphocytes) – mediated cytotoxicity) (Zhang W. et al. 2002; Itoh Y. et al. 1995).

In this work we cloned IP-10 or TNFα in the parvoviral vectors. This allows taking advantage of the properties of parvoviruses (NS1 cytotoxicity) as well as of the intrinsic transgene properties (antiangiogenesis and immunestimulation for IP-10 and immunestimulation, antiangiogenesis and toxicity for TNFα).

1.5 Aims of work

Malignant gliomas are refractory to most standard therapies, including resection, radiation therapy and chemotherapy. A variety of different gene therapy approaches was done within the last decades. For glioma treatment there are many viral vectors being currently under test. Gliomas are described as particularly well vascularized and immunosuppressing tumors. Thus, we constructed new recombinant MVMp- and H1 – based vectors expressing human IP-10 or mouse TNFα in order to test their antiangiogenic and immunostimulatory properties in two animal subcutaneous glioblastoma models.

In vitro experiments have been planned to evaluate the efficacy of parvovirus-transduced transgene production in different human (A172, U87, U373 and U138) and mouse (MT539 and Gl261) glioblastoma cell lines as well as the sensitivity of different glioma cell lines to parvoviral infection.

In vivo studies consist of establishing subcutaneous mouse models allowing evaluation of antitumoral effects of parvoviruses on glioma-derived tumors and testing the antitumoral effects mediated by recombinant and wild type parvoviruses (MVMp and H1) in the animal models. Human U87 and murine Gl261 cell lines were chosen for *in vivo* investigations.

Our study is based on the hypothesis that antitumoral effects of recombinant parvoviruses could be based on their intrinsic cytotoxic properties combined with antiangiogenic/immunestimulatory properties of IP-10 and immunestimulating, antiangiogenic and cytotoxic properties of TNFα.

To evaluate the molecular mechanisms of antitumoral effects *in vivo* we decided to perform histological analyses and MRI measurement showing changes in treated tumors. Investigation of morphological changes upon therapy with recombinant parvoviruses has been planned to get knowledge about therapy consequences.

2 Materials and Methods

2.1 Microbiological Methods

2.1.1 Culture and Cryopreservation of *Escherichia coli*

Bacteria were growing over night at 37°C on agar plates or as suspension culture (shaked 200 rpm). At these conditions after 14-16 h the bacteria were in lag growth phase. In order to amplify the plasmid DNA a chloramphecnicol was added (to final concentration 34 µg/l) and the bacteria were cultured for further 6 h.

For the cryopreservation to 1 ml of bacteria culture 0,5 ml of 50% sterile glycerol was added and obtained glycerol stock was stored at -80°.

Bacterial strains	*E. coli* SURE (Stratagen, Germany)
	E. coli XL-1Blue (Stratagen, Germany) – pBK-CMV_VP
	(H1 helper plasmid)
	E. coli JM109 (Invitrogen, Germany) – pcDNA1-1_VP
	(MVMp helper plasmid)
LB medium	5g NaCl
	5g yeast extract
	5g bacto-trypton
	ad 1000 ml H_2O, pH 7,0, autoclaved
LB agar	LB medium with 1,5% bacto agar
Antibiotics	100 µg/ml ampicilin
	12,5 µg/ml tetracycline
	25 µg/ml kanamycin

2.1.2 Culturing of Electro Competent Bacteria

An overnight E.coli (SURE strain, Stratagene) culture was diluted 1:100 in 1 l LB medium and cultured at 200 rpm, 37°C to OD_{600} 0,6-0,8. Afterwards the culture was cooled down on ice and centrifuged in pre-cooled rotor 10 min. at 6000 rpm. Subsequently the cells were washed with 500 ml ice-cold water and centrifuged again (10 min. at 6000 rpm).

Then the pellet was resuspended in 2-3 ml of LB medium with 10% w/v glycerol and 200 µl aliquots were frozen at -80°.

2.1.3 Transformation of E.coli with Plasmid DNA – Electroporation

A stock of electro competent bacteria was thawn on ice. 40 µl of bacteria suspension were mixed with 1-2 µl (10-100 ng) DNA solution and transferred in an ice-cold electroporation cuvette. The sample was than placed in an E.coli pulser (Biorad) and electroporated at 200Ω, 1,8 kV and 25 MFd. Subsequently, 1 ml of antibiotic-free LB medium was added to the sample and the bacteria were incubated for 40 min. at 37°C, 200 rpm. Then the bacteria were centrifuged for 1 min. at 8000 rpm., resuspended in 100 µl LB medium, transferred on LB agar plates containing selective antibiotics and cultured over night at 37°C.

2.2 Molecular Biology Methods

2.2.1 DNA Purification, Analysis and Modification

2.2.1.1 Plasmids

Plasmid name	Description	Restriction enzymes	Fragments (bp)
Chi-H1/Δ800	H1/MVMp chimeric vector without transgene	XbaI AflIII	1474, 4945 120, 225, 984, 1410, 1732, 1948
Chi-H1/EGFP	Expression vector, EGFP in basic vector CIIIΔ800	NcoI AflIII	567, 964, 1071, 4583 120, 225, 984, 1732, 1913, 2200
Chi-H1/IP-10	Expression vector, human IP-10 in basic vector CIIIΔ800	BglII PstI	610, 1932, 4234 1714, 5105
Chi-H1/TNF	Expression vector, murine TNF in basic vector CIIIΔ800	SpeI ScaI	760, 2280, 3380 669, 2266, 4184
Chi-MVMp/Δ800	MVMp/H1 chimeric vector without transgene	EcoRI/BamHI BglII	1836, 4784 584, 6036
Chi-MVMp/EGFP	Expression vector, EGFP in basic vector C4Δ800	AflIII NcoI	225, 1491, 2716, 2924 967, 1638, 4751
Chi-MVMp/IP-10	Expression vector, human IP-10 in basic vector C4Δ800	BglII HpaI	548, 6036 1555, 5429
Chi-MVMp/TNF	Expression vector, murine TNF in basic vector C4Δ800	SpeI ScaI	2280, 4304 669, 2101, 4514
pBK CMV VP(H1)	Helper plasmid for H1 recombinants	NcoI AflIII	289, 703, 1373, 1904, 2718 1031, 2924, 3032
pCMV VP (MVMp)	Helper plasmid for MVMp recombinants	SpeI HpaI	1000, 1700, 5000 1815, 5885

2.2.1.2 Mini-, Maxi- and Mega-Isolation and Purification of Plasmid DNA

Plasmid DNA isolation and purification from 5, 250 or 1000 ml bacteria culture was performed with Qiagen Mini, Maxi or Mega Kit (Qiagen), respectively, according to the instructions of the manufacturer.

2.2.1.3 DNA Quantification Using UV Spectrophotometer

The DNA concentration is obtained by multiplying the absorbance at 260nm by a constant. The DNA purity is measured by dividing the absorbance at 260nm by the absorbance at 280nm.

DNA at a concentration of 50 ug/ml has an Absorbance260 = 1. Solving this equation for unknown concentration, the concentration (ug/ml) = A260 x 50. To conserve DNA and to get accurate readings, the original sample was diluted, e.g. 1:20 in 1X TE. Hence, the final concentration equation is:

DNA concentration (ug/ml) = A260 x Dilution Factor x 50

A260:A280 ratio: phenol absorbs light at 270 nm. Its spectrum has a slight shoulder that appears at approximately 272 nm. Proteins, which possess tyrosine residues, absorb light at 280 nm. If the sample is contaminated with any of these molecules, the absorbance at 280 nm will increase. The calculated ratio of A260 over A280 for pure DNA should fall between 1.75 - 2.10.

2.2.1.4 Restriction Digestion of Plasmid DNA

DNA can be specificly fragmented by restriction enzymes. This process is used for quality control of plasmid DNA or for further isolation of the fragment of interest.
A general rule of is to use 0,5 µg DNA/20 µl in the final digest reaction mix.

Reaction mix	2,0 µl	10x enzyme buffer
	1,0 µl	1 unit restriction enzyme
	x µl	0,5 µg DNA
	ad 20 µl with sterile dH_2O	

Digestion process was taking place for 1h at 37°C (heating block or water bath), and then stopped by transferring the sample to -20°C. Afterwards the digest has been visualized on an ethidium bromide-stained agarose gel.

2.2.1.5 PCR (Polymerase Chain Reaction)

The polymerase chain reaction (PCR) is a method for oligonucleotide primer directed enzymatic amplification of a specific DNA sequence of interest. This technique is capable of amplifying a sequence 105 to 106-fold from nanogram amounts of template DNA within a large background of irrelevant sequences (e.g. from total genomic DNA).

The PCR product was amplified from the cDNA templates (see also 2.2.3) using a heat-stable DNA polymerase from Thermus aquaticus (Taq DNA polymerase, Invitrogen) and using an automated thermal cycler to put the reaction through 35 cycles of denaturing, annealing of primers, and polymerization. After amplification by PCR, the products were separated by agarose gel electrophoresis and were directly visualized after staining with ethidium bromide.

Reaction mix	5,0 µl	10x PCR enzyme buffer (Invitrogen)	
		0,2 µl Taq DNA polymerase (Invitrogen)	
		4,0 µl specific primer mix	pro 10 µl DNA sample
		4,0 µl 2,5 mM dNTP mix (Sigma)	
		3,0 µl 2,5M $MgCl_2$ (Invitrogen)	
		26 µl H_2O	

Reaction conditions: 4 min. 95°C, 30 sec. 94°C, 30 sec anneling temp., 1 min. 72°C, 7 min. 72°C, hold 4°C

PCR product was stored at -20°C.

Primer	Sequence 5'-3' sens/antisens	Anneling temp. (°C)	PCR product length (bp)
β actin murine	CACGTCACACTTCATGATGG ATGTTTGAGACCTTCAACAC	58	489
NS1	CTGAATGGAAAAGATATCGGATGGAATAG GCCTCCGTCTCTTGGTGG	58	569
IP-10 human	TCTAGAACCGTACGCTGTACCTGC CTGGTTTTAAGGAGATCTTTT	58	230
TNFα murine	ATGAGCACAGAAAGCAGTATCCGC CCAAAGTAGACCTGCCCGGACTC	58	700
Perforin	GGTGGAGTGGAGGTTTTTGTACC CAGAATGCAAGCAGAAGCACAAG	58	486
CD64	TGCAAAGGAAGTCTAGGAAGG GCAGAAGAGTCTTGAGTTGGG	60	510
iNOS	CATGGCTTGCCCCTGGAAGTTTCTCTTCAAAG GCAGCATCCCCTCTGATGGTGCCATCG	60	828
Granzyme A	CCTGAAGGAGGCTGTGAAAGAATC CCCTGCACAAATCATGTTTAGTCC	60	526
Granzyme B	ACTCAAACACGCTCAAAGA ATCCAGGATAAGAAACTC	58	253
IFNγ	CAAGGCTGTGAGAAGGAAACC CCCATGATAAAGAATAGTAGA	60	237
NK1.1	CTACCTCGGTTTAAAGCCACC GAAGCACAGCTCTCAGGATCAC	60	576

2.2.2 RNA Isolation and Analysis

2.2.2.1 Total RNA Isolation from Tissue

For the isolation of total RNA a tissue fragment of interest was transferred into the matrix-tube (Lysing Matrix D®, Qbiogene) and treated with Trizol® (Invitrogene). To resuspend the tissue, the sample was placed in tissue disrupter (Qbiogene) for 3 x 20 sec. cycles at speed 5. Subsequently, per 1 ml Trizol, 0,2 ml chlorophorm (Sigma) was added, mixed and the sample was incubated at RT for 2-3 min. Afterwards the sample was centrifuged for 15 min. at 13000 rpm at 4°C. The upper phase, containing RNA, was collected in another tube and the same amount of isopropanol was added in order to precipitate RNA. After 10 min. incubation the sample was centrifuged 10 min. at 11000 and the supernatant was discarded. RNA pellet was washed with 70% ethanol, centrifuged 10 min. at 1100 rpm and air-dried for 10 min.

The pellet was then diluted in 200 µl 1mM EDTA and stored frozen at -80°C.

2.2.2.2 RNA Quantitation Using UV Spectrophotometer and Agarose Gel Analysis

After isolation a small aliquot of RNA sample (10 µl) was diluted 1:20 with 1mM EDTA buffer, transferred into a UV cuvette (Eurogenetec) and spectrophotometer measurement was done. RNA at a concentration of 40 ug/ml has an Absorbance260 = 1 (see also 1.4.3). For the electrophoresis 10 µl RNA (or 5µl+5µl EDTA) +2 µl loading buffer was runned shortly on 1,5% agarose gel. For not degraded RNA sample two bands – 18S and 28S RNA could be observed.

2.2.2.3 RT-PCR (Reverse Transcription- Polymerase Chain Reaction)

RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantification currently available. It allows detecting the expression of a gene of interest. In this method, RNA will be first transcribed into complementary cDNA and then DNA will be multiplied.

As a first step 1 µg RNA was treated with 1U DNaseI (Invitrogen) in provided buffer (Invitrogen) for 10 min. at RT in order to eliminate contaminating DNA molecules. Total sample volume was 10 µl (adjusted with sterile dH_2O). The reaction was stopped by addition of 1,5 µl 25mM EDTA per sample. Then the probe was incubated 5 min. at 70°C and placed on ice to deactivate DnaseI. During the step of reverse transcription RNA was transcribed into cDNA.

Reaction mix	5,0 µl	10x RT enzyme buffer (Promega)	
		1,0 µl 1 unit RT enzyme (Promega)	
		1,0 µl oligo dT primer (Invitrogen)	pro 10 µl RNA sample
		0,5 µl 0,5U Rnasin (Promega)	
		2,5 µl 2,5 mM dNTP mix (Sigma)	

Reaction conditions: 60 min. at 37°C, 5 min. at 90°C, kept at 4°C.

Ready cDNA sample was diluted 1: 10 with H_2O and stored at -20°C.

2.3 Biochemical Methods

2.3.1 Protein Isolation from Mammalian Cells

For the detection of viral NS1 and VP proteins a protein extract from $1x10^6$ cells cultured in 10 cm dish was prepared. The medium was removed; cells were washed with PBS and subsequently lysed with RIPA buffer. The lysate was collected,

transferred into a 1,5 ml eppendorf tube and placed on ice for 30 min. After that the sample was centrifuged for 10 min. at 14000 rpm in a table centrifuge. The supernatant was transferred into a fresh tube and stored at -20°C.

RIPA buffer 10 mM Tris pH 7,5
150 mM NaCl
1 mM EDTA pH 8,0
1% NP-40
0,5% Na-Deoxycholate
0,1% SDS

2.3.2 Protein Determination by the Bradford Assay

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change

10 ul of protein extract was diluted with 990 µl of Bradford assay reagent (BioRad).

The absorbance was read at 595 nm and readings were compared to a standard curve produced using protein concentrations ranging from 2 to 14 µg/µl.

2.3.3 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length - i.e.: the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2-mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. Determination of molecular weight is done by running a known molecular weight marker (Rainbow™marker, Amersham) along with the protein.

A desired amount of protein extract (containing 20 µg, 40 µg of total proteins) was mixed 1:1 with loading buffer, denaturated 5 min. at 95°C and placed on ice. After loading on a gel, electrophoresis was performed over night at 70V, 100mA.

Stacking gel	(5% acrylamide)
	1,9 ml Tris 0,5M pH 6,8
	2,25 ml acrylamide 30% (BioRad)
	10,7 ml H_2O
	150 µl 10% SDS
	150 µl 10% APS (BioRad)
	15 µl TEMED (BioRad)
Resolving gel	(10% acrylamide)
	12,5 ml Tris 1,5M pH 8,8
	16,7 ml acrylamide 30% (BioRad)
	19, ml H_2O
	500 µl 10% SDS
	500 µl 10% APS (BioRad)
	20 µl TEMED (BioRad)
Electrophoresis	192 mM glycine
buffer	0,1% (w/v) SDS
	25 mM Tris-HCl pH 8,3
	made by diluting a 10x stock solution
Sample buffer	125 mM Tris-HCl pH 6,8
	10% 2-mercaptoethanol
	10% SDS
	10% glycerol
	0,1% bromophenol blue

2.3.4 Western Blot Analysis

Western blot analysis contains of following working steps. The proteins were separated of using SDS-polyacrylamide gel electrophoresis. A nitrocellulose membrane was placed on the gel and, and using electrophoresis (2h at 0,4mA), the negatively charged protein bands were driven onto the nitrocellulose membrane. This gave a nitrocellulose membrane that is imprinted with the same protein bands as the gel. The membrane was blocked with blocking buffer at RT for 1h. The nitrocellulose membrane was incubated

with a primary antibody over night at 4°C. The membrane was washed 3x 10 min. with washing buffer. The membrane was incubated 1 h at RT with a secondary antibody (an antibody-enzyme conjugate). The washing 3x 10 min. with washing buffer was performed. ECL (enhanced chemiluminescence) reaction was done with ECL-kit (Amersham). The membrane was exposed of to an x-ray film (Kodak).

Blocking solution	5% skim milk powder	
	0,5% Tween in PBS	
Washing solution	0,5% Tween in PBS	
Transfer buffer	50 mM Tris	
	380 mM glycin	
	0,1% SDS	
	20% methanol	
Primary antibodies		
	Anti NS1 : SP8 (polyclonal, rabbit)	Faisst et al., 1995
	Anti VP : αH1 peptide (polyclonal, rabbit)	Kestler et a. 1999
Secondary antibodies		
	goat anti rabbit (HRP conjugated)	BioRad

2.4 Cell Culture Methods

2.4.1 Maintenance of Cell Lines

Mammalian cells (human and murine) were maintained as monolayer cultures – all used cell lines are adherent. The cells were growing on 10 cm cell culture plates (Greiner or Nunclon) at 37°C, 5% CO_2 and 90% humidity. Dependently on the growth rate the cells were splited 1 or 2 times per week. The medium was removed; cells were washed with PBS and treated with trypsin (Invitrogen). When a single cell suspension has been obtained, 5 times volume of complete media as trypsin solution was added. 5-10% of cell suspension was transferred into a new dish and supplemented with appropriate medium to 10 ml total volume. Before use, trypsin and media were warmed up in 37°C water bath. The media were supplemented with fetal bovine serum (Gibco) and 100 µg/ml penicilin/streptomycin (Invitrogen).

Cell line	Description	Medium
A9	murine fibroblastic cells (Littlefield J.W. 1996)	MEM 5% FBS
NB324K	human fetal kidney cells, transformed with SV40 (Corsini J. 2004)	MEM 5% FBS
A172 MG	human gliblastoma (Balzarotti M. 2004)	DMEM 10% FBS
U87 MG	human gliblastoma (Goldbrunner R.H. 2000)	DMEM 10% FBS
U373 MG	human gliblastoma (Palma C. et al. 2000)	MEM 10% FBS
U138 MG	human gliblastoma (Jiang Z. 2004)	RPMI 10% FBS
Gl 261	murine gliblastoma (Seligman A.M., Shear M.J. 1939)	DMEM 10% FBS
MT539	murine glioblastoma (Hughes B.W. 1998)	DMEM (low glucose) 10% FBS
293T	human kidney cells, transformed with SV 40 and Ad5 (Teramoto H. 1996)	DMEM 10% FBS

2.4.2 Freezing and Thawing Mammalian Cells

For freezing, the cells from the confluent culture were collected using trypsin, centrifuged and resuspended in FBS containing 10% w/v DMSO (Sigma). The cell suspension was transferred into cryovials (Nunclon) and frozen in a cell freezing box (Nalgene) at -80°C.

For thawing, a cryovial was kept at 37°C until the content was melt. The cell suspension was transferred into a 15 ml falcon tube containing 10 ml proper medium. Cells were centrifuged at 1500 rpm for 10 min. The supernatant was removed, cells resuspended in 10 ml fresh medium and placed in a cell culture dish in the incubator.

2.4.3 Methods of Assessing Cell Proliferation and Viability

2.4.3.1 Trypan blue staining

Trypan blue is the most common stain used to distinguish viable cells from nonviable cells; only non-viable cells absorb the dye and appear blue and may also appear asymmetrical. Conversely, live, healthy cells appear round and refractive without absorbing the blue-coloured dye. The use of this stain, however, is time- sensitive. Viable cells absorb Trypan blue over time, and can affect counting and viability results. A small sample of the cell suspension was diluted in 0.4% (w/v) Trypan blue. The number of cells per millilitre and total number of cells was determined using the following calculations:

cells/ml = # of cells counted in a square x 10^4 x dilution factor
total cells = cells/ml x vol. of original cell suspension

2.4.3.2 AlamarBlue™ Reduction

AlamarBlue™ is a safe, non-toxic aqueous dye that is used to assess cell viability and cell proliferation. The internal environment of the proliferating cell is more reduced than that of non-proliferating cells. Specifically, the ratios of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD, increase during proliferation. Compounds such as tetrazolium salts and alamarBlue™, which can be reduced by these metabolic intermediates, are useful in monitoring cell proliferation because their reduction is accompanied by a measurable shift in color. As alamarBlueTM accepts electrons from these compounds, it changes from the oxidized indigo blue, non-fluorescing state to the reduced fluorescent pink state. The cytotoxicity tests with alamarBlue™ were done on 96-well plates (Nunclon). For the measurement, 10 µl of alamarBlue™ were added per 1 well (to 100 µl of total volume), incubated at 37°C in 5% CO_2. The absorbance was measured after 3-5 h (depending on the cell line) at double wavelength 540 and 620 nm.

2.4.3.3 Neutral Red Staining

Neutral Red stains viable cells. This dye is absorbed by viable cells and is concentrated in the lysosomes. Quantitation of Neutral Red staining therefore has utility in monitoring cytotoxicity assays . Determination of percentage Neutral Red staining requires only a light microscope. In this work neutral red staining has been used for plaque assay. 3% NR (Sigma) solution was mixed with 1,7% or 2% agar and staining buffer and applied on the agar surface. Staining was performed for 8 to 10h.

2.4.3.4 Crystal Violet Inclusion

Crystal violet inclusion detects cell lysis. This dye stains viable cells that adhere to their culture vessel. Lysed cells simply fall away from their vessel surface and are not stained by this dye. In this work crystal violet inclusion was used for clonogenicity assay. After removing the medium, the cells were washed with ice-cold acetic acid-ethanol mixture (1:3) and fixed for 20 min. at 4°C. Then the cells were incubated with ethanol for 20 min.

at RT and following washed with water. Incubation with crystal violet solution took place for 30 min. at RT. Afterwards the cells were washed with water and visible, violet-coloured cell clones could be counted (see also 4.4.3.5).

2.4.3.5 Clonogenicity Assay

The cells were seeded 2,5 x 106 cells/6 cm plate in 4 ml medium. One day later the infection was performed as usually, then the cells were incubated at least 4 hours. After this time, all the cells were harversted, diluted to the desired concentration and desired amount of cells (10000, 4000, 2000, 1000, 500) was seeded on the cell culture dish and supplemented with proper medium. Incubation took 5 – 14 days (depending on the efficiency and speed of colony forming). Afterwards the crystal violet staining was performed.

2.5 Cell Biology Methods

2.5.1 FACS (Fluorescence Activated Cell Sorting)

FACS is a powerful method used to study and purify cells. It has a wide application in immunology and cell biology and other fields of biology.

Individual cells held in a thin stream of fluid are passed through one or more laser beams cause light to scatter and fluorescent dyes to emit light at various frequencies. Photomultiplier tubes (PMT) convert light to electrical signals and cell data is collected. Cell sub-populations are identified and sorted at high purity (~100%). FACS instruments generate three types of data: 1) Forward scatter (FSc) - approximate cell size 2) Side or Orthogonal scatter (SSc) - cell complexity or granularity 3) Fluorescence - fluorescent labeling is used to investigate cell structure and function. In this work, FACS was used to detect gene expression in cells infected with recombinant viruses containing a reporter gene of EGFP (Enhanced Green Fluorescing Protein). At different time points after infection the cells were collected, washed once with PBS, passaged through a nylon mesh and fixed with 1% formaline solution in PBS. For one sample, $1x10^6$ cells were investigated (Flow Cytometry FACSort, Becton Dickinson and Co.). A Cell QuestTM computer program has been used for data collection and anlysis (Becton Dickinson and Co.).

2.5.2 Cytotoxicity Assay

The cells were applied on a 96-well plate (Nunclon); $2x10^4$ cells in 100 µl medium per well. 24 h later serial dilutions of TNFα (Sigma) (0,1-10^4 U/ml) were added to the cells and incubated for 24 or 48 h. Cells not treated with TNFα were positive survival control. 3h before the end of the test AlamarBlue™ was added to the cells and after this incubation time the results were read with an ELISA reader (see also 4.3.2).

2.5.2.1 Cytotoxicity Assay in the Presence of Cycloheximide

The cells were applied on a 96-well plate (Nunclon); $2x10^4$ cells in 100 µl medium per well. 24 h later serial dilutions of TNFα (Sigma) (0,1-10^4 U/ml) and cycloheximide (Sigma) at concentration 2µg/ml or 20µg/ml were added to the cells and incubated for 20h. Cells not treated with TNFα, but treated with cycloheximide (2 or 20µg/ml) were positive survival control. 3h before the end of the test AlamarBlue™ was added to the cells and after this incubation time the results were read with an ELISA reader (see also 4.3.2).

2.5.3 In Vitro Generation of Mouse Dendritic Cells from Bone Marrow

Bone marrow was isolated from the femurs and tibias of hind legs 7-12 weeks old C57Bl/6 female mice. The erythrocytes were removed by incubation of the cell pellet with 0,84% solution of ammonium chloride for 5 min. at RT. After washing with RPMI+10% FBS medium, cells were incubated with anti-$CD34^+$ antibodies (MEC14.7) for 1h at 4°C. Subsequently, the cells were mixed with magnetic MiniMACS microbeads anti-rat IgG (Miltenyi Biotec) according to the manual and incubated for 30 min. at 4°C. Afterwards, $CD34^+$ cells were separated with assistance of MS columns (Miltenyi Biotec) according to the manual. Purified cells were transferred on 6-well plate (~$3x10^5$ cells in 3 ml/well). DC were cultured in complete RPMI medium (5% FBS, 40 ng/ml GM-CSF, FLT-3L 100 ng/ml) at 37°C in 5% CO_2 atmosphere.

2.5.4 Measurement of FITC-Dextran Uptake

Efficient accumulation of FITC-dextran is a specific property of cultured DC, which is not shared by other cell types such as macrophages, monocytes, fibroblasts and lymphocytes. FITC-dextran appears to be taken up by the mannose receptor. The ability to uptake dextran decreases with the maturation of DC.

Cells were resuspended in RPMI+5% FCS. FITC dextran (MW 40000, Sigma) was added at a final concentration of 1 mg/ml. Cells were then incubated either at 0°C and 37°C, washed two times with cold PBS, fixed in PBS +1% formaline and analysed on a flow cytometer (see also 5.1).

The experiments with dendritic cells were performed in collaboration with Dr. A. Vecchi and Dr. S. Sozzani at the Pharmacological Institute Mario Negri Milano, Italy.

2.6 Virological Methods

2.6.1 Production of Recombinant Viruses

Recombinant viruses were produced in 293T cells upon transfection with calcium phosphate. For 1 production cycle $1x10^8$ cells were seeded on 20 15 cm cell culture dishes (Greiner) ($5x10^6$ cells/dish). For the transfection per 1 dish 7,5 µg of viral genome DNA and 15 µg of capside protein genome was used. DNA was mixed with $CaCl_2$ solution (250mM in H_2O) in 459 µl of total volume (per 1 dish). The same volume of 2xHBSS buffer was added dropwise to DNA/CaCl2 solution. After 10 min. incubation precipitates were added to the cell culture medium and by rocking spread over all the cells. The cells were collected 3 days after transfection by scraping and centrifugation for 5 min. at 1500 rpm. The supernatant was removed and cell pellet resuspended in 10 ml of VTE buffer. Subsequently, 3 freeze-thaw cycles were performed in order to release the virus from the cells. The cell debris were centrifuged down for 10 min. at 3500 rpm and virus-containing supernatant was collected in a falcon tube and stored at 4°C (so called crude extract). 10 ml of VTE buffer were added to the pellet and the freeze-thaw cycles were repeated for additional 2 times. Obtained crude extracts were pooled together (total volume 20 ml) and stored at 4°C.

2 x HBSS	1,5 mM Na_2HPO_4
	10 mM KCl
	280 mM NaCl
	12 mM glucose
	50mM Hepes
	add water to 500 ml pH 7,05
VTE buffer	50mM Tris-HCl pH 8,7
	0,5mM EDTA pH 8,7

2.6.2 Production of Wild Type Viruses

Wild type MVMp virus has been produced upon infection of A9 cells. Therefore, $6,6 \times 10^7$ cells were seeded on 20 10 cm cell culture dishes. The cells were infected with MOI 3×10^{-3} (MVMp wt virus). At least 4 h after infection the cells were trypsinized and the content of one 10 cm dish was transferred to one 15 cm dish. The harvesting of progeny viruses took place at day 3-5 after infection, when a visible cytolysis occurred. Scraping and centrifugation for 5 min. at 1500 rpm collected the cells. The supernatant was removed and cell pellet resuspended in 10 ml of VTE buffer. Subsequently, 3 freeze-thaw cycles were performed in order to release the virus from the cells. The cell debris was centrifuged down for 10 min. at 3500 rpm. and virus containing supernatant was collected in a falcon tube and stored at 4°C (so called crude extract). 10 ml of VTE buffer were added to the pellet and the freeze-thaw cycles were repeated for additional 2 times. Obtained crude extracts were pooled together (total volume 20 ml) and stored at 4°C.

2.6.3 Purification and Concentration of Virus Stocks

2.6.3.1 Caesium Chloride Density Gradient

This method has been used for the purification of wild type virus stocks. In one centrifuge tube (Beckmann) 5 ml of CsCl (density 1,4 g/cm^3) was applied followed by 1 ml 1M saccharose (in VTE) solution. The gradient was covered by 5 ml of crude extract. The centrifugation was performed for 20h at 39000 rpm at 10°C. Subsequently, a content of the gradient was fractioned and the optical density of every single fraction was examined by refraction measurement of 2 µl of each sample. The density was calculated according to the following formula:

ρ=10,5416xn-13,059 [kg/l] (where n=refraction). The full viral particles are in the fractions of density ranging between 1,38-1,42 g/cm^3. The presence of virions in particular fractions was verified by hemaglutination assay. Afterwards the samples were dialysed against VTE buffer and virus stocks were stored at 4°C.

2.6.3.2 Iodixanol Density Gradient

This method has been used for purification of recombinant virus stocks.
Iodixanol, $C_{35}H_{44}I_6N_6O_{15}$; molar mass: 1550,31 g/mol (all chiral centres R) is an iodinated, water-soluble, nonionic dimeric radiographic contrast medium. OptiPrep™ (Sigma) is a 60% (w/v) iodixanol solution in water (g=1,32 g/cm^3). For the gradient, the phases were applied in a 40 ml centrifuge tube (Beckmann) as follows: 20 ml of crude extract, 7 ml of 15% iodixanol solution, 5 ml of 25% iodixanol solution, 4 ml of 40% iodixanol solution, 4 ml of 60% iodixanol solution. As diluting medium for iodixanol PBS-MK was used. For the differentiation (colouring) of particular phases a phenol red solution was used (0,01 µg/ml). The gradient was centrifuged for 2h at 50000 rpm at 10°C. The full virions accumulate in the 40% phase, which after centrifugation was isolated with the assistance of syringe with needle. The purified virus suspension was stored at 4°C.

PBS-MK	500 ml 1x PBS
	500 µl 1M $MgCl_2$
	500 µl 2,5M KCl

2.6.3.3 Virus Stock Concentration

This method was used for concentrating of iodixanol stocks in order to receive a high concentrated stock diluted in PBS, what was crucial for the use of virus for the animal experiment. The iodixanol virus stock was diluted at least 1:4 with PBS and applied in a Vivaspin® (Vivascience) 20 ml centrifugal concentrator (pore size 10K). This concentrator contains twin vertical filtrating polythersulfone membranes. After filtration and washing steps concentrated virus was recovered from the tube and diluted in PBS to the desired amount.

2.6.4 Hemaglutination Assay

The hemaglutination assay was performed in order to verify the presence of virions in particular fractions of CsCl density gradient (see also 6.2.1). This assay is based on the property of parvoviruses to inhibit the sedimentation of diluted guinea pig erythrocytes. In a V-shaped microtiter plate a series of 1:2 dilutions of virus fraction with 2% erythrocytes solution (in PBS) was done. The plate was incubated 1-2h at 4°C. When the erythrocytes were mixed with virus- containing fraction, hemaglutination occurred. In the virus-free fraction erythrocytes sedimented in the middle of the well.

2.6.5 Virus Titration Methods

2.6.5.1 Infection of Adherent Cells

The cells were always seeded one day before infection was taking place. The virus was diluted to desired amount in MEM medium with antibiotics, without serum. Per one 6 cm cell culture dish 0,4 ml inoculum was used, per one 10 cm cell culture dish – 1 ml. For the titration, serial dilutions of virus stock were prepared (10^{-4}-10^{-8}). When the titer was known, the cells were infected at certain MOI (Multiplicity Of Infection). MOI is a number of replication competent viruses per one cell. It is calculated according to the formula:
MOI = (ml of virus stock x titer/ml)/ cell number
For infection, the cell culture medium was removed and the inoculum was applied on the cell monolayer. The dishes were placed in the incubator and gently rocked every 10 min., what allowed the inoculum to spread over the whole dish. After 1 hour the cells were supplied with usual cell culture medium and placed in the incubator.

2.6.5.2 Hybridization Assay

Virus titration was always performed on reference cell lines. For the MVMp-based viruses (and for the MVMp wild type) mouse A9 cells were used. For the H1-based viruses (and for the H1 wild type) human NB324K were used. In the hybridization assay a replication of viral DNA was detected and the titer was expressed in replication units per millilitre (RU/ml). Per 6 cm cell culture dish $2{,}5x10^5$ A9 or $5x10^5$ NB324 cells was seeded. The cells were infected with serial dilutions of virus stock ranging from 10^{-4} to 10^{-8}. After 48h the medium was removed, cells were washed with PBS and a nitrocellulose membrane filter (Schleicher&Schüll) was put on every dish. The filters

(with fixed on their surface cells) were described and subsequently put upside down on a Whatman paper satiated with denaturating solution. After 5 min. the filters were transferred on a Whatman paper satiated with neutralizing solution. Then DNA was fixed on the filters by backing for 2h at 80°C. To avoid an unspecific hybridization the filters were blocked for 1h at 65°C with 0,2 ng of denaturated herrings sperm DNA (Roche). The radioactive DNA probe was produced on the base of NS1-containing DNA sequence of the viral genome. This fragment was obtained by digestion of viral DNA (H1 or MVMp chimeras) with EcoRV (cuts at 381) and SpeI (cuts at 915) enzymes, agarose gel electrophoresis and following purification with Qiagen Gel Extraction Kit (Qiagen). The fragment had a length of 534 nt and could be used for titration H1-based as well as MVMp-based viruses. The genomes of H1-based and MVMp-based recombinant viruses have identical sequence from nt 1 to nt 991. The radioactive labelling of desired amount of the fragment (25 ng) was obtained through its incubation with random oligomers, dNTPs, ^{32}P-dCTP and 2U of Klenow polymerase (Megaprime™ DNA Labelling System, Amersham) in the provided buffer for 1h at 37°C. Not bound nucleotides were removed by a passage of the probe through a Sephadex50 column (centrifugation for 10 min. at 2000 rpm). Subsequently, the probe was denaturated for 5 min. at 95°C. and added to the filters for over night incubation. After that, the filters were washed twice for 30 min., one time with washing solution 1 and one time with washing solution 2. The filters were transferred into a photo cassette and exposed to an X-ray film (Kodak) for 48h at -80°C.

Every black point was considered as one replication centre. The titer of the virus stock was calculated according to the formula: titer (RU/ml)= a number of replication centres x 7,5 (a ratio filter surface/dish surface) x proper dilution.

Hybrydization solution	20 x SSC	150 ml
	10% SDS	100 ml
	0,5M EDTA	10 ml
	100 x Denhardts	100 ml
	H_2O	to 1000 ml

Denhardts x 100	2% bovine serum albumine	10g
	2% Ficoll 400	10g
	2% PVP	10 g
	H_2O	to 500 ml

Washing solution 1	20 x SSC	150 ml (3x)
	10% SDS	100 ml (1%)
	H_2O	to 1000 ml
Washing solution 2	20 x SSC	15 ml (0,3x)
	10% SDS	100 ml (1%)
	H_2O	to 1000 ml

2.6.5.3 Plaque Assay

Plaque assay is a quantitative infectivity assay; it determines the number of infectious particles. This is a standard method for the titration of wild type viruses; it can be used as well for the detection of RCV (Replication Competent Viruses) in recombinant stocks. The method is based on the property of parvoviruses to lyse the infected cells. After that, progeny viruses are released, infect neighbouring cells and lyse them. Several cytolytic cycles lead to a formation of round clear areas within the cell monolayer. These areas are called plaques. They can be visualised by a neutral red staining (see 4.3.3). The spreading of progeny viruses is restricted to the surrounding cells by use of a half-solid medium, which is applied on the cell monolayer directly after initial infection with diluted virus stock. The recombinant viruses are not able to form plaques, because they do not give progeny virions.

One day before infection, $5x10^5$ NB324K or $2,5x10^5$ A9 cells per 6 cm cell culture dish were sown. On the next day the cells were infected with serial dilutions of virus stock. Per one dilution step two dishes were used. After infection time the inoculum was removed and the cells were covered with MEM/agar mixture. After the medium got solid, the cells were placed in the incubator for 6 days. On the day 6 the neutral red staining was performed and the plaques were counted. Every single plaque was considered as coming from one viral particle. The titer was calculated according to the formula:

pfu/ml=(number of the plaques for one dilution step x dilution)/inoculum volume
pfu (plaque forming unit)

overlay medium A9	MEM2x, complete: agar 2% = 4:3
overlay medium NB324	MEM2x, complete: agar 1,7%=5:3
	agar was pre-warmed at 48°C, MEM2x complete at 37°C
MEM2x complete	MEM2x (Gibco) 77%
	FBS 17%
	L-glutamine 2%
	Nystatine 2%

2.7 Immunological Methods

2.7.1 ELISA (Enzyme-Linked Immunosorbent Assay)

Cytokine sandwich ELISA is sensitive enzyme immunoassay that can specifically detect and measure the concentration of soluble cytokine and chemokine proteins. The basic cytokine sandwich ELISA method makes use of highly purified anti-cytokine antibodies (capture antibodies), which are noncovalently adsorbed (“coated” – primarily as a result of hydrophobic interactions) onto plastic microwell plates. After plate washings, the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples that were applied to the plate. After washing away unbound material, the captured cytokine proteins are detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labeled avidin or streptavidin stage. Following the addition of a chromogenic substrate-containing solution, the level of coloured product generated by the bound, enzyme-linked detection reagents can be conveniently measured spectrophotometrically using an ELISA plate reader at an appropriate optical density (OD). By including serial dilutions of a standard cytokine protein solution of known concentration, the sandwich ELISA supports the development of standard curves.

Standard curves (“calibration curves”) are generally plotted as the standard cytokine protein concentration (typically ng or pg of cytokine/ml) versus the corresponding mean OD value of replicates. The concentrations of the putative cytokine-containing samples can be interpolated from the standard curve.

2.7.1.1 Mouse TNFα ELISA

Concentration of mouse TNFα in the cell culture supernatants was determined with assistance of the Mouse TNFα Module Set BMS607MST (Bender MedSystems) according to the manual. 96-well MaxiSorp™ (Nunclon) plates were used. For data evaluation SoftMax Pro® Software (Molecular Devices) was used.

2.7.1.2 Human IP-10 ELISA

Concentration of human IP-10 in the cell culture supernatants was determined in the collaboration with Dr. S. Stryuf and Prof. Jo Van Damme (Rega Institute in Leuven, Belgium).

2.7.2 ELISPOT Technique

ELISPOT (Enzyme-Linked ImmunoSPOT) technique was originally used to enumerate antibody secreting B cells. In the current technique, cells are deposited onto a membrane coated with one antibody specific for a protein followed by an appropriate incubation period. Subsequently, the protein of interest is detected in the environment immediately surrounding the cell secreting it, with another antibody specific for a different epitope of the protein. The signal detected by the HRP enzyme/substrate results in a colorimetric footprint of the cells and can be quantitated by visual scoring or specialized plate-readers. For cytokines, ELISPOT may in general be more sensitive than sandwich ELISA, since it takes advantage of the higher concentration of the secreted cytokine close to its source. In addition, ELISPOT allows quantitation of cytokine secretion on a per cell basis. ELISPOT plate (Millipore) was coated with antibodies: rαm IFNγ diluted in PBS (final conc. 200 µg/µl), 100 µl/well and left over night at +4°C. The antibodies were removed and the plate was washed 1x with PBS+0,05% Tween. Afterwards the plate was blocked 1-2h with RPMI 10%FCS 1%PS medium in the incubator (374°C, 5% CO_2). Medium was removed and proper dilutions of spleen cells to designated wells were applied. The plate was incubated for 20h. Subsequently the cells were removed and plate washed 6x with PBS+0,05% Tween. Antibodies: rαm IFNγ - biotin diluted in PBS (final conc. 200 µg/µl), 100 µl/well were applied and the plate was placed over night at +4°C. On the next day the antibodies

were removed and the plate washed 4x with PBS+0,05% Tween. Streptavidine Alkaline Phosphatase (SAP) (final conc. 200 µg/µl), 100 µl/well was applied and incubated 2h at RT. The wells were washed 4x with PBS+0,05% Tween. Afterwards the substrate was applied: BCIP/Nitro Blue – 50 µl/well and developed approx. 5 min. in the darkness. When the points were visible, the plate was rinsed well with tap water. The plate was drying over night at RT in the darkness and at the next day the measurement was done.

2.7.3 Immunohistochemistry

Tumor tissue was isolated, fixed with TissueTec (Sakura) and frozen immediately in liquid nitrogene. Tissue was stored at -80°C. For paraffin sections tumor material was fixed with 4% paraformaldehyde over night, washed 2 times with PBS and finally stored in 70% ethanol at 4°C. For immunohistochemistry frozen sections were cut and fixed with ice cold 4% paraformaldehyde. After 2 washing steps (5 min. PBS) the slices were blocked with 1% in PBS normal swine serum (10 min). The first antibodies (αCD31, rat α mouse, Pharmingen) at the dilution 1:200 were applied on the tissue and incubated at 37°C for 1 hour. After 2 washing steps (5 min. PBS) secondary antibodies (biotynylated α rat Ig, Pharmingen) were applied on the slice at the dilution 1:100 (in PBS) and incubated for 30 min. at 37°C. Subsequently the tissue was washed 2 times (5min.) with Tris buffer (50mM, pH 7,6). Sterptavidin-coupled alkalic phosphatase was applied on the tissue and incubated for 20 min at 37°C. The enzyme was diluted 1:150 in Tris buffer containing 1mM Levamisol (an inhibitor of endogenous alkalic phosphatase). Afterwards 3 washing steps (5 min.) with Tris buffer were performed. The substrate for alkalic phosphatase, Neufuchsin (DAKO) was applied on the sections. The staining took place for 2 min. and washing with Tris buffer stopped the reaction. The sections were counterstained with hematoxylin according to Mayer's protocol. After 2 washing steps (5 min. aqua dest.) the slices were dried and coverslipped using Mounting medium (DAKO).

Necrotic areas were determined by morphological changes via hematoxylin staining. The largest cross-sectional diameter of each tumor was evaluated. The different sections of the tumor section were traced manually. The percentage of necrotic tumor area was calculated as the ratio of the necrotic area to the total tumor area x 100.

The cross-sectioned vessels were quantified counting CD31-postive vessels (CD31 is a surface marker endothelial cells that are present in the lumen of blood vessels).

The vessels were visually identified at 20-fold magnification and manually counted. Using a custom-developed computer-aided image analysis device for assistance the area of each slide preparation was determined.

The vessel density was manually determined for each slide preparation, and expressed as vessel number per mm^2.

The author, who was "blinded" to the subtype of the tumors, performed all counts. Immunohistochemistry was done at the Department of Anatomy III, University of Heidelberg under the supervision of Dr. Ralf Kinscherf.

2.8 MRI Analysis

Magnetic resonance imaging (MRI) is a non-invasive imaging modality, which yields high spatial resolution and excellent soft tissue contrast. Especially in tumors, MRI is a powerful tool to visualize vessel morphology and function.

2.8.1 Measurement technique

All MRI measurements were performed at a clinical 1.5 T MR scanner (Magnetom Vision, Siemens, Erlangen, Germany) (Fig. 1a) using a home-built animal resonator (Figure 1b). For morphological imaging six transversal slices were acquired at a spatial resolution of (0.2×0.2×2.0) mm^3 (Figure 1c) using a T_2-weighted spinecho sequence (TR/TE=4000ms/96ms). Physiological parameters were assessed by dynamic contrast-enhanced magnetic resonance imaging (DCE MRI). Using a T_1-weighted spoiled gradient echo sequence (TR/TE/α=46ms/7ms/40°) two transversal slices of (0.3×0.3×2.0) mm^3 resolution were acquired. Reaching an imaging time of 7 s the measurement was repeated successively in order to cover a total measurement time of 10.5 min. After the third repetition a gadolinium-based contrast agent (Omniscan®, Amersham) was injected into the tail vein at a dose D = 0.1 mmol/kg body weight over an infusion time of τ = 5 s. Before imaging, each mouse was anesthetized by inhalation of 3% isofluorane in a NO/O_2 mixture (1:2) and an intravenous catheter was inserted into the tail vein for contrast agent administration.

This work was performed in collaboration with Dr. M. Heilmann and Dr. F. Kießling at the Department of Radiology DKFZ Heidelberg.

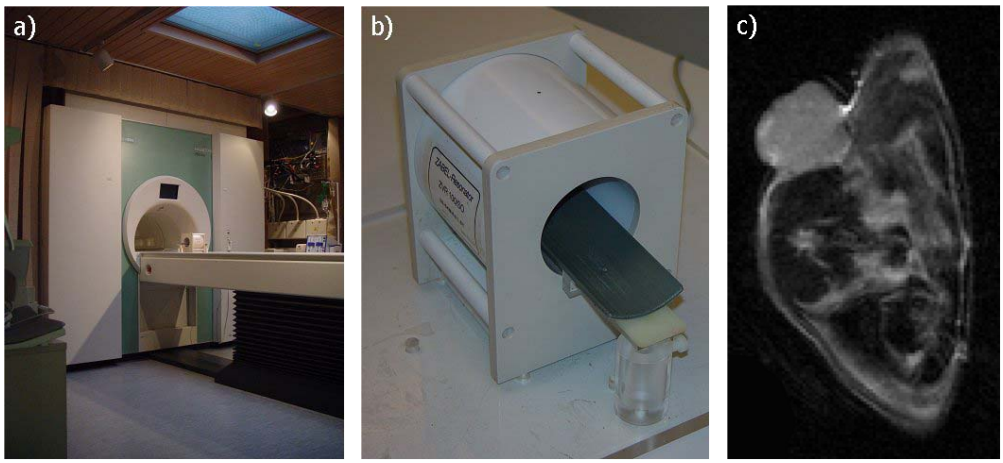


Figure 1 MRI measurements were performed at a clinical 1.5 T MR scanner (a) using a home-built animal resonator (b). Tumor morphology was imaged resulting in transversal sections of the whole mouse body including the tumor (c).

2.8.2 Data Analysis

DCE MRI data were analyzed by assuming a bidirectional contrast agent (CA) exchange between blood (plasma volume) and tissue (extravascular extracellular space) compartment (Figure 2a). In the MR images for each pixel signal intensity was measured during course of time yielding a signal-time-course. Figure 2b shows a schematic represenetation of signal-time-course. For each image pixel, the contrast agent enhancement in tumor tissue was determined by calculating the parameters amplitude A (relative signal enhancement with respect to the pre-contrast value) and the exchange rate constant k_{ep}, which is a mixture of CA exchange velocity and tissue perfusion.

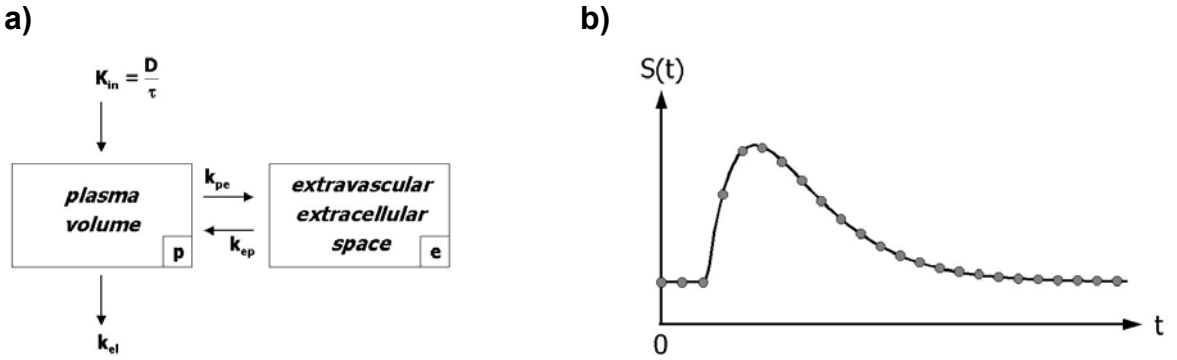


Figure 2 MRI data were analyzed by assuming a bidirectional contrast agent (CA) exchange between blood (plasma volume) and tissue (extravascular extracellular space) compartment (a). In the MR images for each pixel signal intensity was measured during course of time yielding a signal-time-course (b). Based on this, CA enhancement in tissue was determined by calculating the relative signal enhancement (*ampliutde A*) and the velocity of CA exchange (*exchange rate constant* k_{ep}).

For comparison of treated with untreated animals, an averaged signal-time-course of the whole tumor was measured in order to calculate amplitude (A) and exchange rate constant (k_{ep}). Finally, average and standard deviaton values of A and k_{ep} were determined for each group (mock, IP-10) and compared to each other.

2.9 Animal Techniques

2.9.1 Experimental Animals

Experimental animals were obtained from Charles River Wiga Company (Sulzbach, Germany). Mice represented strains C57Bl/6 and Swiss cd 1 nu/nu. 5-6 weeks old female mice were grouped up to 5 animals per cage. The cages were placed in the isolator at 21-24°C, 40-60% humidity. All animals were housed under the same conditions (food and water ad libitum throughout the experiment, 12 h dark-light). Animals were sacrificed by an overdose of CO_2. The Referat Veterinärwesen at the Regierungspräsidium Karlsruhe, Germany approved the animal studies.

2.9.1.1 Injection of In Vitro Infected Tumor Cells

Infection of the tumor cells was done like described previously (see 6.4.1). Four hours after infection the cells were trypsinized, collected, washed two times with Dulbecco's PBS and counted. Desired amount of cells was resuspended in proper amount of PBS. 100 µl of cell suspension was injected subcutaneously in the right flank of each animal.

Growing tumors were examined and measured every 2-3 days. Animals were sacrificed when the tumor volume exceeded 1,5-2,0 cm^3 or in case of necrosis.

2.9.1.2 Injection of Virus Suspension

For the injections virus stock was diluted to desired concentration of viral particles in Dulbecco's PBS containing calcium. Virus suspension was injected in the tumor surrounding to avoid mechanical damage of the tumor. 100 µl of virus suspension was applied in 2-3 injections. Growing tumors were examined and measured every 2-3 days. Animals were sacrificed when the tumor volume exceeded 1,5-2,0 cm^3 or in case of necrosis.

2.9.1.3 Tumor Volume

For the evaluation of tumor volume tumors' length, breadth and height were measured 2-3 times per week with the assistance of a caliper. Tumor volume was calculated for an ellipsoid according to the formula $V= \pi/6 *L*B*H$.

Animals were sacrificed when the tumor volume exceeded 1,5-2,0 cm^3, in case of necrosis or any other disorders.

2.10 Statistical Methods

Statistical analyses were performed using Sigma Stat (SPSS, Chicago, USA) and MedCalc (MedCalc Software, Mariakerke, Belgium) software packages. Statistical differences were determined using the Student's *t* test, the nonparametric Mann-Whitney rank sum test and the longrank test for trend. A $P<0.05$ was considered significant.

3 List of Abbreviations

α	anti
AAV	adeno-associated virus
APC	antigen presenting cell
BCNU	bischloroethyl nitrosourea
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
βTG	beta-thromboglobulin
CNS	central nervous system
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMEM	Dulbeco's Minimum Essential Medium
ds DNA	double stranded DNA
ECL	enhanced chemiluminescence
EDTA	ethylendiamintetraacetic acid
EGFP	enhanced green fluorescent protein
EGF-R	epithelial growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ENA	epithelial-derived NAP
EtBr	ethidiumbromide
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GBM	glioblastoma multiforme
GDEPT	gene-directed enzyme prodrug therapy
GM-CSF	granulocyte-macrophage colony stimulating factor
GRO	growth-related oncogene
HBSS	Hank's buffered salt solution
HSV	herpes simplex virus
HSV-tk	herpes simplex virus thymidinkinase

IGF-1	insulin-like growth factor 1
IL	interleukin
iNOS	inducible nitric oxide synthase
INFγ	interferon γ
IP-10	interferon γ-inducible protein
ITAC	interferon inducible T cell α chemoattractant
kb	kilobases
kD	kilodalton
LB	Luria-Bertani medium
LPS	lipopolysaccharide
mA	miliamper
MCP	monocyte chemotactic protein
MEM	minimum essential medium eagle
MHC	major histocompatibility complex
MOI	multiplicity of infection
MTD	maximally tolerated dose
MVMp	minute virus of mice prototype strain
NAP	neutrophil-activating peptide
NDV	Newcastle disease virus
NK	natural killer cells
NS	non-structural protein
OD	optical density
ODNs	oligodeoxynucleotides
PBP	platelet basic protein
PF	platelet factor
PBS	phosphate buffered saline
PCV	procarbazine, carmustine, vincristine
PDGF	platelet-derived growth factor
PFU	plaque forming unit
PKC	protein kinase C
PMV	paramyxovirus
PTN	pleiotrophin

RB	retinoblastoma
RCV	replication competent virus
RF	replicative form
rpm	rotation per minute
RT	room temperature
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
ssDNA	single stranded DNA
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor α
VEGF	vascular endothelial growth factor
VP	viral protein
WHO	World Health Organisation

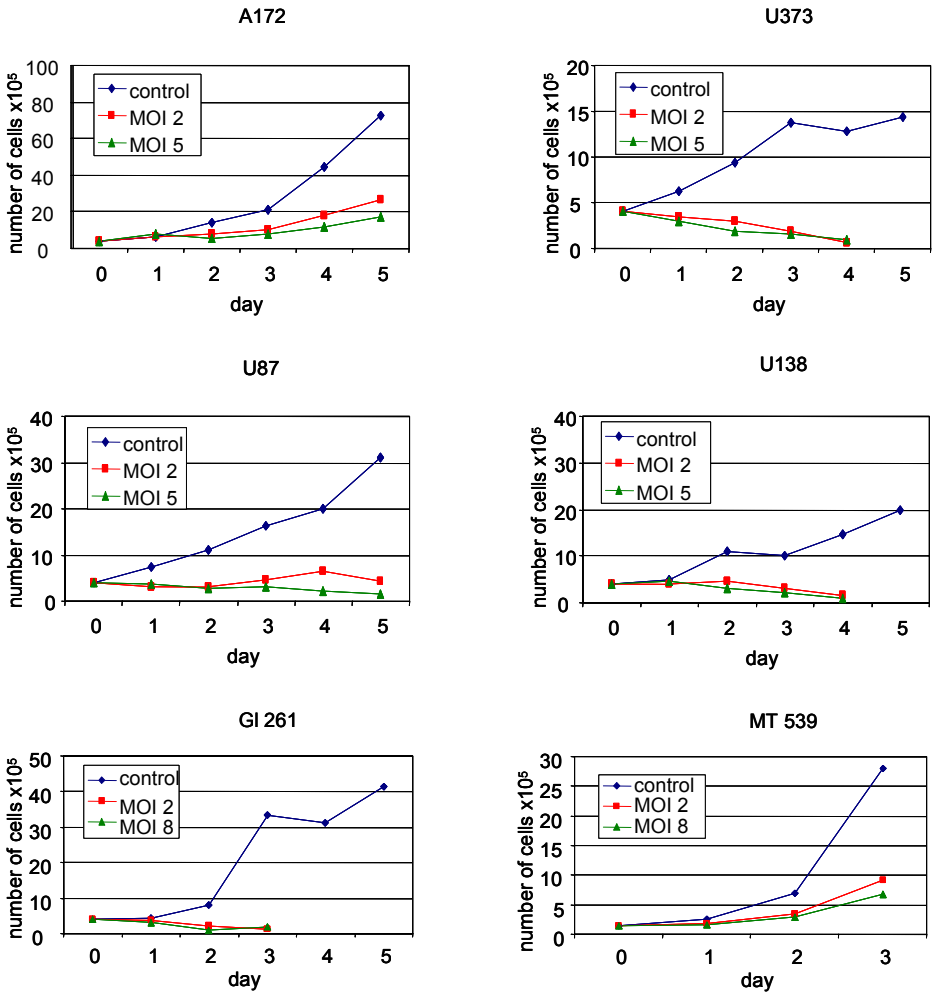
4 Results

4.1 Characterization of parvoviral infection in glioblastoma cells

4.1.1 Sensitivity of glioblastoma cells to the infection with wild type parvoviruses

In order to evaluate whether glioma cells might be used as tumor models for cancer therapy with parvoviruses, the cells of four different human (A172, U87, U138 and U373) and two mouse (Gl261 and MT539) glioblastoma cell lines were examined for their sensitivity *in vitro* to wild type parvoviruses. Human cells were infected with H1 virus (rat virus) and mouse cells with MVMp virus. Figure 1 shows growth curves of cells infected at different multiplicities of infection (MOI) compared to control (not infected) cells.

a)



b)

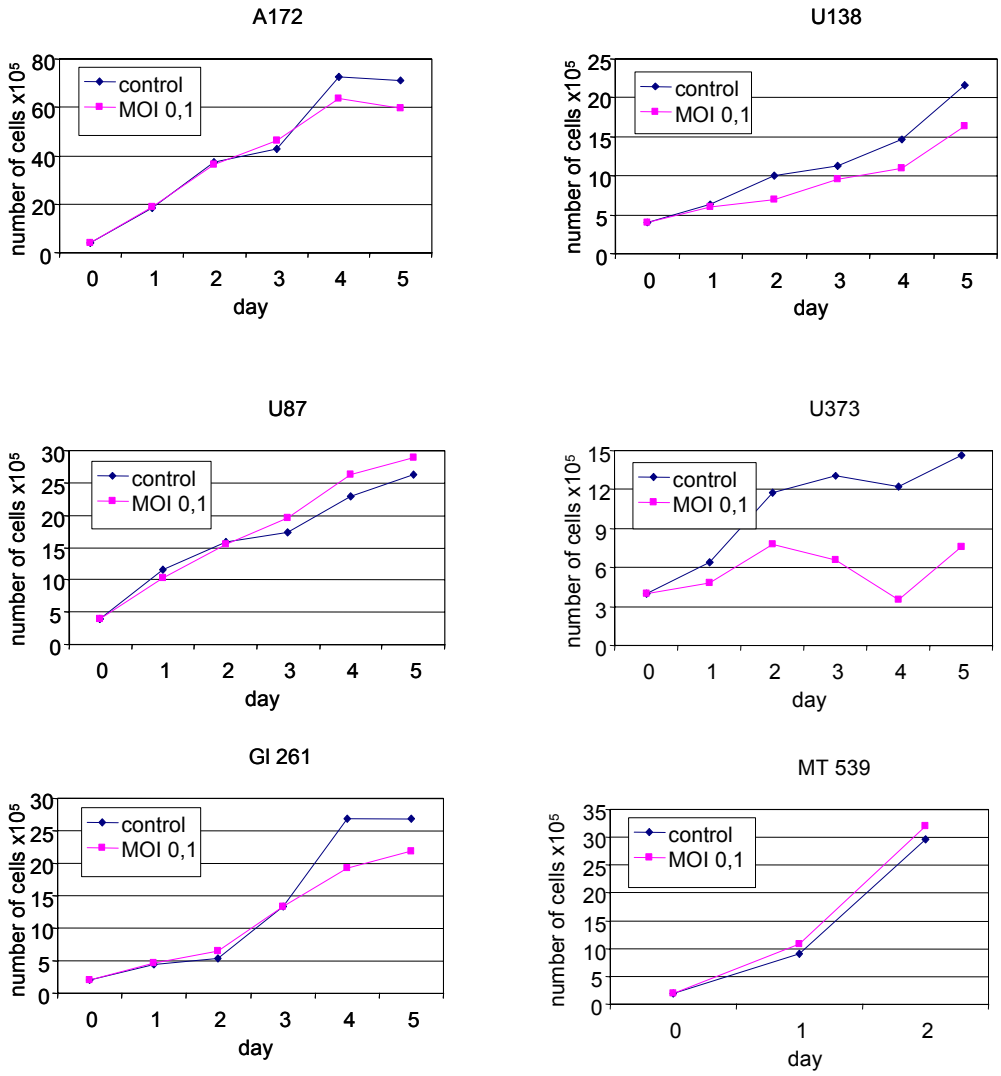


Figure 1 Sensitivity of glioblastoma cells to wild type parvoviruses.
a) 4x10^5 cells were infected with H1 virus at MOI 2 or 5 (human cells) or MVMp virus at MOI 2 or 8 (murine cells). The number of alive cells was obtained using the Trypan Blue exclusion method.
b) 4x10^5 cells were infected with H1 virus at MOI 0,1 (human cells) or a MVMp virus at MOI 0,1 (murine cells). The number of alive cells was obtained using the Trypan Blue exclusion method.

The cells of examined lines showed to be different in their sensitivity to the infection. The growth of cell population slows down (A172, MT539); number of cells stays at the same level (U87) or significantly decreases (U138, U373, Gl261) in comparison to non-infected control cells. A complete damage of U373, U138 and Gl261 cell populations can be observed at day 4 post infection. There is no difference between effects observed at MOI 2 and 5 (see Figure 1a). Infection at MOI 0,1 has either no effect on cell population growth (A172, U87, MT539) or slightly slows down the growth of the most sensitive cells (U138, U373, Gl261) (see Figure 1b).

For murine cells a clonogenicity assay was performed in order to discriminate between the cytotoxic and cytostatic effect of MVMp virus on cell survival. The results show a high sensitivity of Gl261 cells to the viral infection – colony formation dropped to 1% (compared to non-infected cells), which suggest a high mortality or strong due to infection. This confirms the results shown with Figure 1a. For MT539 cells an only 50% reduction on colony formation can be observed (see Table 1).

Gl261 survival				**MT539 survival**	
MOI 2	$4x10^3$	cells	0,1%	MOI 2	56%
MOI 2	$1x10^4$	cells	1,09%	MOI 8	42%
MOI 5	$4x10^3$	cells	0,2%		
MOI 5	$1x10^4$	cells	0,6%		

Table 1 Clonogenicity assay on Gl261 and MT539 cells.

4.1.2 Production of progeny wild type viruses by infected glioblastoma cells

Production of progeny viruses by infected tumor cells must be considered individually for each cell line because there are big differences even between the cells of the same origin (Kayser T. 2004). The production of new viruses upon infection can increase the cytotoxic effect of the virus by allowing secondary infections.

In the majority of tested cell lines the amount of virus measured 5 days post infection was lower than or similar to the amont of input virus. This means that those cells do not produce progeny viruses. A degradation of the input virus in those cells might take place. U373 and Gl261 samples show an increased amount of the virus compared to the input, thus indicating that they are potent for the production of progeny virions (see Figure 2).

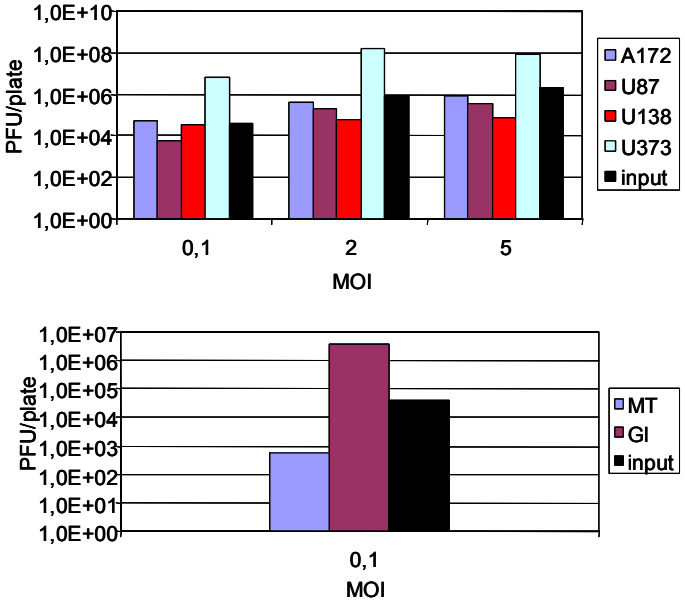


Figure 2 Progeny virus production in glioblastoma cells.
4x10^5 cells were infected with the wild type parvovirus (H1 – human cells; MVMp – mouse cells) at MOI 2; 5 or 0,1. After five days the cells with the medium were collected and 3 freeze-thaw cycles were performed. Virus amount in obtained supernatant was evaluated by plaque assay.

4.1.3 Infectability of glioblastoma cells

In order to determine the efficiency of transduction after infection of glioma cells with parvoviral vectors, infection with the vector expressing a reporter gene was performed. Infectability can be estimated by determining the expression of a marker gene, for example encoding EGFP. The percentage of cells positive for EGFP expression was measured by FACS (see Figure 3).

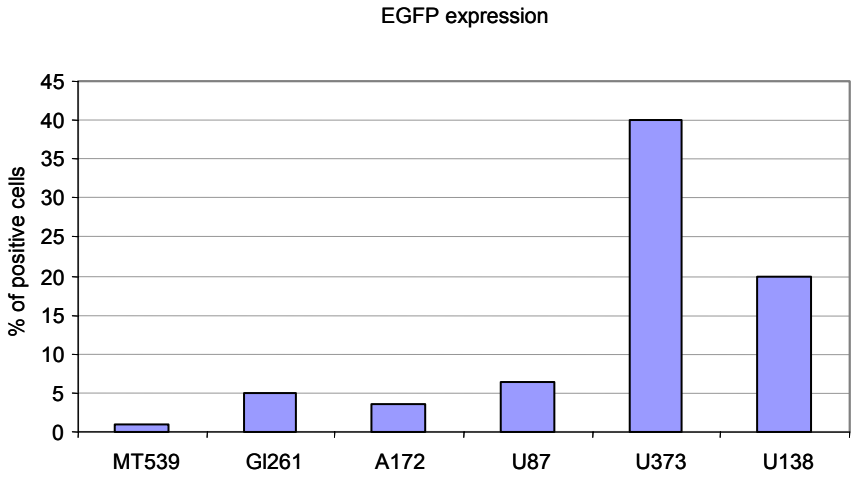


Figure 3 FACS analysis of the cells infected with EGFP-expressing vector.
Glioblastoma cells were infected at MOI 2 with a Chi-hH1/EGFP or a Chi-MVMp/EGFP virus. 2 days post infection the cells were collected and FACS analysis was done.

The percentage of EGFP-positive cells was variable between the cell lines. The best infectable among murine cells are Gl261. Among human cells the highest percentage of EGFP-positive cells could be measured for U373 cells (see Figure 3). The results show that all the tested cell lines are permissive for parvoviral infection.

4.2 Cloning of the transgenes into the parvoviral vectors

4.2.1 Basic vectors

Basic parvoviral vectors used in this work are MVMp- and H1- based chimeras – Chi-MVMp/Δ800 and Chi-hH1/Δ800. Chimeric recombinant vector genomes were designed by replacing the right-hand region of the H-1 virus DNA with that of the closely related MVMp virus DNA and conversely Wrzesinski et al., 2003). Genes coding for the capsid proteins (VP) have been partially deleted and may be replaced by different transgenes.

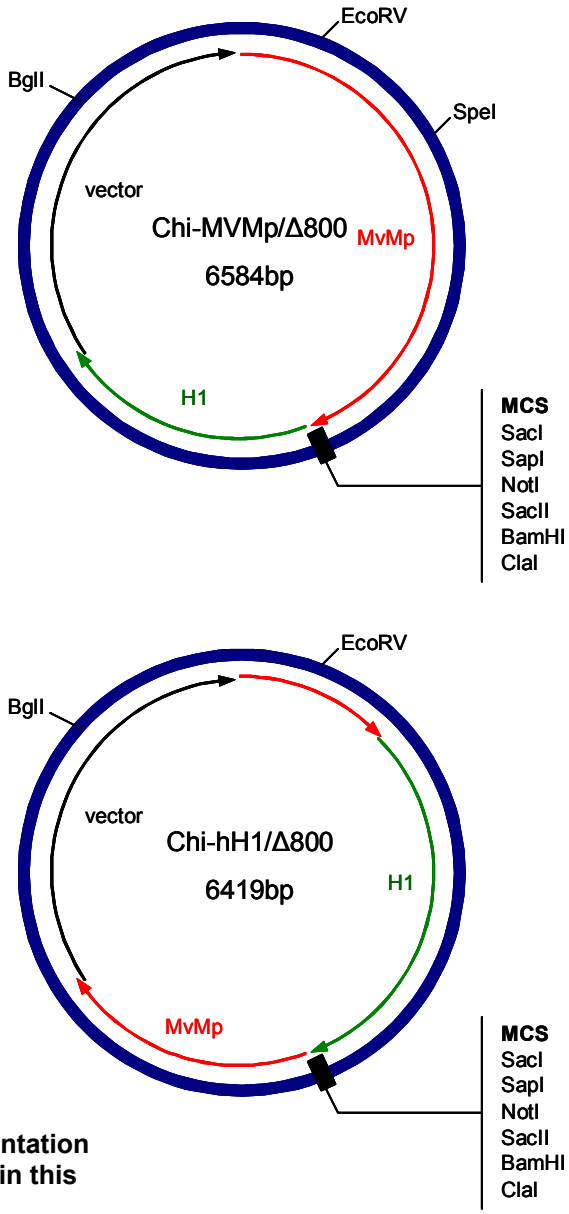


Figure 4 Schematic presentation of chimeric vectors used in this work

The transgenes used in this work are human IP-10 and murine TNFα and were cloned as 400 bp and 700 bp inserts into BamHI/ClaI and NotI/BamHI sites, respectively.

4.2.2 Cloning of human IP-10

Human IP-10 was chosen due to its antiangiogenic and immunostimulatory properties. The levels of IP-10 in cell culture supernatants could be monitored by ELISA. Human IP-10 ELISA measurements were performed in collaboration with Dr. S. Stryuf and Prof. J. Van Damme who established an appropriate system (Abu El-asrar A. M. et al. 2004).

Repeated restriction digestions controlled the quality of the plasmid of origin and basic vectors. 3 µg of plasmid DNA was digested with BamHI/ClaI enzymes to obtain a 400 bp insert. The insert was separated on 1% agarose gel, purified, and its concentration was estimated by comparing it to a DNA marker. 4 µg of Chi-MVMp/Δ800 and 4 µg of basic Chi-hH1/Δ800 plasmids were digested with BamHI/ClaI enzymes. Digested plasmids were separated on 1% agarose gel, purified, and their concentration was estimated by comparing them to a DNA marker. 90 ng of basic vector was mixed with 50 ng of insert and ligated over night at 12°C. Sure bacteria were transformed with obtained constructs. Restriction digestions confirmed the presence of an insert in parvoviral vectors.

4.2.3 Cloning of mouse TNFα

Mouse TNFα was chosen as a potent immunestimulatory and potentially antiangiogenic factor. These properties are of advantage in glioma treatment, which are described as well-vascularized tumors demonstrating immunesupressing features.

Repeated restriction digestions controlled the quality of the plasmid of origin. 10 µg of plasmid DNA was digested with SalI enzyme to obtain a 998 bp insert. The insert was separated on 1% agarose gel, purified, and its concentration was estimated by comparing it to a DNA marker. The maximal insert length for parvoviral vectors is 800 bp. For that reason and to avoid the insertion of plasmid DNA sequences of pSV23SMTNFinto the viral genome, it was necessary to design specific primers to synthesize by PCR a 700 bp cDNA fragment with NotI-BamHI linkers for the insertion into parvoviral vectors.

NotITNF primer

NotI recognition and cut site

5’ GGG CGG CCG CGC GGC CGC ATG AGC ACA GAA AGC ATG ATC 3’

BamHITNF primer

BamHI recognition and cut site

5’ GGG GAT CCG GAT CCT CAC AGA GCA ATG ACT CCA AAG TAG ACC TG 3’

In a PCR reaction the fragment of interest was multiplied. Afterwards it was separated on 1% agarose gel, purified, and the concentration was estimated by comparing it to a DNA marker. 150 ng of the fragment was digested with BamHI/NotI enzymes, which resulted in “sticky ends”. For the ligation of basic vectors Chi-MVMp/Δ800 and Chi-hH1/Δ800, which were previously digested with BamHI/NotI enzymes, 38 ng of insert was used. Sure bacteria were transformed with obtained constructs. Restriction digestions confirmed the presence of an insert in parvoviral vectors.

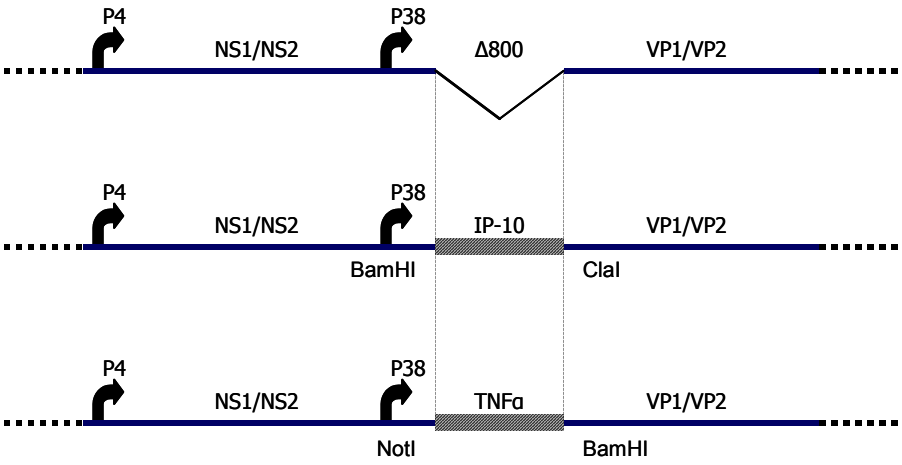


Figure 5 Schematic presentation of parvoviral vectors with cloned transgenes.

The viruses were produced by cotransfection of 293T cells with vector DNA and the corresponding helper plasmid, using the calcium phosphate procedure. Virus stocks were harvested 72 h posttransfection by removing the medium, washing the cultures, and lysing the cells by three rounds of freezing and thawing as previously described (Haag A. et al. 2000). Cell debris was removed by low-speed centrifugation, and viruses were purified by nonionic iodixanol gradient centrifugation (a modified version of the method described by Zolotukhin, S. et al. 1999). Virus stocks were diluted with medium or PBS before use. The titers of viral stocks ranged from $1x10^6$-$1,5x10^7$ RU/ml for MVMp-based vectors and $2,4x10^6$-$2,4x10^7$ RU/ml. The titers of pseudotyped vectors varied between $4,6x10^6$-$1x10^8$ RU/ml. Recombinant parvoviruses used in this work are presented in Table 2.

Virus	**Characteristics**
Chi-MVMp/Δ800	MVMp-based chimeric vector (Wrzesinski C. et al. 2003)
Chi-MVMp/EGFP	MVMp-based chimeric vector with EGFP sequence (Wrzesinski C. et al. 2003)
Chi-MVMp/IP-10	MVMp-based chimeric vector with human IP-10 sequence
Chi-MVMp/TNF	MVMp-based chimeric vector with murine TNFα sequence
Chi-hH1/Δ800	H1-based chimeric vector (Wrzesinski C. et al. 2003)
Chi-hH1/EGFP	H1-based chimeric vector with EGFP sequence (Wrzesinski C. et al. 2003)
Chi-hH1/IP-10	H1-based chimeric vector with human IP-10 sequence
Chi-hH1/TNF	H1-based chimeric vector with murine TNFα sequence
Chi-MVMp/Δ800(H1)	MVMp-based chimeric genome in H1 capsid
Chi-MVMp/IP-10(H1)	MVMp-based chimeric genome with human IP-10 sequence in H1 capsid
Chi-MVMp/TNF(H1)	MVMp-based chimeric genome with murine TNFα sequence in H1 capsid

Table 2 Recombinant parvoviruses used in this work

4.2.4 RCV contamination

By the production of recombinant vectors due to a homologous recombination between a helper plasmid and a recombinant genome the formation of replication competent viruses (RCVs) is possible. RCVs are able to build the capsids and produce progeny virions. For safety reasons the purity of recombinant stocks should be controlled. This can be done using the plaque assay method. At least 3 independently produced stocks of each recombinant virus were tested for the presence of RCVs. A variable amount of RCVs could be detected, ranging from 0,002% to 0,0026% for the chimeric vectors and from 0,13% to 1,7% for the pseudotyped vectors (MVMp-based genomes packaged in H1 capsid).

4.3 Major viral protein NS1 expression in glioblastoma cells

4.3.1 Major viral protein NS1 expression

NS1 is a 83 kDa, cytotoxic, multifunctional nuclear protein with nickase, helicase and ATPase activities (Palmer G.A. 2000). H-1- and MVMp – based vectors developed in our laboratory remain the early promoter P4 and the sequences encoding the NS proteins. The transgene is expressed under control of the internal P38 promoter that is strongly activated by the vector-encoded NS1 protein (Wetzel K. et al. 2001). NS1 expression is necessary for the transgene induction. NS1 expression in glioblastoma cells was analyzed by Western blot.

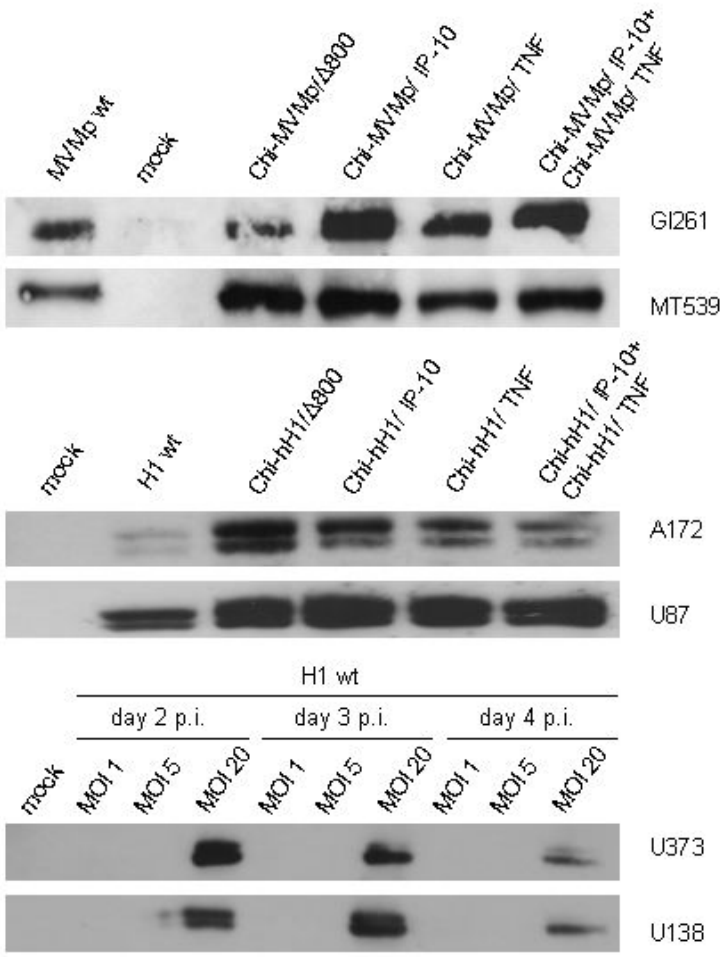


Figure 6 NS1 expression in glioblastoma cells
$1x10^6$ cells per experimental point were infected at MOI 1. For the combination of a Chi-MVMp/IP-10virus with a Chi-MVMp/TNF virus or a Chi-hH1/IP-10 virus with a Chi-hH1/TNF virus the combination MOI 0,5 and MOI 0,5 was used. Cells infected with recombinant viruses were collected 2 days post infection. U373 and U138 infected with H1wt virus were collected 2, 3 and 4 days post infection. 20 µg of protein extract was applied on each lane.

As shown in Figure 6, NS1 expression can be detected in mouse Gl261 and MT539 cells infected with all recombinant virus stocks. The NS1 amount is similar for each recombinant virus as well as for the combination. In human A172, U87, U373 and U138 cells two NS1 bands (hyperphosphorylated and non-phosphorylated forms) are visible. For the recombinants the NS1 levels were similar.

U373 and U138 cells infected with wild type H-1 virus represent different accumulation of NS1 protein. No NS1 was detectable when the cells were infected with recombinant viruses at MOI1 and collected after 2 days. Infection with the H1wt at MOI1 and MOI5 does not lead to NS1 expression detectable after 2, 3 or 4 days post infection. At these time points the NS1 expression is detectable only when the cells were infected with MOI20 (see Figure 6).

It is possible that in these cells NS1 protein undergoes rapid degradation and infection at MOI 1 or MOI5 does not lead to the accumulation of NS1 protein at detectable levels.

Obtained data demonstrate that infection with recombinant MVMp-, H1- based vectors or H1wt virus results with NS1 protein expression in cells of all tested glioma lines. This suggests that upon infection with recombinant vectors NS1 would facilitate amplification of the recombinant genome, increasing the copy number of the transgene and transactivate P38 promoter to drive expression of the transgene. NS1 expression could not be demonstrated while U373 and U138 cells were infected with recombinant vectors at MOI1 (data not shown). Nevertheless, as shown in Figures 7a-b, infection at MOI1 with vectors encoding IP-10 or TNFα lead to efficient transgene production.

4.4 Transgene expression in glioblastoma cells

4.4.1 Transgene expression evaluated by ELISA

An effective transgene expression is, besides the sensitivity of the cells to the virus, an important parameter for the gene therapy of cancer. A high transgene expression (a high concentration of the secreted protein in a cell culture medium) is desired. In order to evaluate the efficacy of transgene expression in glioblastoma cells upon infection with parvoviral vectors containing a human IP-10 or a mouse TNFα transgene, glioblastoma cells were infected with the virus at MOI 1. Cell culture supernatants were collected and examined by ELISA.

NBK and A9 cells are reference cell lines - NBK for human cells, A9 for mouse cells. Cells of these lines are highly infectable and produce a high amount of proteins by means of parvovirus-mediated transduction. The amount of secreted protein for each glioblastoma cell line was high, especially for murine cells Gl261 (up to 1 µg IP-10 per $2x10^5$ cells at day 3 post infection) (Figure 7c). A production peak may be observed usually at day 2 post infection (see “daily production”). Proteins seem to be stable in a cell culture medium up to day 4 post infection (reduction of the protein level can not be observed; see “accumulation”). A transgene expression upon infection with a pseudotyped virus (TNFps) was at least as efficient as an expression reached upon infection with a Chi-hH1/TNF construct (a pseudotyped virus is a Chi-MVMp/TNF genome in hH1 capsid.) Endogenous production of TNFα in tested cells did not reach a measurable level (Figures 7b and 7d).

The human IP-10 transgene expression evaluated by ELISA was done by the group of Professor Jo Van Damme (Leuven, Belgium) (see Figures 7a-d) in the frame of EU scientific collaboration.

a)

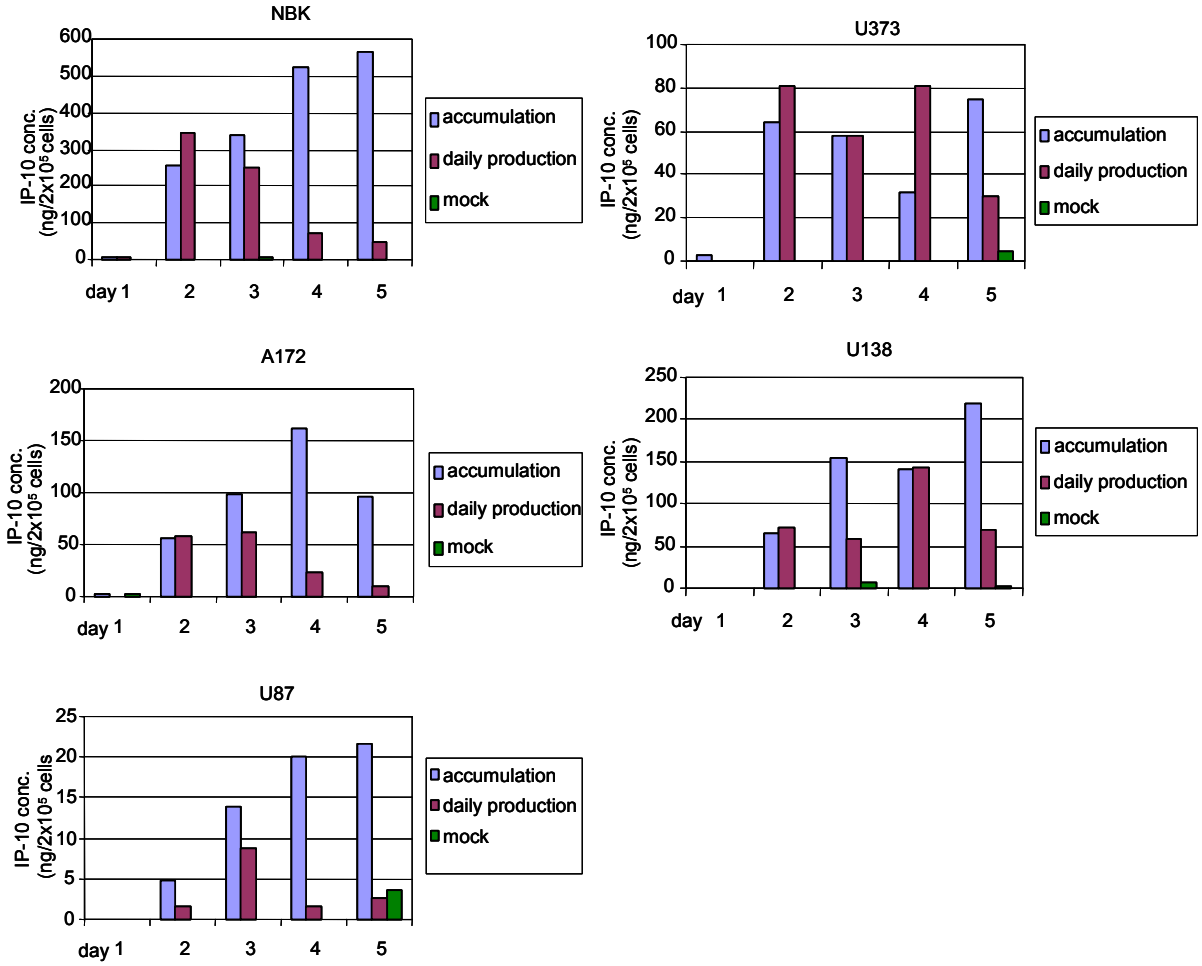


Figure 7a Human glioblastoma cells produce high amount of transgene protein (IP-10)
$2x10^5$ cells were infected at MOI 1 with a Chi-hH1/IP-10 virus. In order to obtain an "accumulation" measurement the cell culture supernatant was collected each day. For a "daily production" the supernatant from two selected plates was collected and replaced with a fresh medium every day.

b)

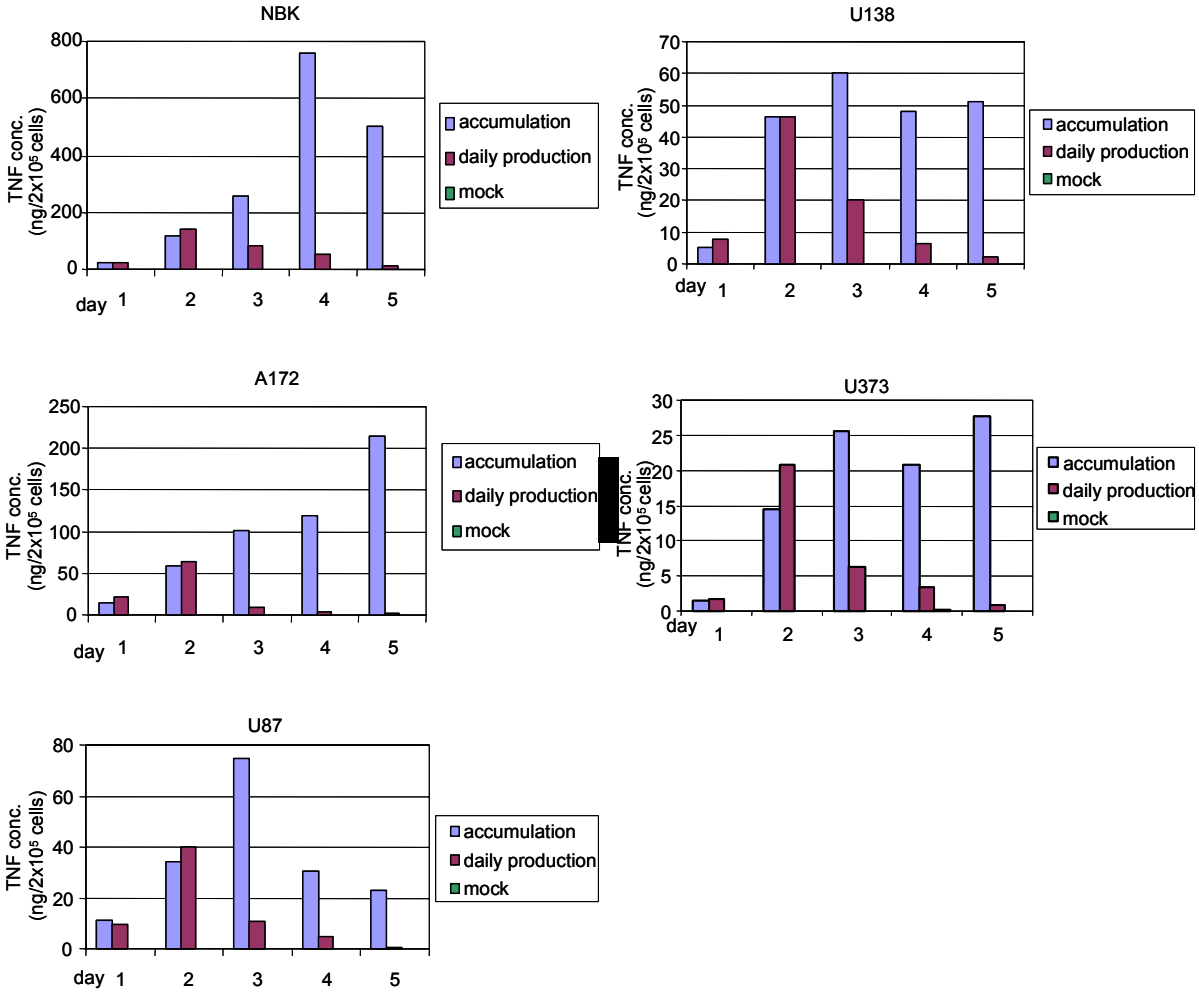


Figure 7b Human glioblastoma cells produce high amount of transgene protein (TNFα)
$2x10^5$ cells were infected at MOI 1 with a Chi-hH1/TNF or a TNF ps virus. In order to obtain an "accumulation" measurement the cell culture supernatant was collected each day. For a "daily production" the supernatant from two selected plates was collected and replaced with a fresh medium every day.

c) **d)**

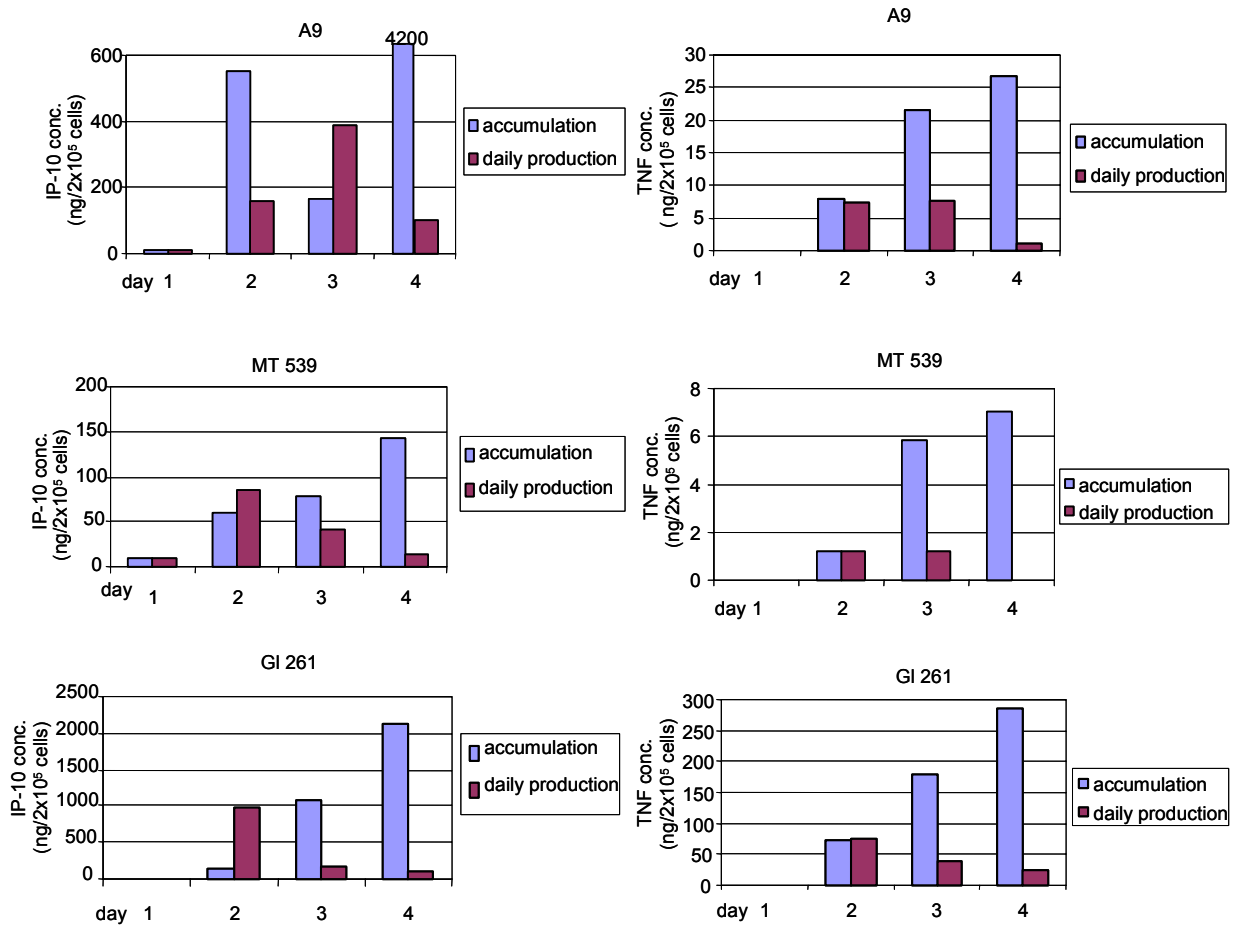


Figure 7c Mouse glioblastoma cells produce high amount of transgene protein (IP-10)
$2x10^5$ cells were infected at MOI 1 with a Chi-MVMp/IP-10 virus. In order to obtain an "accumulation" measurement the cell culture supernatant was collected each day. For a "daily production" the supernatant from two selected plates was collected and replaced with a fresh medium each day.

Figure 7d Mouse glioblastoma cells produce high amount of transgene protein (TNFα)
$2x10^5$ cells were infected at MOI 1 with a Chi-MVMp/TNF virus. In order to obtain an "accumulation" measurement the cell culture supernatant was collected each day. For a "daily production" the supernatant from two selected plates was collected and replaced with a fresh medium each day.

Obtained data demonstrate that upon infection with recombinant parvoviruses expressing IP-10 or TNFα cells of all tested glioblastoma lines produced transiently high amounts of recombinant proteins. However, obtained levels were variable and different for particular cell lines.

Transgene expression induced by parvoviral vectors is transient, since the genome of autonomous parvoviruses does not integrate in the host cell genome and the expression is limited in time, as shown previously (Wetzel K. et al 2001).

Cytotoxic NS1 protein whose expression is maintained in the vectors might contribute to cell death and in this way may limit the time of transgene expression. High, but transient expression of the chemokines or cytokines is an advantage for cancer therapy, because their permanent expression might disrupt chemokine milieu and lead to severe side effects (Carmeliet P. 2000).

4.4.1.1 Cytotoxic effect of recombinant parvoviruses

In addition to the expression of transgenes, the influence of infection with recombinant viruses on cell population growth was monitored. After the supernatant was collected, living cells were counted using the Trypan Blue exclusion method (see Figures 8 a-d).

At MOI 1 a Chi-hH1/IP-10 virus seemed not to have a cytotoxic effect (defined as a decrease in the number of living cells) on human A172, U87 and U138 glioblastoma cells. A slight difference between the number of infected and non- infected cells in the case of NBK and U373 could be caused by a higher sensitivity of these cells to the viral infection, in particular to the cytotoxic activity of the NS1 protein (see Figure 8a). When a TNF-expressing virus was used, the number of infected cells for all tested lines remained similar to the number of non-infected cells. Again, the difference between mock-treated and infected cells (observed for NBK and U138) might be caused by a high sensitivity of these cells to the viral infection itself. There was no difference between U87 cells treated with a Chi-hH1/TNF or a TNF pseudotyped virus (see Figure 8b).

For mouse cells a cytotoxic effect of the Chi-MVMp/IP-10 viruscould not be observed. A growth delay for Gl261 and reference A9 cells might be an effect of the NS1 protein (see Figure 8c). When a Chi-MVMp/TNF virus was used there was a clear cytotoxic effect for Gl261 and A9 cells. Many dead cells could be observed in the culture. It was probably caused by the additive toxicity of the NS1 protein and the transgene. A9 and Gl261 cells are sensitive to cytotoxic action of TNFα (see Figure 9b). There was no decrease in the number of MT539 cells upon infection. These cells are also sensitive to TNF, but less infectable than Gl261 and also much faster growing. Taken together it could be that a high number of cells that have not been infected have overgrown and masked the cytotoxicity caused by a transgene expression. (see Figure 8d).

a)

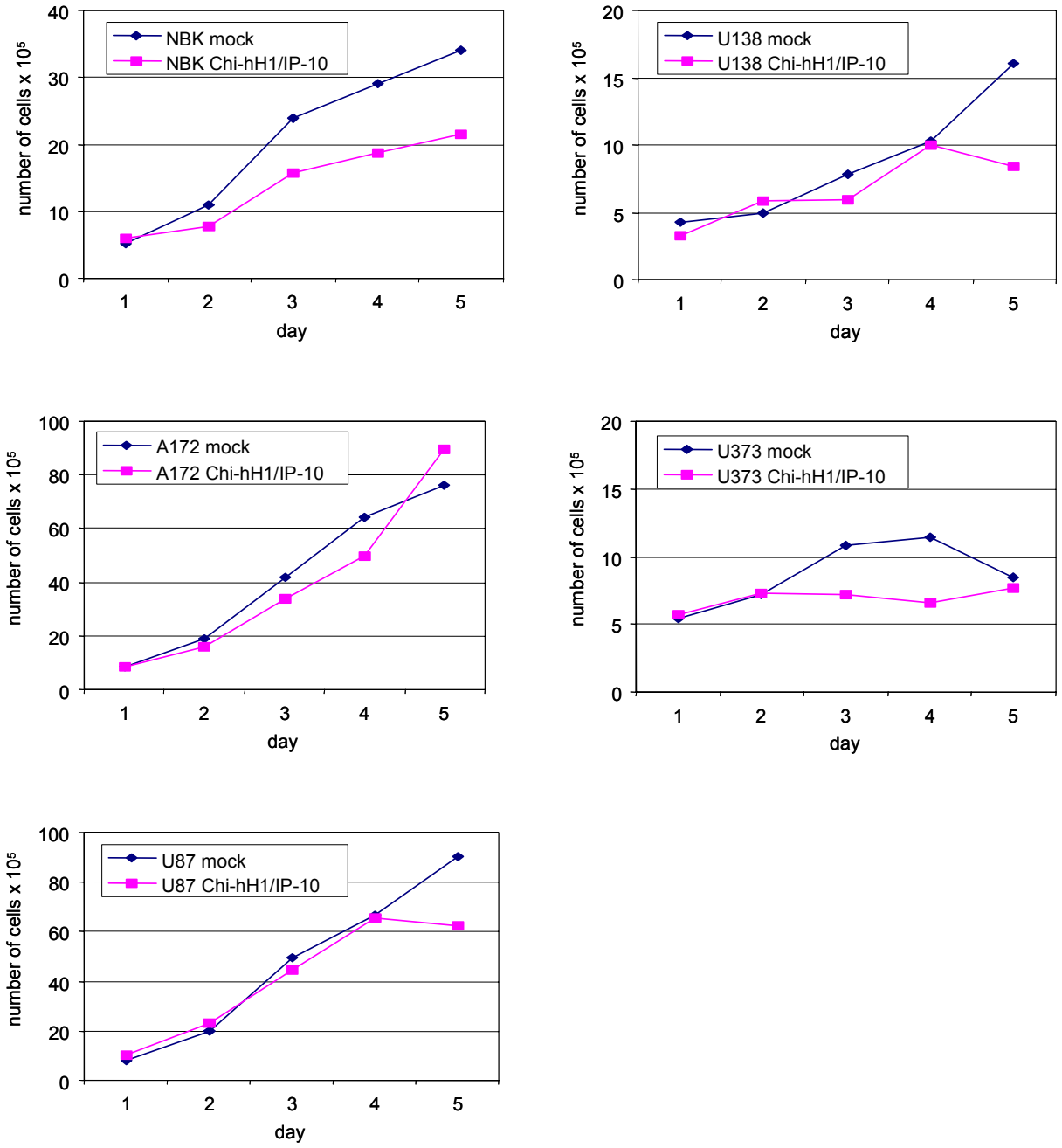


Figure 8a Growth curves of human glioma cells infected with Chi-hH1/IP-10 virus
$2x10^5$ cells were infected at MOI 1 with a Chi-hH1/IP-10 virus. The cells were counted every day using the Trypan Blue exclusion method

b)

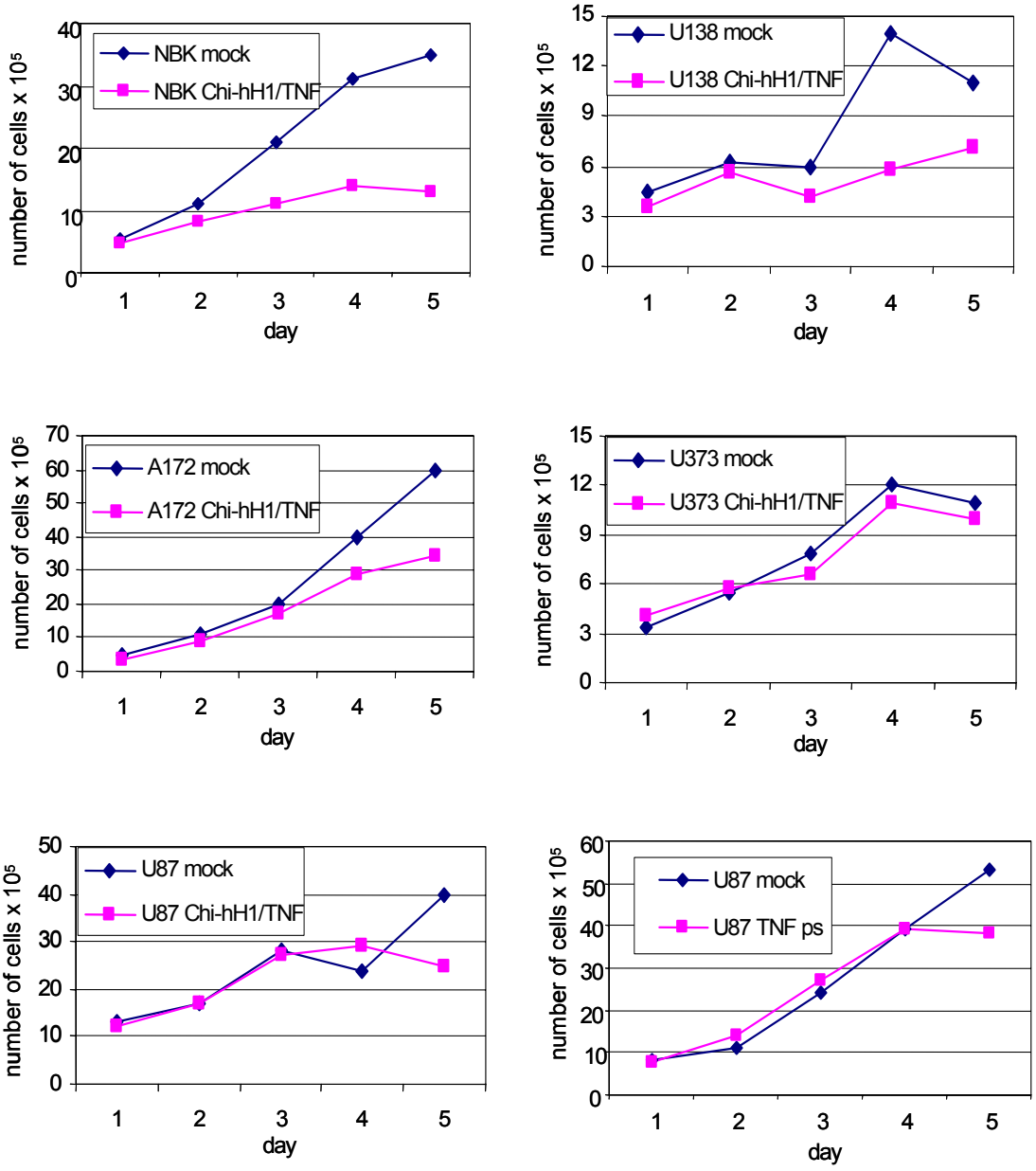


Figure 8b Growth curves of human glioma cells infected with Chi-hH1/TNF virus
$2x10^5$ cells were infected at MOI 1 with a Chi-hH1/TNF virus. The cells were counted every day using the Trypan Blue exclusion method

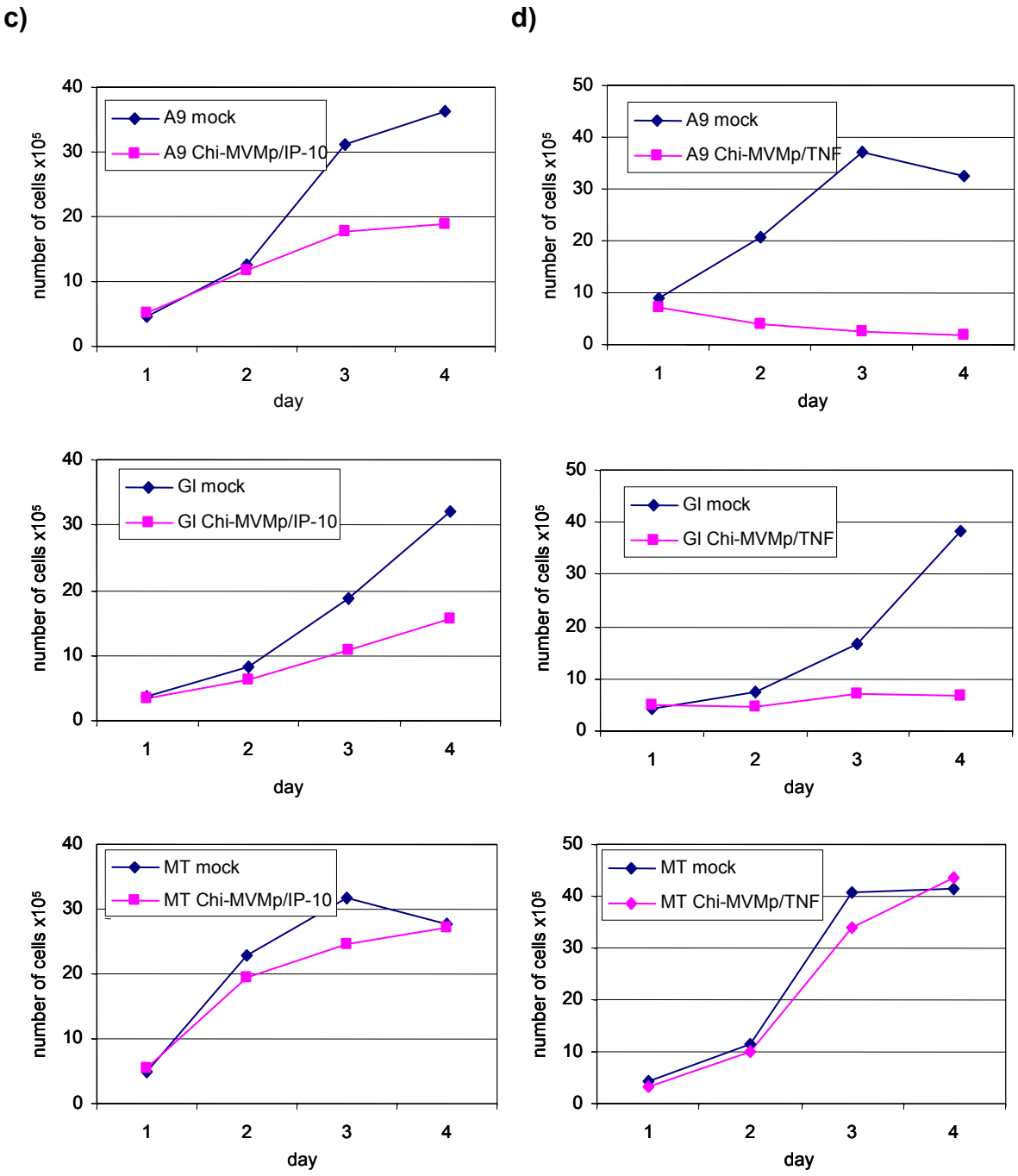


Figure 8c Growth curves of mouse glioma cells infected with Chi-hH1/IP-10 virus
$2x10^5$ cells were infected at MOI 1 with a Chi-MVMp/IP-10 virus. The cells were counted every day using the Trypan Blue exclusion method

Figure 8d Growth curves of mouse glioma cells infected with Chi-hH1/TNF virus
$2x10^5$ cells were infected at MOI 1 with a Chi-hH1/TNF virus. The cells were counted every day using the Trypan Blue exclusion method

4.5 Sensitivity of the glioblastoma cells to TNFα

One of the transgenes expressed by recombinant parvoviruses generated in this work is the mouse TNFα. This cytokine is known to have cytotoxic activity with different tumor cells and potential immunostimulatory properties (Aggarwal,B.B. 1996). A possible antitumoral effect *in vivo* could be directly caused by cytotoxic activity of TNFα or by an antitumor immune response and/or by antiangiogenesis stimulated by this cytokine

4.5.1 Cytotoxicity tests

To evaluate glioblastoma sensitivity to TNFα cytotoxicity assays were performed. For each cell line, $2x10^4$ cells per well were seeded on a 96-well plate and cultured in the presence of serial dilutions of TNFα for 24 or 48 hours. Human cells were treated with a human recombinant cytokine produced in bacteria, mouse cells with a mouse recombinant TNFα. Cells that were incubated in the medium only were set as 100% survival. The assays were developed using the AlamarBlue™ reduction method (see Figures 9a-d).

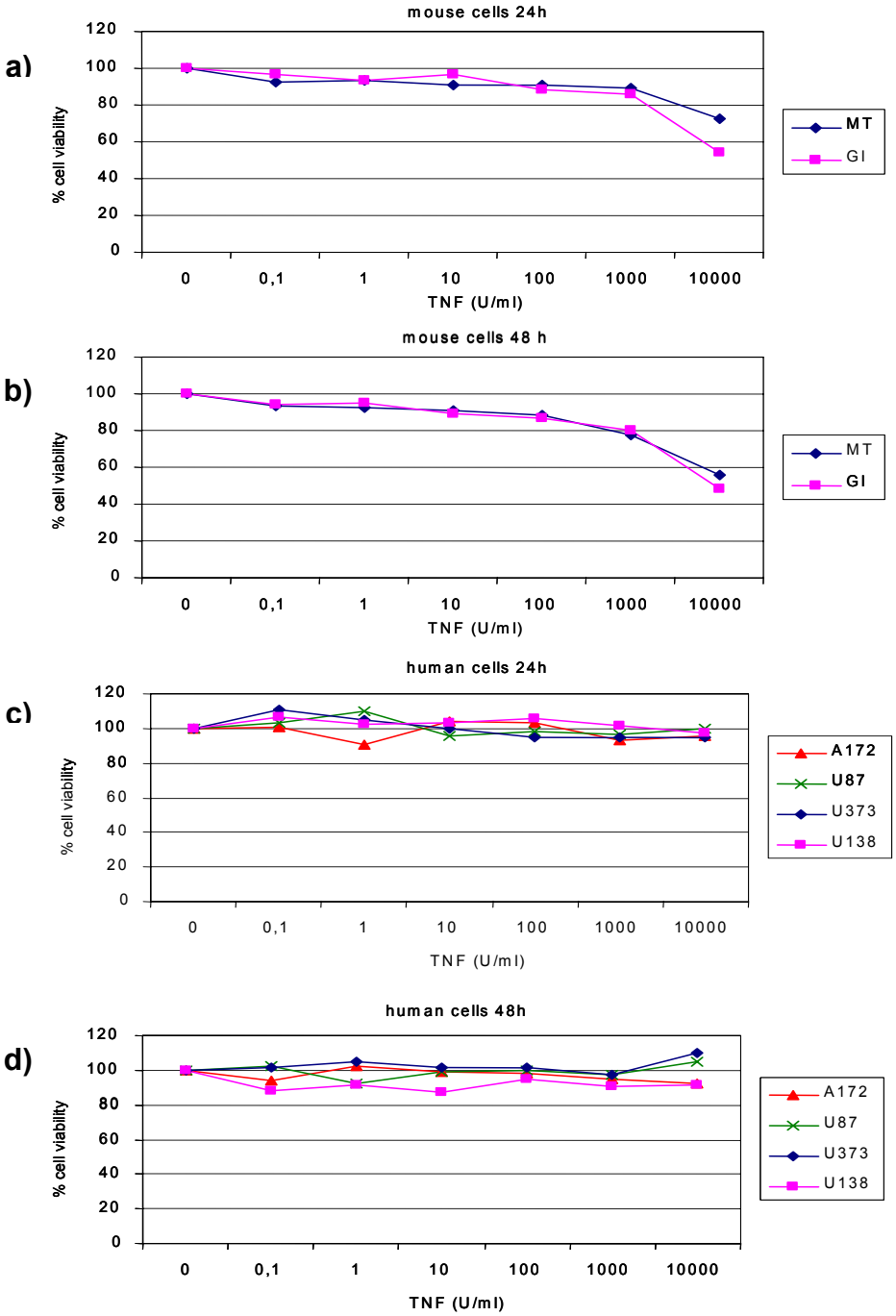


Figure 9 Sensitivity of the glioblastoma cells to TNFα
a) 24 hour cytotoxicity test on mouse glioblastoma cells. $2x10^4$ Gl261 or MT539 cells were incubated with serial dilutions of TNFα (10^{-1}-10^4 U/ml) for 24 hours. Test was developed using the AlamarBlue™ reduction method.
b) 48 hour cytotoxicity test on mouse glioblastoma cells. $2x10^4$ Gl261 or MT539 cells were incubated with serial dilutions of TNFα (10^{-1}-10^4 U/ml) for 48 hours. Test was developed using the AlamarBlue™ reduction method.
c) 24 hour cytotoxicity test on human glioblastoma cells. $2x10^4$ A172, U87, U373 or U138 cells were incubated with serial dilutions of TNFα (10^{-1}-10^4 U/ml) for 24 hours. Test was developed using the AlamarBlue™ reduction method.
d) 48 hour cytotoxicity test on human glioblastoma cells. $2x10^4$ A172, U87, U373 or U138 cells were incubated with serial dilutions of TNFα (10^{-1}-10^4 U/ml) for 48 hours. Test was developed using the AlamarBlue™ reduction method.

Murine glioblastoma cells are sensitive to cytotoxic activity of TNFα. In the presence of 10^4 U/ml cell viability was reduced to 55-70% after 24h and to 50% after 48h. This result explains also the reduction in cell number during the transgene production experiment (see 3.1.1 Figure 7d). For *in vivo* experiments this means that TNFα cytotoxicity might contribute to an antitumoral effect. Human cells used in this study are not sensitive to TNFα. Cell viability amounts to 100% for all tested cytokine concentrations during 24 and 48 hours. This suggests that the sensitivity of human cells to murine TNFα is unlikely because this cytokine from different species is known to have a reduced activity (Aggarwal,B.B. 1996). Thus, An antitumoral effect, *in vivo,* should not be caused by direct transgene toxicity, but by stimulated immune response and/or antiangiogenesis.

4.5.1.1 Human cells are not susceptible to TNFα even in the presence of protein synthesis inhibitor.

Cycloheximide, a protein synthesis inhibitor, is known to sensitize some kinds of tumor cells to TNFα cytotoxicity (Kaszubowska L. et al. 2001). The sensitization of human glioblastoma cells could produce the advantage of intrinsic antitumoral properties of the transgene.

Reference cells, A9, are sensitive to TNFα alone only at the highest concentration tested (10^4 U/ml). Cell viability is around 75%. In the presence of cycloheximide (2 µg/ml or 20 µg/ml) the cells become sensitive to TNFα at every concentration. Cytotoxic effect is dependent on the dose of cycloheximide; more pronounced at higher concentrations (see Figure 10a).

Human glioblastoma cells do not become sensitive to TNFα at any tested cycloheximide concentration. Decrease of cell viability can be observed neither at 2 µg/ml, nor at 20 µg/ml of protein synthesis inhibitor (see Figures 10b and c). The used concentration of cycloheximide decreased cell survival up to 50-80% compared to cells incubated with medium only. For that reason higher concentration of cycloheximide or a prolonged incubation time was not tested.

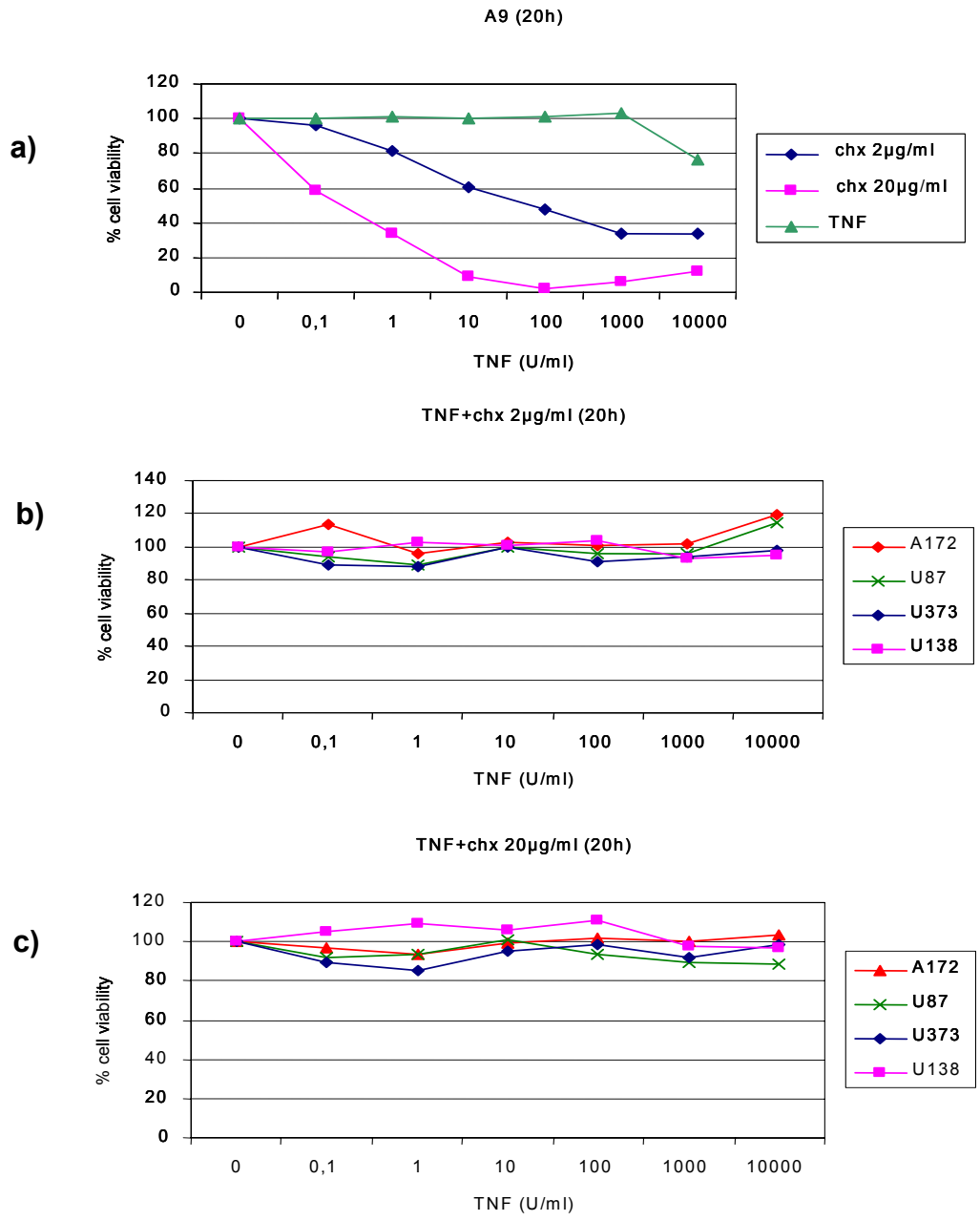


Figure 10 Cytotoxicity test in the presence of cycloheximide
a) $2x10^4$ A9 cells were incubated with serial dilutions of human TNFα (10^{-1}-10^4 U/ml) in the presence of cycloheximide (2 µg/ml or 20 µg/ml) for 20 hours. Test was developed using the AlamarBlue™ reduction method.
b) $2x10^4$ A172, U87, U373 or U138 cells were incubated with serial dilutions of human TNFα (10^{-1}-10^4 U/ml) in the presence of cycloheximide (2 µg/ml) for 20 hours. Test was developed using the AlamarBlue™ reduction method.
c) $2x10^4$ A172, U87, U373 or U138 cells were incubated with serial dilutions of human TNFα (10^{-1}-10^4 U/ml) in the presence of cycloheximide (20 µg/ml) for 20 hours. Test was developed using the AlamarBlue™ reduction method.

In conclusion these data suggest that *in vivo* sensitization of human glioma cells to cytotoxic action of TNFα by the use of cycloheximide would be not possible. An antitumoral effect *in vivo* would then result from the stimulation of the immune system or

inhibition of angiogenesis, rather than direct killing of tumor cells by parvovirus-delivered TNFα.

4.5.2 Endogenous expression of TNFα by glioblastoma cells

We showed that glioma cells were not sensitive to TNFα added in the medium (see Figure 10). Yet, many cell types can secrete TNFα. A potential endogenous secretion of this cytokine would interfere with a vector-transduced cytokine release. In order to evaluate if human glioblastoma cells can secrete significant amounts of TNFα, cell culture supernatants were collected and then applied on reference cells A9. The assay was done in the presence of cycloheximide, which makes A9 cells susceptible to human TNFα.

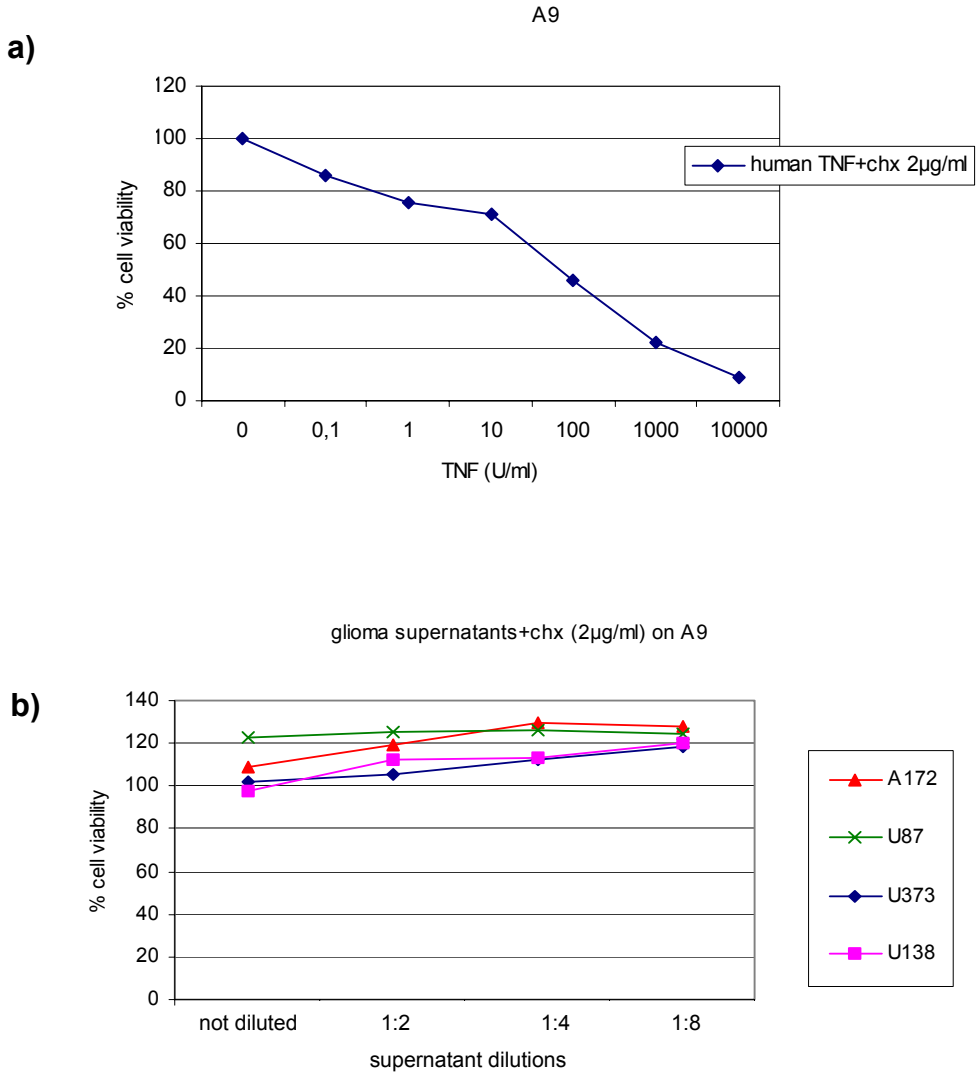


Figure 11 A9 cells do not die upon incubation with human glioblastoma cells supernatants

a) $2x10^4$ A9 cells per well were sown on a 96-well plate. A9 cells were cultured with human TNFα in the presence of cycloheximide (2 µg/ml) for 20 hours. Test was developed using the AlamarBlue™ reduction method.
b) glioblastoma supernatants were obtained from $1x10^6$ cells after 48 hours culturing. Dilutions were prepared in MEM 5% FBS medium. A9 cells were cultured with supernatants in the presence of cycloheximide (2 µg/ml) for 20 hours. Test was developed using the AlamarBlue™ reduction method.

Control A9 cells incubated with serial dilutions of human TNFα in the presence of cycloheximide (2 µg/ml) were sensitive to its cytotoxic activity. Cell viability dropped up to 10% at the highest cytokine concentration (see Figure 11a). The survival of A9 cells, incubated with dilutions of glioblastoma supernatants, remained unchanged even when undiluted supernatants were used (see Figure 11b). This suggests that tested human glioma cells do not secrete detectable (or any) amounts of TNFα.

We observed that upon infection with recombinant parvoviruses expressing TNFα glioma cells expressed high amounts of this cytokine (see Figures 7a-d). Since endogenous expression of TNFα could not be detected, potential antitumoral effects *in vivo* would be mediated by vector-transduced expression of this cytokine.

4.6 Animal experiments

4.6.1 Analysis of growth of human cells in nude mice

We showed *in vitro* that different glioblastoma cells were efficiently infected by parvoviruses (see Figure 3) and expressed high amounts of transgene proteins upon infection with recombinant vectors (see Figures 7 a-d).

We performed animal experiments in order to evaluate the potential use of parvoviruses in the gene therapy of glioblastoma. The initial characterization of parvoviral infection in glioblastoma cells was done by *in vitro* experiments. Four human cell lines were characterized: A172, U87, U373 and U138. Their high sensitivity to the parvoviral infection was shown by high percentage of infected cells and high transgene production. Among them U373and U138 cell lines were especially sensitive and therefore considered promising for animal experiments. First of all we wanted to find out the tumorigenicity of subcutaneously injected cells and identify the cell lines for use in gene therapy experiments.

4.6.1.1 U373 cells in nude mice

U373 is a human glioblastoma cell line obtained from a patient with a grade III brain tumor. This line was a good candidate for use in animal experiments due to its positive *in vitro* characteristics (high sensitivity to H1 wild type virus, high infectability and high levels of produced transgenes). $2x10^5$, $5x10^5$, $1x10^6$ and $2x10^6$ cells per animal were injected subcutaneously into the right flank of cd1 swiss nu/nu mice. Tumor formation and growth was monitored every 2-3 days.

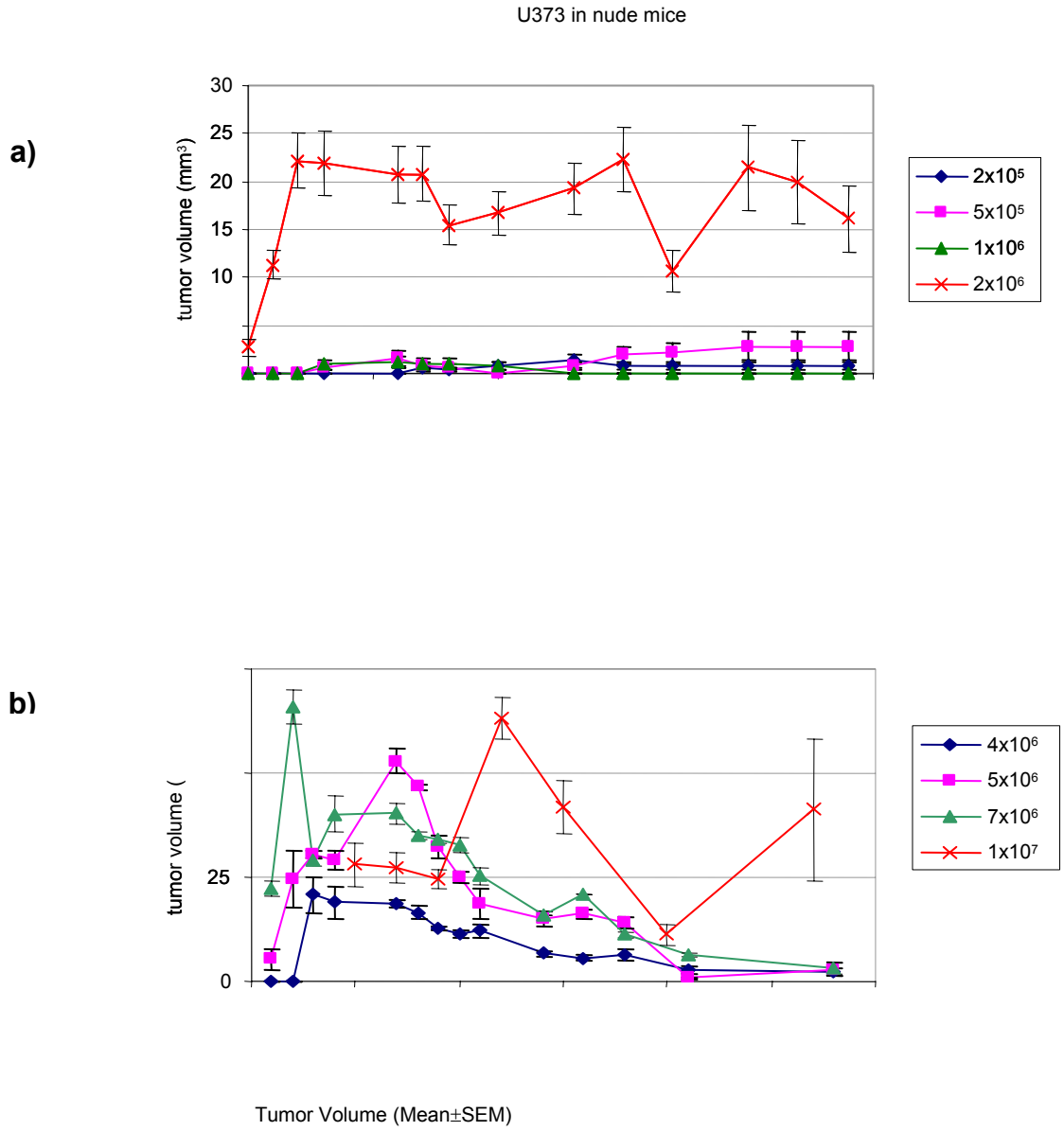


Figure 12 Tumor growth following subcutaneous injection of human U373 cells in cd1 swiss nu/nu mice
a) $2x10^5$, $5x10^5$, $1x10^6$ or $2x10^6$ cells per mouse were injected subcutaneously into the right flank. 5 animals per group were used.
b) $4x10^6$, $5x10^6$, $7x10^6$ or $1x10^7$ cells per mouse were injected subcutaneously into the right flank. 5 animals per group were used.

Injection of U373 cells did not lead to efficient tumor formation. In particular groups some animals did not develop tumors. The growth rate of tumors between individual animals was very different. Tumor formation in the animals injected with $2x10^6$ cells was very variable and lead to the development of small tumors (see Figure 12a). An increase in the number of cells to $4x10^6$, $5x10^6$, $7x10^6$ or $1x10^7$ did not allow an effective tumor growth. A strong inflammatory reaction, a few days post implantation, could be observed. It caused a rapid increase in tumor volume. After the inflammatory reaction

disappeared, the tumors systematically decreased in size until they disappeared (see Figure 12b). An increase in the mean tumor volume for the "1x10^7" group was caused by rapid tumor growth in only one animal. One month after the implantation of tumor cells, almost all animals from all groups had lost their tumors. The remaining tumors showed a decrease in volume.

Although the U373 line could be an attractive model, the lack of effective tumor formation in nude mice excludes the use of these cells in animal experiments.

4.6.1.2 U138 cells in nude mice

In vitro characteristics: high sensitivity to the virus, high percentage of infected cells and high transgene production, made the U138 cell line another potentially promising candidate for animal experiments.

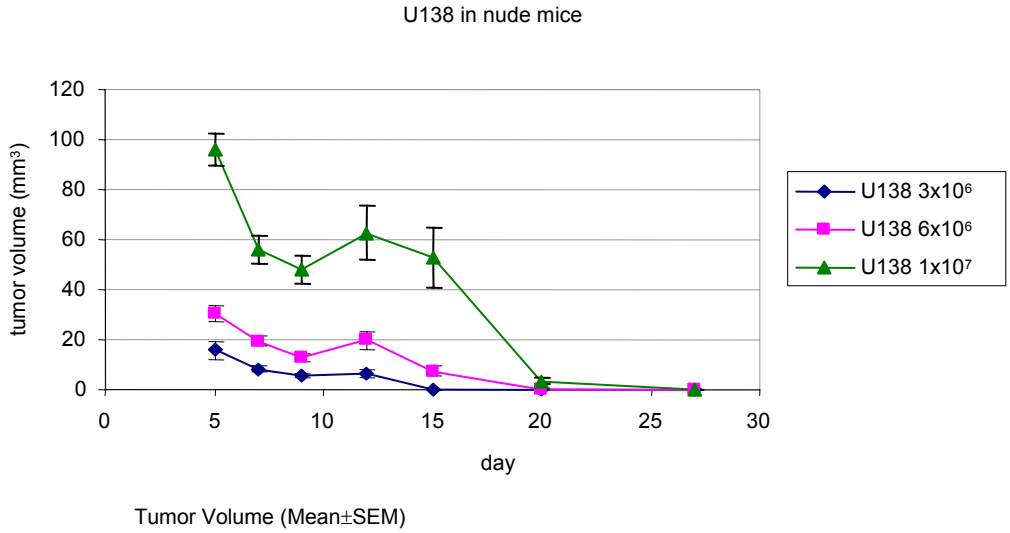


Figure 13 Tumor growth following subcutaneous injection of human U138 cells in cd1 swiss nu/nu mice
3x10^6, 6x10^6 or 1x10^7 cells per mouse were injected subcutaneously into the right flank. 5 animals per group were used.

An injection of 3x10^6, 6x10^6 or 1x10^7 tumor cells did not lead to tumor growth. Tumor volume shortly after injection was large due, not yet to the tumor, but to the injected cells. Glioblastoma cells are relatively large and 1x10^7 cells formed a cluster under the animal's skin that could be measured. However, a few days after injection the tumor volume started to reduce in size; within 3 weeks it was gone (see Figure13). The lack of effective tumor formation in nude mice excludes the use of the U138 cell line in animal experiments.

4.6.1.3 A172 and U87 cells in nude mice

After U373 and U138 cell lines were discovered not to be useful for animal experiments, A172 and U87 tumor growth in nude mice was tested.

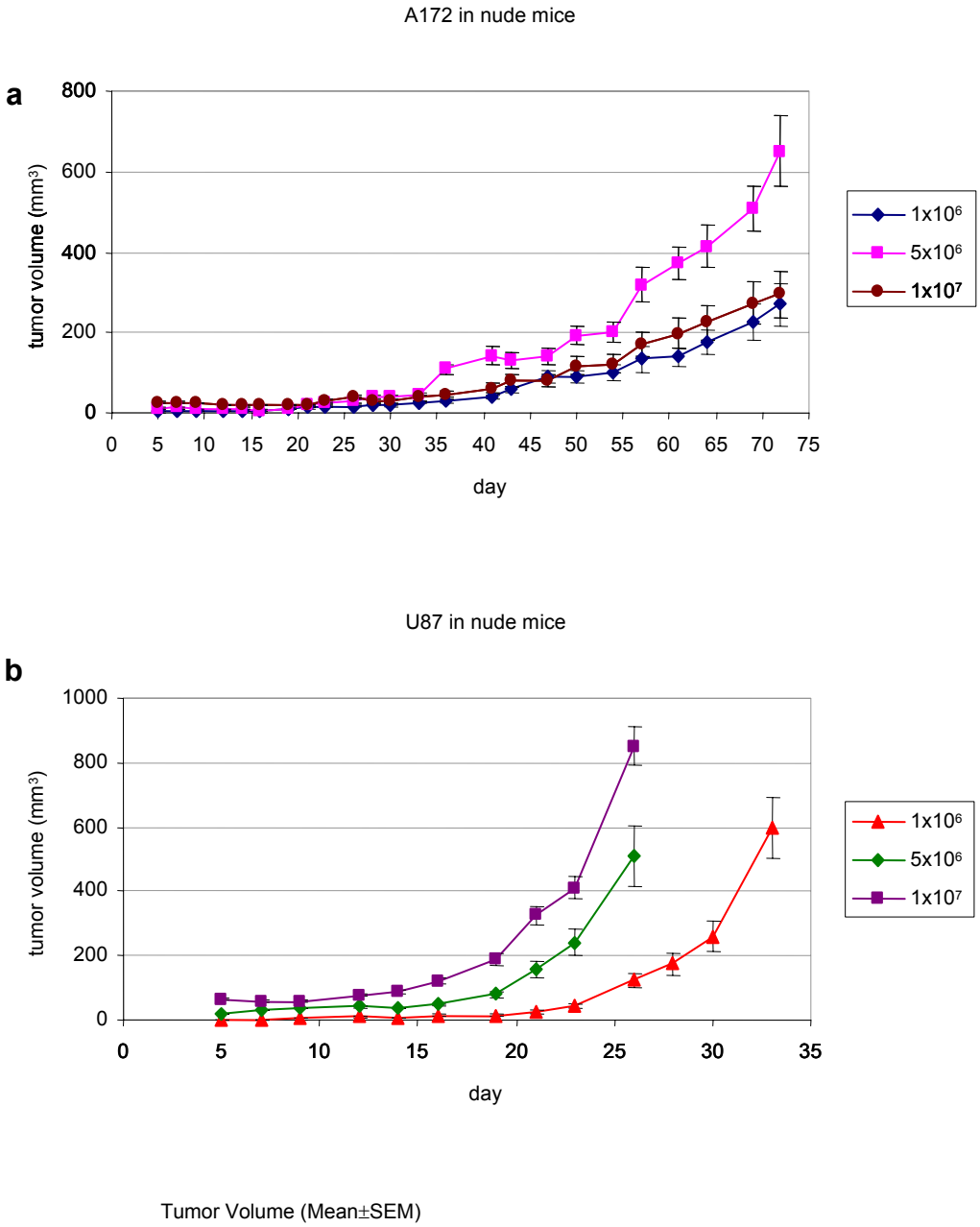


Figure 14 Tumor growth following subcutaneous injection of human A172 or U87 cells in cd1 swiss nu/nu mice
a) $1x10^6$, $5x10^6$or $1x10^7$ A172 cells per mouse were injected subcutaneously into the right flank. 5 animals per group were used.
b) $1x10^6$, $5x10^6$or $1x10^7$ U87 cells per mouse were injected subcutaneously into the right flank. 5 animals per group were used.

Injection of A172 and U87 cells subcutaneously into nude mice caused tumor growth. A172 tumors grew very slowly. All animals injected with these cells survived up to day 72 post injection. The first differences between the groups were visible from day 35. The most intensive tumor growth was observed in the "$5x10^6$" group, but not the "$1x10^7$" group, so there was no correlation between growth rate and the number of injected tumor cells (see Figure 14a). The time factor also plays an important role. It takes too long to see the differences between the groups and too long before the animals are to be sacrificed. Transgene expression induced by parvoviral vectors is transient, thus at this low growth ratio it would be difficult to see its effect. The A172 cell line, therefore, is considered not to be the optimal one for animal experiments.

An injection of U87 cells lead to a required, moderate tumor growth. The enlargement ratio was proportional to the number of implanted cells (see Figure 14b). Tumor development was similar for the animals belonging to one group. No inflammatory reaction was observed.

Based on these results the U87 cell line was considered for future animal studies where $5x10^6$ cells per animal should be implanted.

4.6.2 Antitumor effect of recombinant parvoviruses on human U87 glioblastoma cells implanted subcutaneously in nude mice

1.) For the evaluation of the antitumor effect of parvoviral vectors expressing IP-10 or TNFα, an animal experiment with *in vitro* infected cells was performed. Human U87 glioblastoma cells were infected with Chi-hH1/IP-10, Chi-hH1/Δ800, and H1 wt or with the combination Chi-hH1/IP-10+TNFα pseudotype at MOI2. For the combination MOI1+MOI1 was used. Infection was performed according to the usual protocol. At 4 hours post infection the cells were collected, washed twice with PBS, diluted to the desired number and volume in PBS and injected subcutaneously into the right flank of the animal. The control group ("mock") was injected with non-infected tumor cells. Eight cd1 swiss nu/nu mice per group were used. Figure 15 illustrates the results.

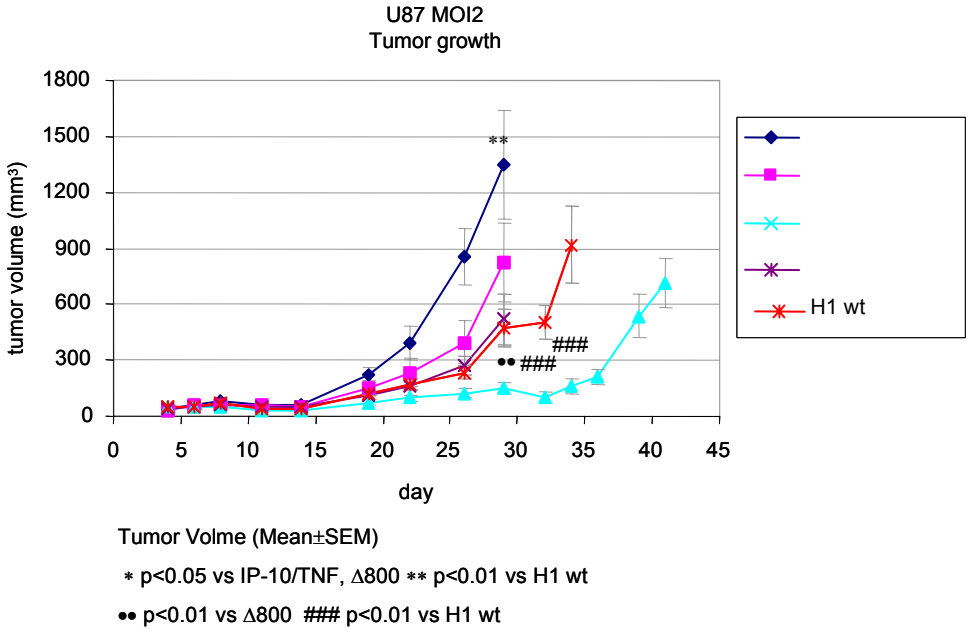


Figure 15 Tumor growth following subcutaneous injection of human U87 cells infected *in vitro* with different H1-based vectors.
$5x10^6$ U87cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-hH1/IP-10, Chi-hH1/Δ800, H1 wt or with the combination Chi-hH1/IP-10+TNF pseudotype at MOI2 (for the combination MOI1+MOI1). 8 animals per group were used.

In comparison with the mock group, a slower tumor growth may be observed for all groups, which received cells, infected with different types of virus. An intermediate reduction of growth rate appeared for “Chi-hH1/IP-10 “, “Chi-hH1/Δ800” and for “H1 wt” groups. Expression of the therapeutic transgene (IP-10) had no effect on tumor growth compared to animals treated with a vector that does not express any transgene (“Chi-hH1/Δ800”). The effect of the wild type H1 virus was very similar to the Chi-hH1/Δ800 vector (see Figure 15), mediated probably by cytotoxic action of NS1 protein. Parallel expression of IP-10 and TNFα had the best therapeutic effect, defined here as the reduced rate of tumor growth. At day 29 the mean tumor volume for “Chi-hH1/IP-10+TNF ps.” and “Chi-hH1/Δ800” group was significantly lower then the mean volume for the “mock” group. The mean tumor volume for “Chi-hH1/IP-10+TNF ps.” was also significantly lower then the value for “Chi-hH1/Δ800” and “H1 wt” groups.

Animal survival was slightly prolonged in the groups injected with infected cells. There was no significant difference between “Chi-hH1/Δ800”, “Chi-hH1/IP-10” and “H1 wt” groups. The most pronounced survival prolongation was observed for the “Chi-hH1/IP-10+TNFps” group (see Figure 16).

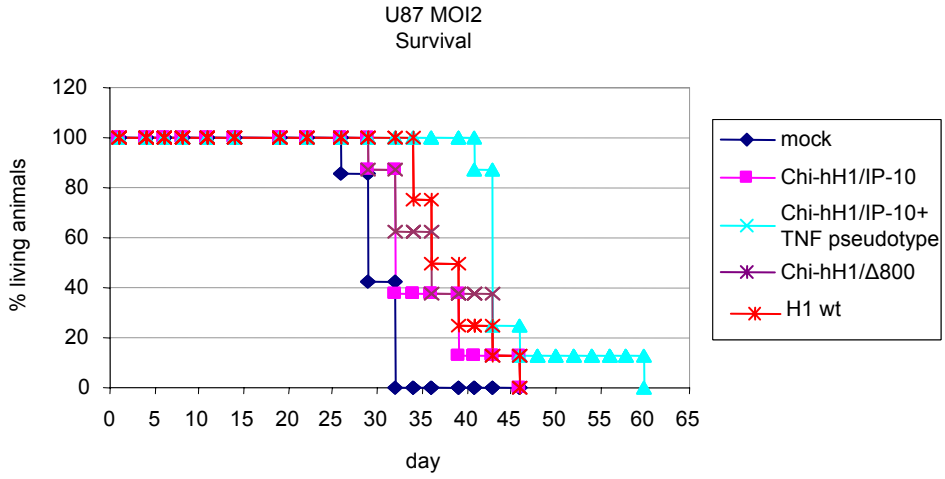


Figure 16 Animal survival after subcutaneous injection of human U87 cells infected *in vitro* with different H1-based vectors.
$5x10^6$ U87cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-hH1/IP-10, Chi-hH1/Δ800, H1 wt or with the combination Chi-hH1/IP-10+TNF pseudotype at MOI1 (for the combination MOI1+MOI1). 8 animals per group were used.

Despite the fact that a strong reduction of tumor growth was observed in the “Chi-hH1/IP-10+TNFps.” group, all animals developed tumors. The clumps of injected cells under the skin could be observed at the very beginning, and tumors started to grow very rapidly. Infection of the cells with different viruses had no effect on tumor formation but reduced their growth rate.

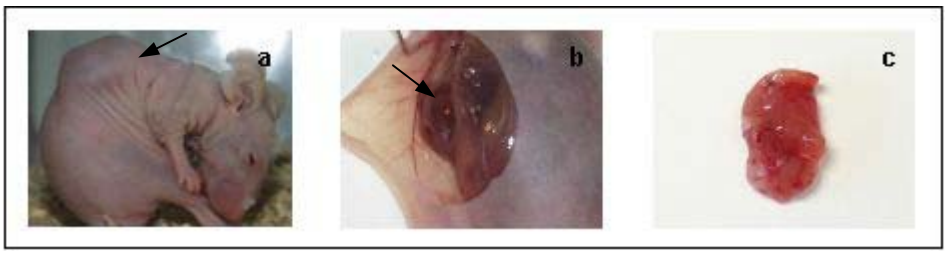


Figure 17 Subcutaneous U87 tumors in nude mice
a) tumor-bearing animal b) subcutaneously localized U87-derived tumor c) U87-derived tumor after dissection

2.) The animal experiment with *in vitro* infected cells was repeated. Human U87 glioblastoma cells were infected with Chi-hH1/IP-10, Chi-hH1/TNF, Chi-hH1/Δ800, H1 wt or with the combination Chi-hH1/IP-10+ Chi-hH1/TNF at MOI3. For the combination MOI1,5+MOI1,5 was used. Infection was performed according to the usual protocol. At 4 hours post infection the cells were collected, washed twice with PBS, diluted to the desired number and volume in PBS and injected subcutaneously into the right flank of the animal. The control group ("mock") was injected with non-infected tumor cells. Eight cd1 swiss nu/nu mice per group were used.

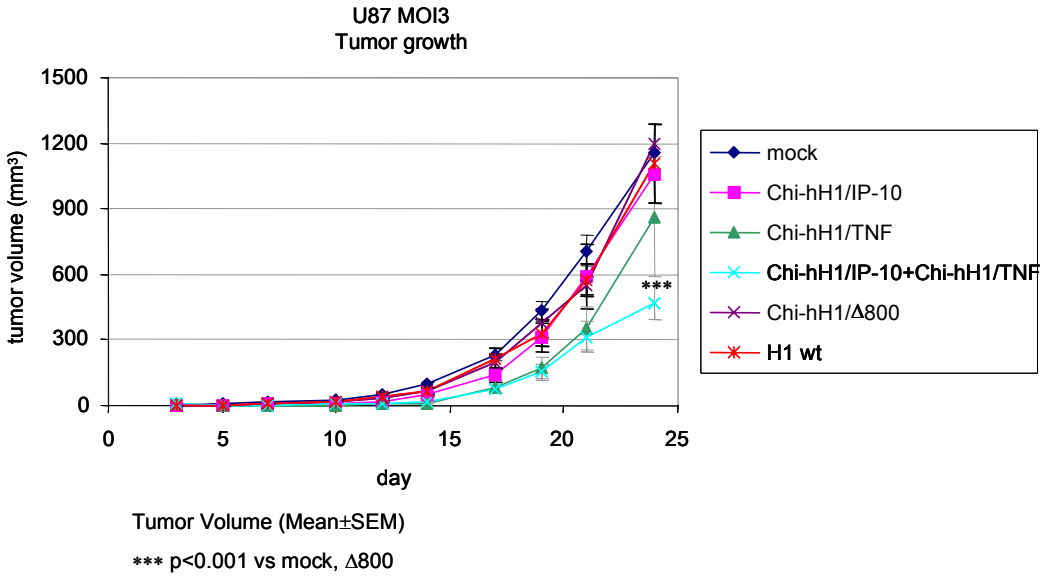


Figure 18 Tumor growth following subcutaneous injection of human U87 cells infected *in vitro* with different H1-based vectors.
$1x10^6$ U87cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-hH1/IP-10, Chi-hH1/TNF, Chi-hH1/Δ800, H1 wt or with the combination Chi-hH1/IP-10+ Chi-hH1/TNF at MOI3 (for the combination MOI1,5+MOI1,5). 8 animals per group were used.

In comparison with the mock group, a slower tumor growth may be observed for "Chi-hH1/TNF" and "Chi-hH1/IP-10+Chi-hH1/TNF" groups. Expression of the therapeutic transgene (TNF or IP-10+TNF) has effect on tumor growth compared to animals treated with a vector that does not express any transgene ("Chi-hH1/Δ800"). Like in the previous experiment, the effect of the wild type H1 virus is very similar to the Chi-hH1/Δ800 vector. Parallel expression of IP-10 and TNFα had again the best therapeutic effect, understood here as the reduced rate of tumor growth. TNFα alone had

a moderate effect on the tumor growth whereas IP-10 alone did not show any influence on tumor progression (see Figure 18).

24 days post tumor cell implantation the animals were sacrificed; the tumors isolated and underwent a histological examination (see chapter “Evaluation of the antitumoral mechanism”). At the end of the study the mean tumor volume for “Chi-hH1/IP-10+Chi-hH1/TNF” group was significantly lower then for “mock” and “Chi-hH1/Δ800” groups.

4.6.2.1 *In vitro* study with U87 cells

Parallel to the animal experiment the viruses used for the animal study were used for *in vitro* study in order to check if cytotoxic effect *in vitro* corresponds to antitumoral effect *in vivo*. $2x10^5$ U87 cells per experimental point were infected *in vitro* at MOI2 with the virus stocks used for the animal experiment. Viable cells were counted with the Trypan blue exclusion method.

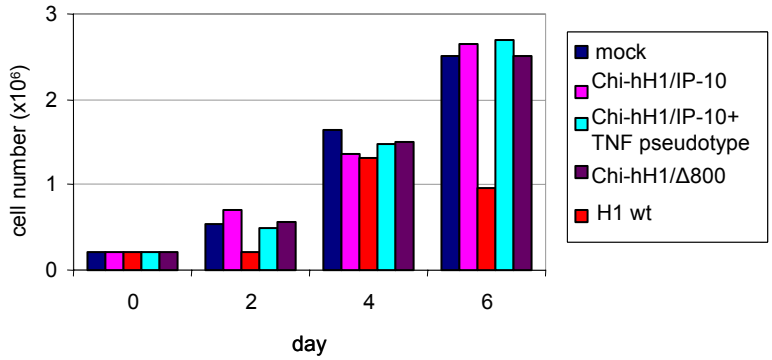


Figure 19 U87 cell growth after infection with different H1-based vectors
$2x10^5$ cells were infected with different H1-based vectors at MOI2. Chi-hH1/IP-10+TNFps. was infected at MOI 0,5+MOI 0,5. Viable cells were counted with the Trypan blue exclusion method.

Recombinant vectors at MOI2 seem not to be toxic for U87 cells. Cell number remained comparable to the number of non-infected cells (“mock”) up to day 6 post infection. This stays in agreement with other observations (see Figures 8a-b), where cytotoxic effect of IP-10 or TNFα could not be observed. The number of cells infected with the wild type H1 virus decreases from day 4 to day 6. In comparison to other experimental points, the number of cells in the “H1 wt” group, at day 6, is around 2,5 times lower. Although the H1 wt virus has a cytotoxic effect *in vitro*, in the animal experiment it has no effect.

Probably *in vivo* conditions provide factors (growth factors, components of extracellular matrix), which support tumor formation by cells that were not infected by the virus.

4.6.2.2 Transgene release in U87 cells

The cells that remained, after the animals were injected with $5x10^6$ U87 cells (infected at MOI2), were seeded on a 10 cm dish and cultured under normal conditions for 4 days. Afterwards the supernatants were collected and analyzed, by ELISA, for the presence of IP-10 and TNFα.

sample	IP-10 conc. (ng/2x10^5 cells)	TNFα conc. (ng/2x10^5 cells)
mock	not detected	not detected
Chi-hH1/Δ800	not detected	not detected
Chi-hH1/IP-10	>500	not detected
Chi-hH1/IP-10+TNFps.	98	>100
H1 wt	not detected	not detected

Table 3 IP-10 and TNFα concentrations in supernatants of U87 cells used for animal experiment.

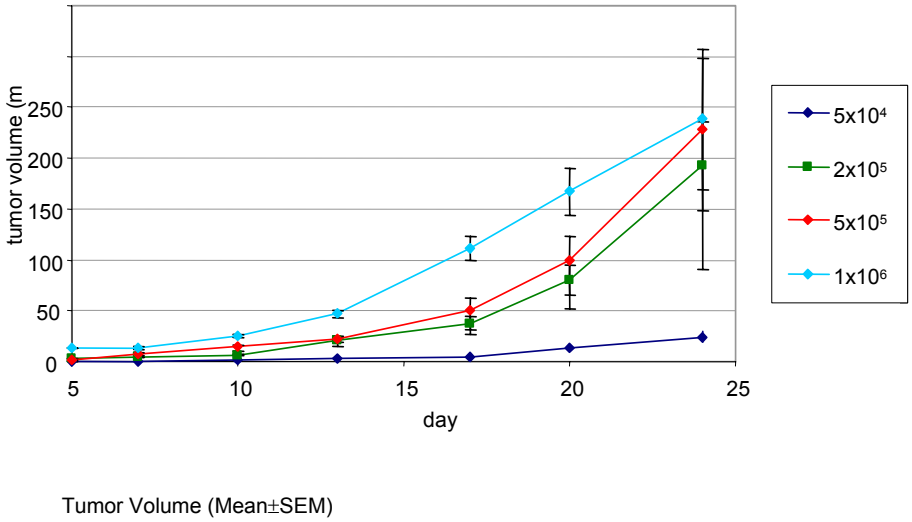
Cells infected with the combination of IP-10 /TNFα expressing viruses secreted both transgene proteins (see Table 3). Cells infected with either IP-10- expressing virus secreted high amount of IP-10. Neither H1 wild type virus nor basic Δ800 vector were able to induce IP-10 or TNFα expression in U87 cells. Obtained results suggest that transgene expression *in vivo* was not impaired.

4.6.3 Antitumoral effect of recombinant parvoviruses on mouse Gl261 glioblastoma cells implanted subcutaneously into immunocompetent mice

In order to assess the antitumoral potential of recombinant parvoviruses expressing IP-10 or TNFα animal experiments were performed. The characterization of parvoviral infection in glioblastoma cells was done by *in vitro* experiments. Among characterized mouse cell lines Gl261 seems to be the appropriate one for use in animal experiments. Cells of this line are very sensitive to the infection with MVMp wild type virus (see Figure 1; Table 1), are infectable to a high percentage (see Figure 3), and produce transgene proteins in high levels (see Figure 7 c-d). This aside Gl261 cells come from a well-defined genetic background – the C57/Bl6 mouse strain.

4.6.3.1 Gl261 cells in C57/Bl6 mice

The Gl261 cell line was originally established from C57/Bl6 mice in 1939 by chemical carcinogenesis (Seligman A.M., Shear M.J. 1939). We determined how many tumor cells, injected subcutaneously, would lead to moderate tumor growth. $5x10^4$, $2x10^5$, $5x10^5$ or $1x10^6$ cells per mouse were injected subcutaneously into the right flank and tumor growth was monitored (see Figure 20).



Tumor Volume (Mean±SEM)

Figure 20 Tumor growth following subcutaneous injection of Gl261 cells into C57/Bl6 mice
$5x10^4$, $2x10^5$, $5x10^5$ or $1x10^6$ cells per mouse were injected subcutaneously into the right flank. 5 animals per group were used

All injected tumor cell doses lead to tumor growth. It was slow for the group injected with $5x10^4$ cells. $2x10^5$ and $5x10^5$ injected cells lead to a similar, moderate effect. The fastest growth could be observed for the "$1x10^6$" group. Therefore the correlation between the number of injected tumor cells and the growth rate can be concluded. For future animal experiments $2x10^5$ and $5x10^5$ cells per animal seems to be the most appropriate.

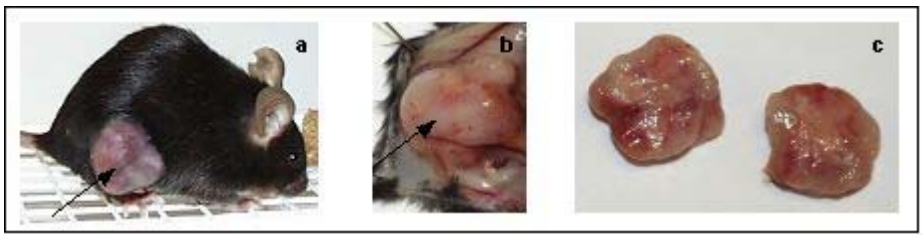


Figure 21 Subcutaneous Gl261-derived tumors in C57/Bl6 mice
a) tumor-bearing animal b) subcutaneously localized Gl216-derived tumor c) Gl261-derived tumor after dissection

4.6.3.2 Animal study with Gl261 cells infected *in vitro* at MOI1

Having established the conditions where all the animals developed tumors after injection of Gl261 cells, we wanted to evaluate the antitumoral effect of recombinant MVMp-based vectors. Mouse glioblastoma Gl261 cells were infected *in vitro* with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI1. (For the combination MOI 0,5+MOI 0,5 was used.) Infection was performed according to the usual protocol. The cells, 4 hours post infection, were collected, washed twice with PBS, diluted to the desired number and volume in PBS and injected subcutaneously into the right flank of the animal. A control group ("mock") was injected with non-infected tumor cells. Eight C57/Bl6 mice per group were used.

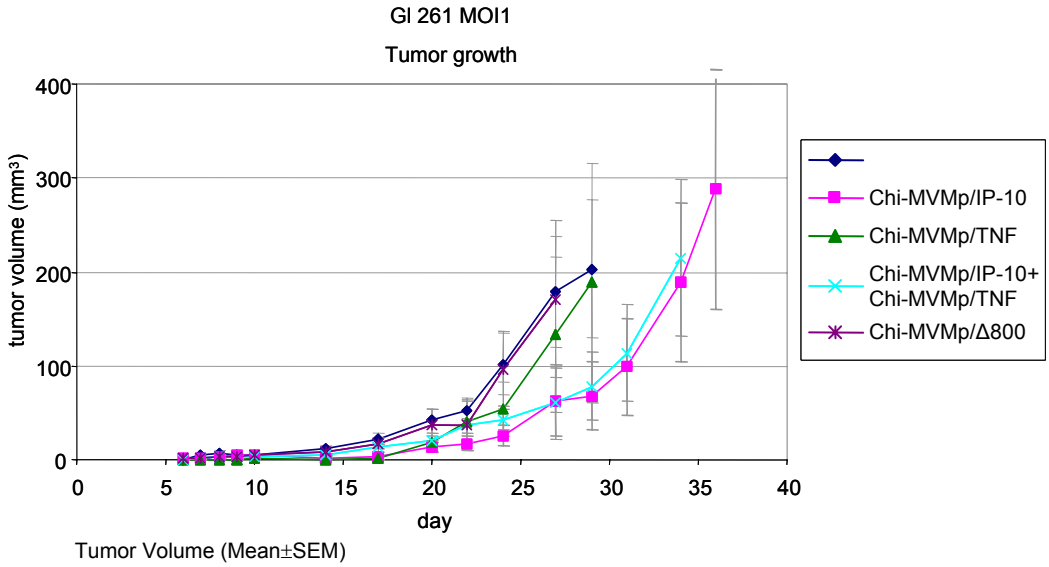


Figure 22 Tumor growth following subcutaneous injection of mouse Gl261 cells infected *in vitro* with different MVMp-based vectors.
$3x10^5$ Gl261cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI1 (for the combination MOI 0,5+MOI 0,5). 8 animals per group were used.

The antitumoural effect of recombinant vectors was moderate. The control vector, Chi-MVMp/Δ800, had no effect on tumor expansion. The growth rate of tumors derived from Chi-MVMp/Δ800 infected cells was the same as for tumors derived from non-infected cells ("mock" group).

A delay in the tumor growth could be observed for "Chi-MVMp/IP-10", "Chi-MVMp/TNF" and "Chi-MVMp/IP-10+Chi-MVMp/TNF" groups. This effect was less pronounced for the "Chi-MVMp/TNF" group. Tumor enlargement was similar for the "Chi-MVMp/IP-10" and "Chi-MVMp/IP-10+Chi-MVMp/TNF" groups. It is therefore likely that, the main role in reduced tumor expansion was due to the IP-10 expression since Chi-MVMp/TNF alone had only marginal effect. Although tumors derived from cells treated with transgene-containing vectors started to grow later then control tumors, in general, tumor growth rates were similar for all experimental groups (see Figure 22). It may be due to the fact that, at MOI1 expression of the therapeutic transgene did not reach a level that was high enough to mediate a strong antitumor effect *in vivo*. Indeed at MOI3 the antitumor effect was much more pronounced, including inhibition of tumor development and growth (see Figure 28).

Infection of tumor cells had no pronounced effect on early tumor development. Tumors appeared in all groups over a similar time period. Only in the “Chi-MVMp/TNF” group tumors appeared later and 3 out of 8 animals remained tumor-free. In the “mock” group 1 animal remained tumor-free, in the “Chi-MVMp/IP-10” group 1, in the “Chi-MVMp/IP-10+Chi-MVMp/TNF” group 1, and in the “Chi-MVMp/Δ800” group 2 (see Figure 23).

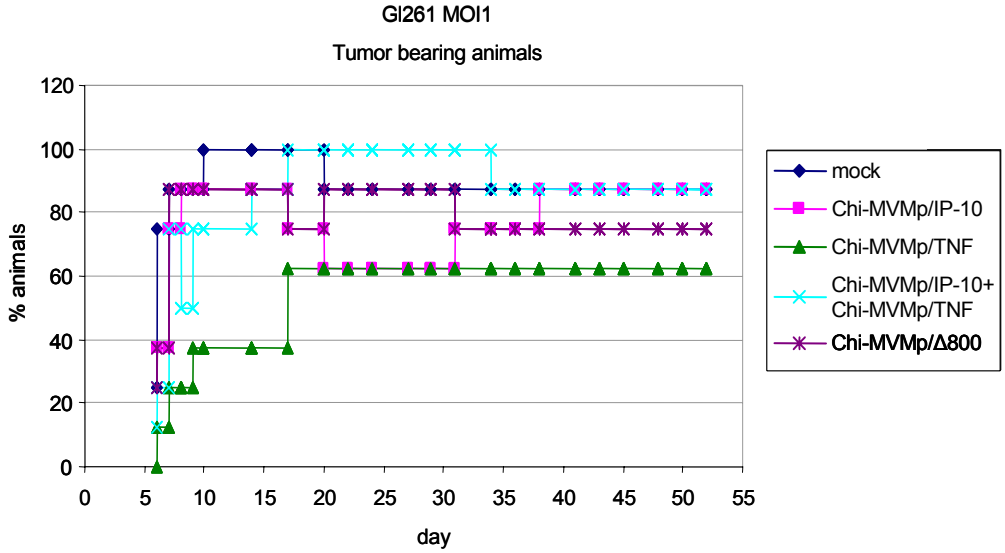


Figure 23 Tumor development after subcutaneous injection of mouse Gl261 cells infected *in vitro* with different MVMp-based vectors.
$3x10^5$ Gl261cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI1 (for the combination MOI 0,5+MOI 0,5). 8 animals per group were used.

The pattern of tumor development does not correspond to the later tumor growth. Although in “Chi-MVMp/IP-10” and “Chi-MVMp/IP-10+Chi-MVMp/TNF” groups almost all animals developed the tumors (7 out of 8 and 8 out of 8, respectively), they grew slower compared to the other groups. In “Chi-MVMp/TNF” group 3 out of 8 animals remained tumor free, but other animals developed rapidly growing tumors.

Animals injected with cells infected with recombinant vectors survived longer than the control group (see Figure 24). There are no statistically significant differences in survival among treated groups ($p>0.05$). Generally, infecting tumor cells at MOI 1 prior to subcutaneous implantation prolongs animal survival, slightly, compared to the control group.

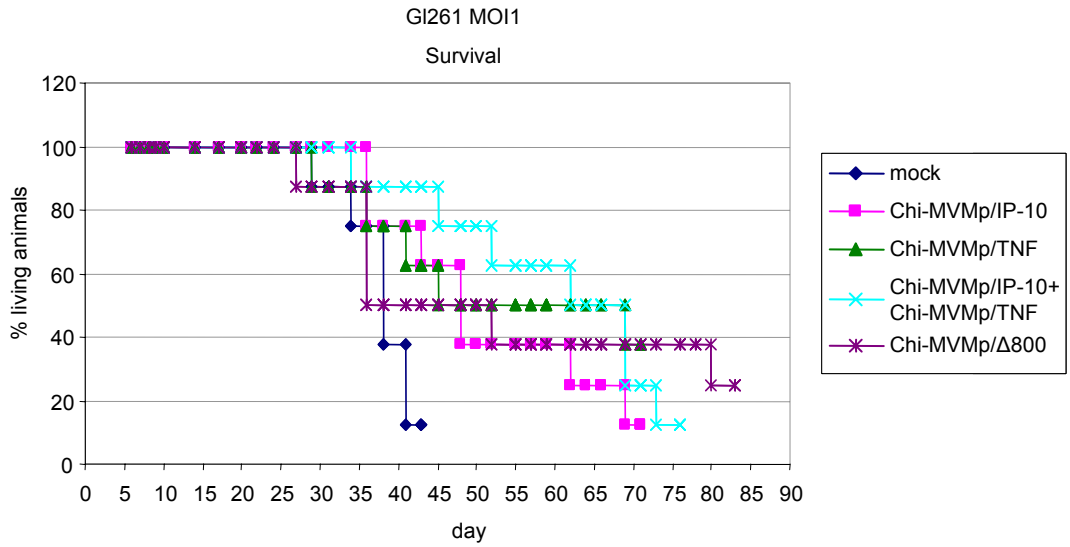


Figure 24 Animal survival after subcutaneous injection of mouse Gl261 cells infected *in vitro* with different MVMp-based vectors.
$3x10^5$ Gl261cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at (the) MOI1 (for the combination MOI 0,5+MOI 0,5). 8 animals per group were used.

4.6.3.2.1 *In vitro* study with Gl261 cells

In order to compare the antitumor effects observed in the animals with the effects induced in the cell culture $2x10^5$ Gl261 cells per experimental point were infected *in vitro* at MOI1 with the virus stocks used for the animal experiment. Viable cells were counted with the Trypan blue exclusion method (see Figure 25).

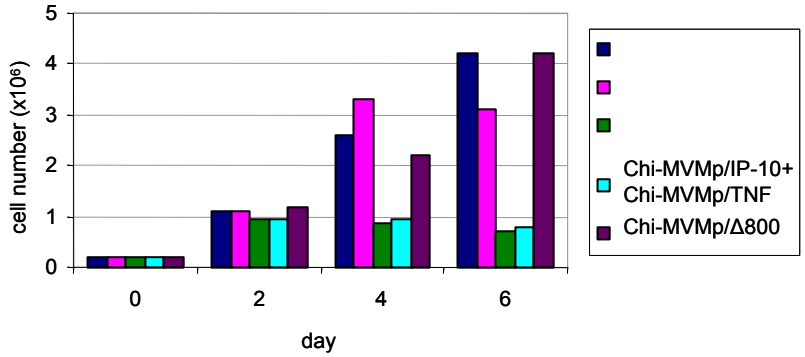


Figure 25 Gl261 cell growth after infection with different MVMp-based vectors.
$2x10^5$ cells were infected with different MVMp-based vectors at MOI1. Chi-MVMp/IP-10+Chi-MVMp/TNF was infected at MOI 0,5+MOI 0,5. Viable cells were counted with the Trypan blue exclusion method.

Recombinant vectors at MOI1 have different effects on the growth of Gl261 cells *in vitro*. Chi-MVMp/IP-10 and Chi-MVMp/Δ800 did not seem to be toxic. Cell number is similar whether cells were virus-infected or not ("mock") up to day 6 post infection (with a slight decrease for Chi-MVMp/IP-10 at day 6). The number of cells infected with the Chi-MVMp/TNF virus or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF decreases from day 2 to day 6. Infection with Chi-MVMp/IP-10 alone had no cytotoxic effect, so it seems likely that cell death is caused by the Chi-MVMp/TNF infection. This may be because Gl261 cells begin to produce high amounts of TNFα 2 days post infection (see Figure 7d) and these cells are TNFα-sensitive (see Figure 9 a-b). Although Chi-MVMp/TNF virus had a cytotoxic effect *in vitro*, in the animal experiment it had no strong antitumoral effect. It is possible that TNFα produced *in vivo* diffused within the tumor mass and tumor surrounding and therefore its local concentration was not high enough to mediate strong antutumor effect. *In vivo* conditions could also provide factors (growth factors, components of extracellular matrix), which supported tumor growth.

4.6.3.2.2 Challenge experiment

In order to verify if surviving animals developed immunity against tumor cells, a challenge experiment was performed. After the implantation of tumor cells some animals remained tumor-free:

“mock”	1 animal
“Chi-MVMp/IP-10”	1 animal
“Chi-MVMp/TNF”	3 animals
“Chi-MVMp/IP-10+Chi-MVMp/TNF”	1 animal
“Chi-MVMp/Δ800”	2 animals

57 days after the first injection these animals were challenged with $3x10^5$ Gl261 cells implanted subcutaneously into the left flank (opposite to the first implantation). As a control group 8 untreated animals from the same delivery were used.

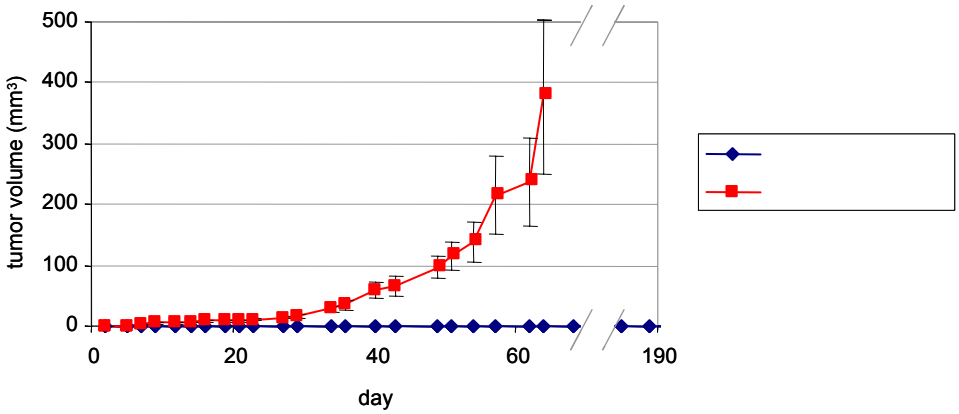


Figure 26 Challenge experiment
Animals were challenged with $3x10^5$ Gl261 cells implanted subcutaneously into the left flank. Control animals received $3x10^5$ Gl261 cells subcutaneously into the right flank.

None of the challenged animals developed tumors. They remained tumor-free over 187 days from the second injection. This suggests a long-lasting antitumoral activity in these animals, probably due to the development of tumor-reactive clones, what was subsequently verified by ELISpot analysis. 3 out of the 8 control animals were also tumor-free. The animals were sacrificed and the spleens, used for ELISpot analysis.

4.6.3.2.3 ELISpot analysis

The secretion of IFNγ after the stimulation of spleen cells with tumor cells (or tumor antigens) demonstrates that the cells specifically recognized tumor cells and exerted specific antitumoral activity. The splenic lymphocytes were separated for ELISpot analysis. The spleenocytes were cultured with irradiated tumor cells (Gl261) on the anti-IFNγ antibody-coated ELISpot plate at 37°C for 20 hours.

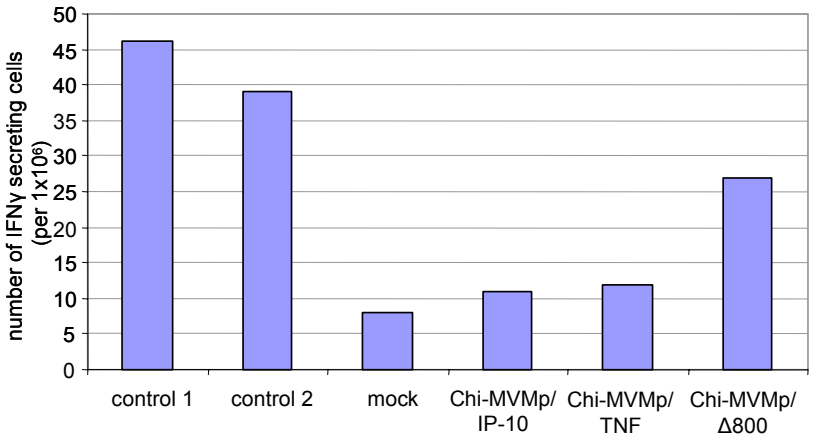


Figure 27 Number of IFNγ-producing cells for challenged animals
Spleenocytes of single animals from different groups were used for ELISpot analysis. As specific stimulus $2x10^4$ irradiated Gl261 cells were used.

The number of cells that secrete IFNγ upon stimulation with Gl261 cells was different for particular animals. Control animals (injected once with tumor cells) showed a higher number of specifically stimulated cells than challenged animals (which were injected twice with tumor cells). Even though the challenged animals belong to different groups there was no big difference between the numbers of IFNγ-secreting cells for each group. The results demonstrated that, C57/Bl6 mice developed immune cells that recognize Gl261 tumor cells.

Formation of antitumoral clones might be higher when naive animals are treated with tumor cells for the first time. It looks unlikely that a second administration of tumor cells would lead to an increase in the number of detectable antitumoral clones.

The age of the animals can also have some influence. Control animals, which received tumor cells for the first time, were 40 weeks old. Challenged animals during the first contact with tumor cells were 6 weeks old. It is possible that in adult animals the formation of tumor-reactive clones is more efficient than in young animals.

4.6.3.3 Animal study with Gl261 cells infected *in vitro* at MOI3

1.) In the previously described study tumor cells were infected at MOI 1 prior to implantation. To increase the antitumoral effect, in the second experiment of this type, tumor cells were infected at MOI 3. Mouse glioblastoma Gl261 cells were infected with Chi-MVMp/IP-10, Chi-MVMp/TNF and Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI 3. For the combination MOI 1,5+MOI 1,5 was used. Infection was performed according to the usual protocol. 4 hours post infection the cells were collected, washed twice with PBS, diluted to the desired number and volume in PBS and injected subcutaneously into the right flank of the animal. A control group ("mock") was injected with non-infected tumor cells. Eight C57/Bl6 mice per group were used.

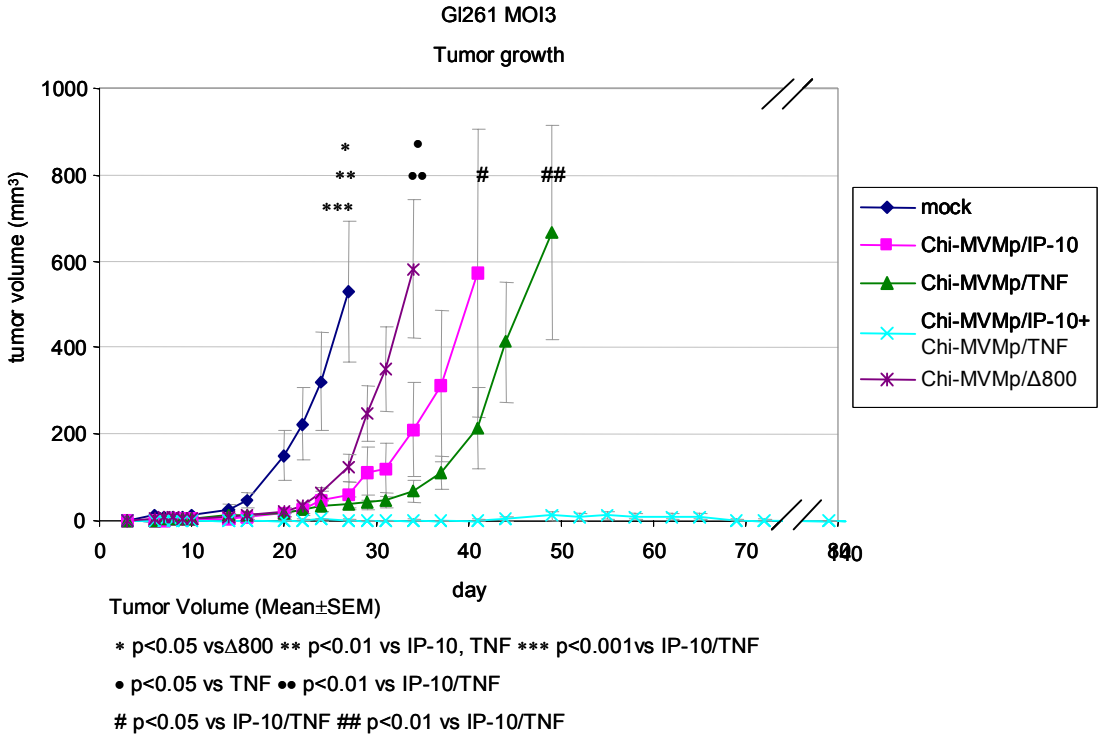


Figure 28 Tumor growth following subcutaneous injection of mouse Gl261 cells infected *in vitro* with different MVMp-based vectors at MOI 3.
$3x10^5$ Gl261cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI3 (for the combination MOI 1,5+MOI 1,5). 8 animals per group were used.

As expected, antitumoral effect of recombinant vectors was more pronounced than in the case of infection at MOI1. A delay in tumor growth could be observed for "Chi-MVMp/Δ800", "Chi-MVMp/TNF" and "Chi-MVMp/IP-10" groups. The control vector

(Chi-MVMp/Δ800) had the weakest effect and can be assigned to the cytotoxicity of the vector. Vectors containing therapeutical transgenes caused a more pronounced delay of tumor growth. For the “Chi-MVMp/TNF” group this effect was stronger than for the “Chi-MVMp/IP-10” group. Strikingly, almost complete inhibition of tumor growth was observed for the “Chi-MVMp/IP-10+Chi-MVMp/TNF” group. 4 out of 8 animals developed very small tumors that disappeared by day 72 post injection (see Figure 28). The parallel expression of IP-10 and TNFα in tumor cells could provide optimal conditions for the development of antitumoral immunity and/or antiangiogenesis.

At the day 27 the mean tumor volume for “mock” group was significantly higher compared to all another groups. At the day 34 this value for “Chi-MVMp/Δ800” was significantly higher compared to “Chi-MVMp/TNF” and “Chi-MVMp/IP-10” groups. At the day 41 and 49 mean tumor volume for “Chi-MVMp/IP-10+Chi-MVMp/TNF” group was significantly lower compared to “Chi-MVMp/IP-10” and “Chi-MVMp/TNF” groups, respectively.

As shown on Figure 29, tumors appeared in all groups over a similar time period. All animals injected with non-infected cells (“mock”) developed tumors. A number of animals remained tumor-free: in the “Chi-MVMp/Δ800” group 1, in the “Chi-MVMp/IP-10” group 2 and in the “Chi-MVMp/TNF” group 1. In the combination “Chi-MVMp/IP-10+Chi-MVMp/TNF” group 4 out of 8 animals developed tumors early after the implantation of cells. Afterwards, the number of tumor-bearing animals in this group was variable, but never higher than 4. Two months after cell implantation tumors in this group started to disappear and the last measurement was done 72 days post injection. All the animals in this group remained tumor-free for more than 140 days post implantation (see Figure 29).

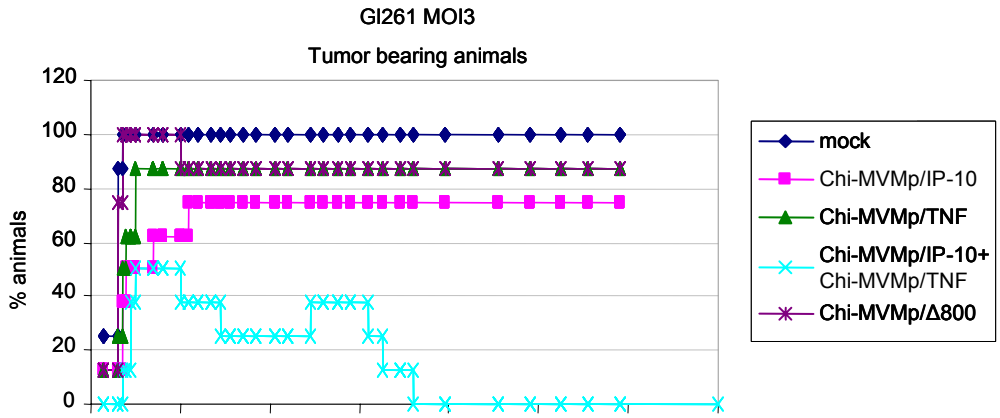


Figure 29 Tumor development after subcutaneous injection of mouse Gl261 cells infected *in vitro* with different MVMp-based vectors at MOI 3.
$3x10^5$ Gl261cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI3 (for the combination MOI 1,5+MOI 1,5). 8 animals per group were used.

This striking effect observed in the group treated with the combination of IP-10- and TNFα-expressing viruses could be mediated both on the stage of tumor formation (4 out of 8 animals did not develop the tumors) and later (tumors disappeared within 72 days). To the best of our knowledge, this is the first example where recombinant viruses expressing a cytokine and a chemokine induced regression of established tumors.

This was reflected on the survival of animals as shown on Figure 30.

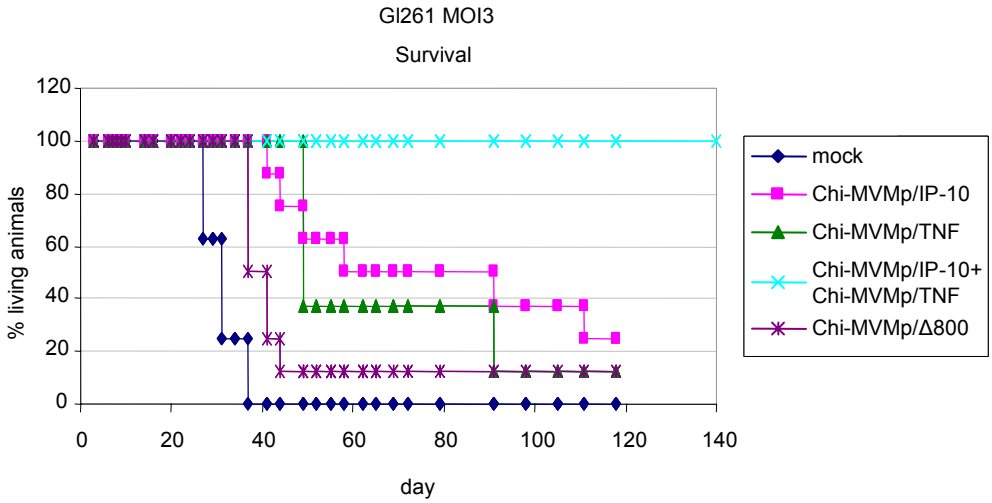


Figure 30 Animal survival after subcutaneous injection of mouse Gl261 cells infected *in vitro* with different MVMp-based vectors at MOI 3.
$3x10^5$ Gl261cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI 3 (for the combination MOI 1,5+MOI 1,5). 8 animals per group were used.

The survival of animals injected with cells infected with the control vector (Chi-MVMp/Δ800) was slightly prolonged compared to the control group ("mock"). More pronounced survival prolongation was observed for the "Chi-MVMp/IP-10" and "Chi-MVMp/TNF" (and "Chi-MVMp/IP-10") groups. The "Chi-MVMp/IP-10+Chi-MVMp/TNF" group showed 100% survival over 140 days post injection (showed the "Chi-MVMp/IP-10+Chi-MVMp/TNF" group) (see Figure 30). Survival curve for "mock" group differs significantly from all other groups (p=0.001). Infection with recombinant parvoviruses at MOI3 prolonged animal survival, although the less pronounced effect could be observed for the control vector. Statistically significant difference was also measured between the curve for "Chi-MVMp/IP-10+Chi-MVMp/TNF" group and all other curves (vs "mock" p=0.001; vs "Chi-MVMp/IP-10" p=0.002; vs "Chi-MVMp/TNF" p=0.005;
vs "Chi-MVMp/Δ800" p=0.004). Treatment with the combination of IP-10- and TNFα-expressing vectors at MOI3 strikingly prolonged animal survival.

4.6.3.3.1 *In vitro* study with Gl261 cells

As for the experiment performed at MOI1 (see Figure 25) the viability of Gl261 cells infected at MOI3 was evaluated *in vitro.* Viable cells were counted with the Trypan blue exclusion method (see Figure 31).

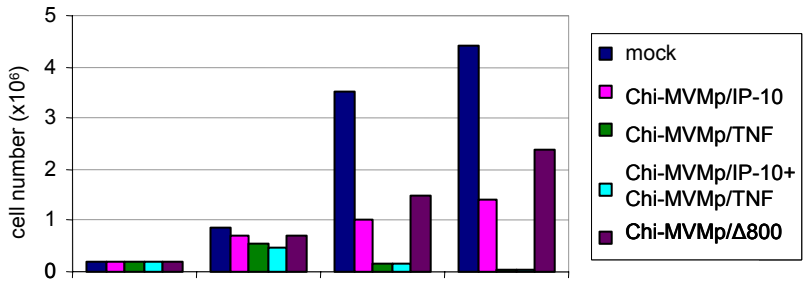


Figure 31 Gl261 cell growth after infection with different MVMp-based vectors.
$2x10^5$ cells were infected with different MVMp-based vectors at MOI3. The combination Chi-MVMp/IP-10+Chi-MVMp/TNF was infected at MOI 1,5+MOI 1,5. Viable cells were counted with the Trypan blue exclusion method.

The virus toxicity was more pronounced at MOI 3 than at MOI 1. The number of viable cells infected with the control vector (Chi-MVMp/Δ800) was less (about 2 fold) in comparison to non-infected cells ("mock"). It is likely to be due to the cytotoxic properties of the NS1 protein. The number of Chi-MVMp/IP-10 - infected cells was about 4 times less than the non-infected cells. However, cell populations infected with Chi-MVMp/TNF or Chi-MVMp/IP-10+Chi-MVMp/TNF viruses dropped dramatically from day 2 post infection. This could be explained by a high TNF release upon infection with the Chi-MVMp/TNF vector and sensitivity of Gl261 cells to the cytotoxic action of TNFα.

4.6.3.3.2 Clonogenicity assay

As suggested by the results described in Figure 31, impaired tumor formation in the "Chi-MVMp/IP-10+Chi-MVMp/TNF" group could be caused by a high mortality of the cells infected with the virus combination. To exclude this possibility, a clonogenicity

assay was done. Gl261 cells were infected at MOI 3 with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 and at (MOI 1,5+1,5) with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF. The cells formed clones 14 days post infection. After staining with crystal violet, the clone number for each group was evaluated (see Figure 32).

Virus	Colony formation
Chi-MVMp/IP-10	26%*
Chi-MVMp/TNF	28%
Chi-MVMp/IP-10+ Chi-MVMp/TNF	32%
Chi-MVMp/Δ800	44%

Figure 32 Colony formation by infected tumor cells
*The number of colonies formed by non-infected cells was set at 100%

Colony formation of Gl261 cells infected with recombinant parvoviruses was reduced when compared to non-infected cells (100%) and amounted to approximately 30%. The number of colonies formed by Chi-MVMp/IP-10+Chi-MVMp/TNF – infected cells was not lower than in other groups. In this case, due to the low cell number TNFα concentration produced upon infection might be not high enough to mediate cytotoxic effect like illustrated with Figure 31. These data suggest that on the single cell level infection with the combination of IP-10- and TNFα-expressing viruses does not affect cell survival. This suggests that, the strong antitumoral effect observed *in vivo* was caused by an immune response against tumor cells rather than by direct killing of the tumor cells.

4.6.3.3.3 Challenge experiment

3 In order to verify if surviving animals developed immunity against tumor cells, a challenge experiment was performed. After the implantation of tumor cells 12 animals remained tumor-free:

“Chi-MVMp/IP-10”	2 animals,
“Chi-MVMp/TNF”	1 animal,
“Chi-MVMp/IP-10+Chi-MVMp/TNF”	8 animals and
“Chi-MVMp/Δ800”	1 animal.

172 days after the first injection (100 days after the last tumor disappeared) these animals were challenged with $1x10^6$ normal Gl261 cells implanted subcutaneously into the left flank (opposite to the first implantation). As a control group 4 untreated animals from the same delivery were used.

Only one (from “Chi-MVMp/IP-10+Chi-MVMp/TNF” group) out of 12 challenged animals developed a tumor. The other animals remained tumor-free for over 100 days after the second injection. This suggests a long-lasting antitumoral activity in these animals. 1 out of 4 control animals also remained tumor-free. The animals were sacrificed and the spleens used for ELISpot analysis.

4.6.3.3.4 ELISpot analysis

At day 105 after the animals were challenged, they received $1x10^5$ Gl261 cells. The cells were applied to immunize the animals and increase the frequency of tumor-reactive immune system cells. 4 days after this injection the animals were sacrificed and the spleenocytes isolated.

Spleen cells were restimulated *in vitro* (co-cultured with irradiated Gl261 cells) for 6 days in order to multiply the tumor-responding cells. The anti-IFNγ. antibody-coated ELISpot plate was incubated with restimulated cells at 37° for 20 hours (see Figure 33).

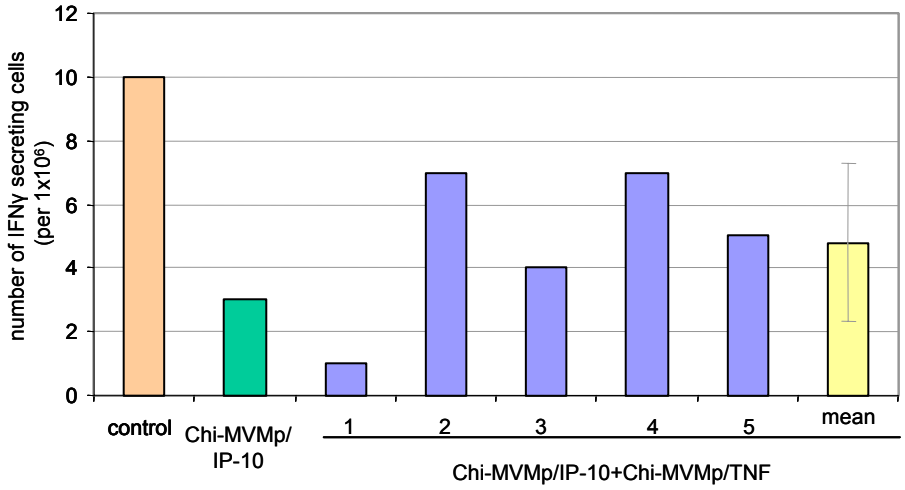


Figure 33 Number of IFNγ-producing cells for challenged animals
Spleenocytes of single animals from different groups were used for ELISpot analysis. As specific stimulus $2x10^4$ irradiated Gl261 cells were used.

The number of cells that secrete IFNγ upon stimulation with Gl261 cells was special for particular animals. The control animal (which received tumor cells only once) showed a higher number of specifically stimulated cells than the challenged animals (which were injected with tumor cells twice). All tested animals developed a specific response against Gl261 tumor cells.

2.) To confirm the antitumoral effect of IP-10/TNF combination in Gl261 model the experiment with infected at MOI3 tumor cells was repeated. In order to evaluate the mechanisms of antitumoral response, parallel to immunocompetent animals nude mice were used. The study included 4 experimental groups: control “immunocomp. mock” – C57/Bl6 mice that received $5x10^5$ bufffer-treated Gl261 cells; “nude mock” – cd1 swiss nu/nu mice that received $5x10^5$ bufffer-treated Gl261 cells; “immunocomp. IP-10/TNF” - C57/Bl6 mice that received $5x10^5$ Gl261 cells infected with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI 3 (MOI 1,5+ MOI 1,5) and “nude IP-10/TNF” - cd1 swiss nu/nu mice that received $5x10^5$ Gl261 cells infected with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI 3 (MOI 1,5+ MOI 1,5).
4 hours post infection the cells were collected, washed twice with PBS, diluted to the desired number and volume in PBS and injected subcutaneously into the right flank of the animal. Eight animals per group were used.

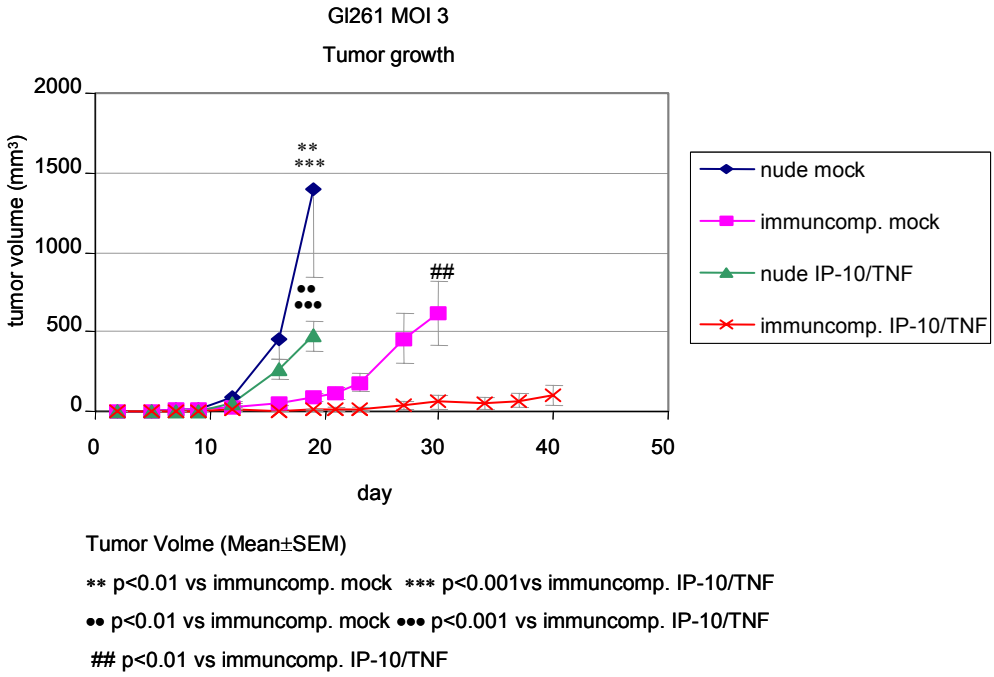


Figure 34 Tumor growth following subcutaneous injection of mouse Gl261 cells infected *in vitro* with MVMp-based vectors at MOI 3.
$5x10^5$ Gl261cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI3 (MOI 1,5+ MOI 1,5). 8 animals per group were used.

Tumor development in nude mice was faster then in immunocompetent animals. The tumors were growing very aggressively; reaching a big volume within short period of time and at day 19 of the study the animals of “nude mock” group had to be sacrificed. Compared to “nude mock” group, animals of “nude IP-10/TNF” group developed smaller tumors. In those groups all the animals developed tumors, similarly in the “imunocomp. mock” group. In contrast, only 3 out of 8 immunocompetent animals that received the cells infected with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF developed the tumors that remained very small, showing a strong antitumoral effect of combined IP-10/TNF expression in immunocompetent animals. In contrast to immunocompetent animals, transgenes expression in nude mice did not mediate such pronounced effect (see Figure 34). The results suggest that this effect may be T cell – dependent.

At day 19 of the study the mean tumor volume for “nude mock” group was significantly higher then for both groups of immunocompetent animals. However, there was no statistically significant difference between the tumor sizes of “nude mock” and “nude IP-10/TNF” groups.

"Nude IP-10/TNF" group mean tumor volume was also was significantly bigger than for both groups of immunocompetent animals. At day 30 this value for "immuncomp. IP-10/TNF" group was significantly lower compared to "immunocomp. mock" group.

The tumors appeared in all the groups over a similar period of time. In "nude mock", "nude IP-10/TNF" and "immunocomp. mock" all the animals developed tumors. In the "immuncomp. IP-10/TNF" in the first phase 7 out of 8 animals developed tumors but 4 tumors regressed so that finally 5 out of 8 animals remained tumor free (see Figure 35).

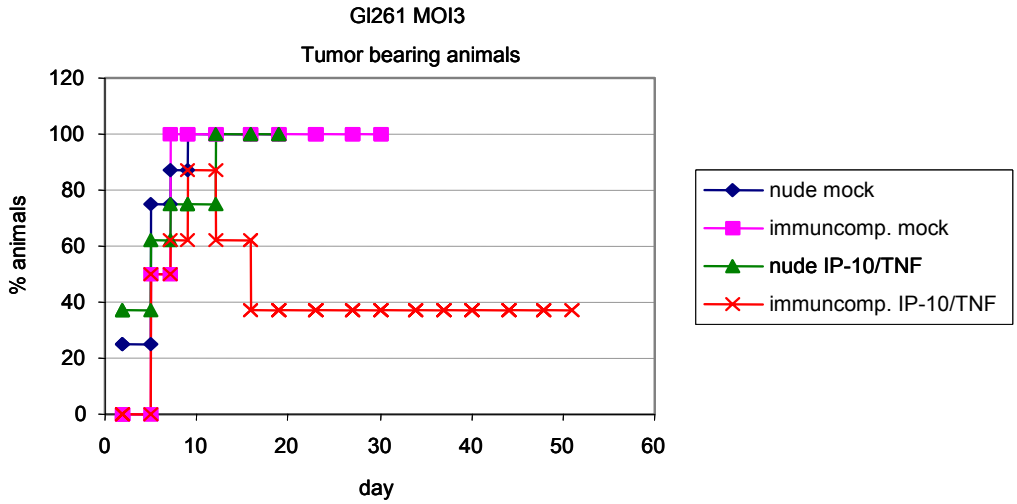


Figure 35 Tumor development after subcutaneous injection of mouse Gl261 cells infected *in vitro* with different MVMp-based vectors at MOI 3.
$3x10^5$ Gl261cells per mouse were injected subcutaneously into the right flank. 4 hours prior to injection the cells were infected with Chi-MVMp/IP-10, Chi-MVMp/TNF, Chi-MVMp/Δ800 or with the combination Chi-MVMp/IP-10+Chi-MVMp/TNF at MOI3 (for the combination MOI 1,5+MOI 1,5). 8 animals per group were used.

Due to the tumor development the animals have been sacrificed at different time points for particular groups. At first the animals belonging to the "nude mock" group (day 19), because the tumors were growing very aggressively and reached huge volumes in a short time. At the same time point "nude IP-10/TNF" animals were sacrificed. The tumors have been smaller than in the control group, however, a histological analysis was planned and tumor isolation at the same time point was desired. All animals from the "immunocomp. mock" group were sacrificed at day 30 of the study. Animals form the "immuncomp. IP-10/TNF" group demonstrated prolonged survival. Two out of 3 tumor-bearing animals were sacrificed at day 44 and 48, respectively. We should mention that the criterion to sacrifice these animals was not the tumor size, but advanced tumor necrosis. These animals developed tumors of moderate sizes, which underwent

massive necrosis. The animals were sacrificed because of progressing hemorrhage and skin injure. Third tumor-bearing animal developed a very small tumor without necrosis symptoms. Its size remained stable up to the day 51 of the experiment; finally the animal was sacrificed at day 81 because of increased tumor volume and necrosis development.

4.6.3.4 *In vivo* treatment of Gl261-derived tumors

It was previously shown in our laboratory that MVMp-based genomes with H1 capsids (pseudotyped viruses) retained the properties of recombinant MVMp but may be useful when successive injections are needed (Wrzesinski C. et al. 2003, Lang S. 2003). Indeed neutralizing antibodies are produced after 14 days in the mice, making rpeated injections of recombinant virus unefficient. Yet, antibodies against H1 capsids do not cross-react with those against MVMp. Therefore the application of pseudotyped recombinant virus (MVMp-based genomes with H1 capsids) makes possible prolonged virus injections without neutralizing it by circulating antibodies. In this study the effect of pseudotyped recombinant parvoviruses (MVMp-based chimeric genome in H-1 capsid) on established subcutaneous tumors was established.

Mice received $1x10^6$ Gl261 cells in the right flank. After the tumors were visible (2-3 mm^3 at day 5 post implantation), 6 virus injections were given: at day 5, 6, 7, 9, 11, and 13 after tumor cell implantation. Each animal received $1x10^6$ RU per injection. The combination group was injected with $0,5x10^6$+$0,5x10^6$ RU. Control (“mock”) animals were injected with 100 µl PBS. Tumor growth was monitored every 2-3 days (see Figure 36).

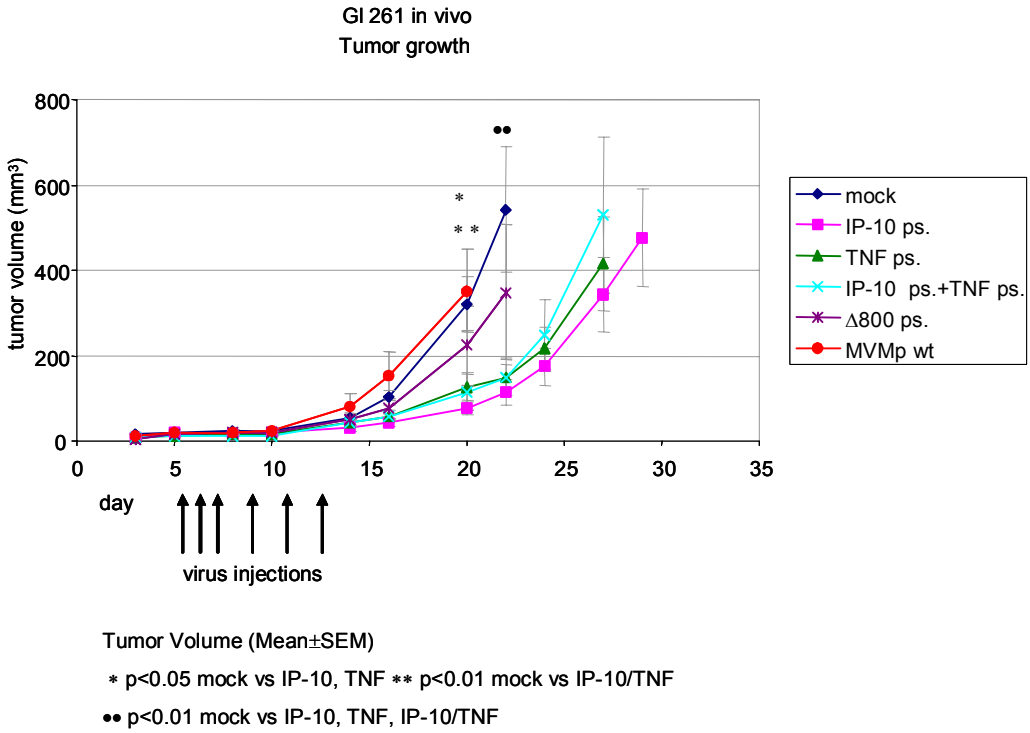


Figure 36 Growth curves of subcutaneous established tumors treated *in vivo* with repeated virus injections.
Animals received subcutaneously 1x10^6 Gl261 cells. At day 5, 6, 7, 9, 11 and 13 virus injections were given. 8 animals per group were used.

In all animals the sizes of the tumors were comparable at the time when the first virus injections were done (day 5 up to day 10). After day 10 the mean tumor size became different for particular groups and the growth curves started to diverse between the different groups. There was a delay in tumor expansion and the growth rate in the groups treated wit vectors expressing a therapeutical transgene. The control vector, Δ800 ps. cause only a moderate effect. There was no difference in the tumor growth between “IP-10 ps.”, “TNF ps.” and “IP-10 ps. + TNF ps.” groups (see Figure 36). At day 20 and 22 the mean tumor volume for ”IP-10 ps.”, “TNF ps.” and “IP-10 ps.+TNF ps.” groups was significantly lower compared to “mock” group. Notably, there was no influence of the wild type MVMp virus on the tumor growth. Gl261 cells are very sensitive to the killing effect of this virus *in vitro* (see Figure 1; Table 1), but in *in vivo* conditions MVMp wt did not inhibit tumor growth. Virus injection in the tumor surrounding might be not as effective as infection of the cells in culture.

The treatment with recombinant vectors expressing therapeutical transgenes significantly prolonged animal survival comparing to the animals treated with MVMp wild type virus (vs IP-10 ps. p=0.007; vs TNF ps. p=0.04; vs IP-10/TNF ps. p=0.03).

Treatment with the IP-10-expressing virus significantly prolonged the survival also comparing to the “mock” group (treated with PBS) (see Figure 37; IP-10 ps. vs mock p=0.003).

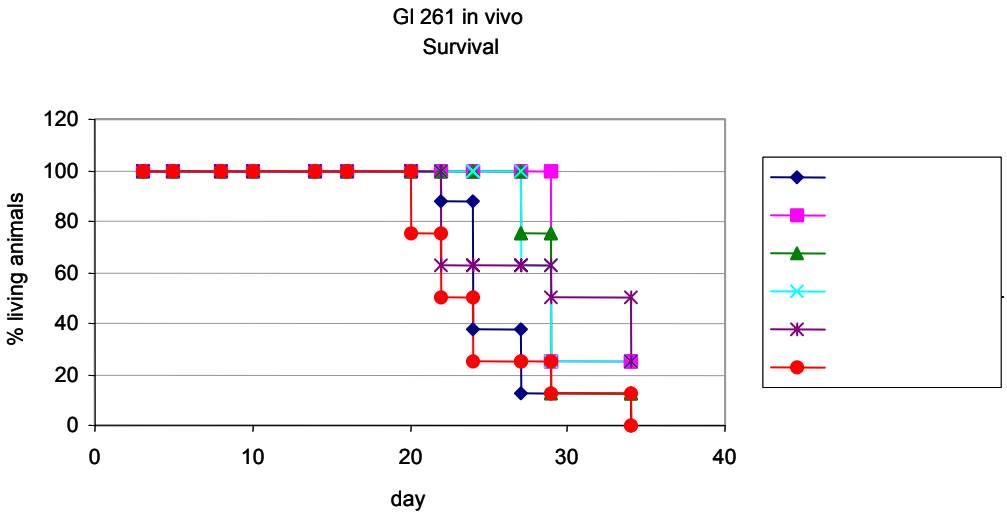


Figure 37 Animal survival after in vivo treatment of subcutaneously implanted Gl261 cells
$1x10^6$ Gl261cells per mouse were injected subcutaneously into the right flank. After tumors appeared, 6 injections of IP-10 ps., TNF ps., Δ800 ps. viruses ($1x10^6$ RU) or the combination IP-10 ps.+TNF ps. ($0,5x10^6$+$0,5x10^6$ RU) were given. 8 animals per group were used.

Compared to cells infected *in vitro* (MOI1 and MOI3 used for above-described animal studies) a dose of $1x10^6$ RU used for *in vivo* treatment is low. At day 5, when the treatment started, tumor mass might contain approximately $8x10^6$ cells. Beside this, in *in vivo* conditions tumor cells are not easily accessible to the infection (virus injected in the surrounding of the tumor, tumor cells form a compact structure). Nevertheless, therapeutic effects (tumor growth delay and prolonged survival) of recombinant parvoviruses expressing IP-10 or TNFα could be demonstrated, showing the promising antitumor potential of this approach.

This experiment initially started with 11 animals per group. 10 days after tumor cell implantation (3 days after the third virus injection) 3 animals per group were sacrificed; the tumors isolated and underwent histological examination (see chapter “Evaluation of the antitumoral mechanism”).

4.7 Dendritic cells and parvoviruses in gene therapy

Dendritic cells (DCs) are one of the most potent cells of the immune system. They collect and process antigens for presentation to T cells, directing them to different types of immune response or to tolerance (Banchereau J. et al. 2000).

A promising strategy would consist in stimulating DCs by virus-infected tumor cells. Such an approach would also be the first assay for the development of a vaccination. DCs co-cultured with infected tumor cells would stimulate T cells to act against infected tumor cells. Direct infection of DCs with parvoviral vectors could lead to the presentation of viral antigens and, again, direct T cells to the response targeted by the virus tumor cells.

In this study we wanted to take advantage of the expression of TNFα by recombinant vectors. Indeed TNFα is known as inducer of DCs maturation (Banchereau J. et al. 2000).

Preliminary experiments to evaluate the potential use of recombinant parvoviruses in DC-directed antitumor response were done in cooperation with the scientific group of Dr. Annunciata Vecchi and Dr. Silvano Sozzani (Pharmacological Institute “Mario Negri”, Milano, Italy).

4.7.1 Stimulation of DCs with infected tumor cells

Two approaches were investigated: stimulation of DCs with tumor cell lysates and co-culture of DCs with tumor cells.

a) Stimulation with tumor cell lysates

$2x10^5$ Gl261 cells were infected at MOI3 with the Chi-MVMp/Δ800 or the Chi-MVMp/TNF virus. 3 days post infection the cells were collected, supernatants discarded and the cell pellet frozen in a 0,5 ml fresh medium. One freeze-thaw cycle was performed and the lysates stored at -20°C. $2x10^6$ DCs were stimulated for 24 hours with the lysate obtained from $1x10^5$ tumor cells. Afterwards phenotype characteristisc of DCs and a pinocytosis assay were performed. The maturation of DCs can change the expression profile of certain surface markers and decrease their pinocytotic activity.

b) Stimulation with whole tumor cells (co-culture)

$1x10^5$ Gl261 cells were infected with the Chi-MVMp/Δ800 or the Chi-MVMp/TNF virus at MOI3. One day post infection tumor cells were collected and co-cultured with DCs at the ratio 1:10 (Gl261: DCs) for 24 hours. As in the above-described approach, phenotype characteristics of DC and a pinocytosis assay were performed.

4.7.1.1 Changes in the pinocytotic activity of DC

For the evaluation of pinocytotic activity DCs were incubated with FITC-coupled dextran ($1,5x10^5$ cells per sample). The samples were analysed by FACS.

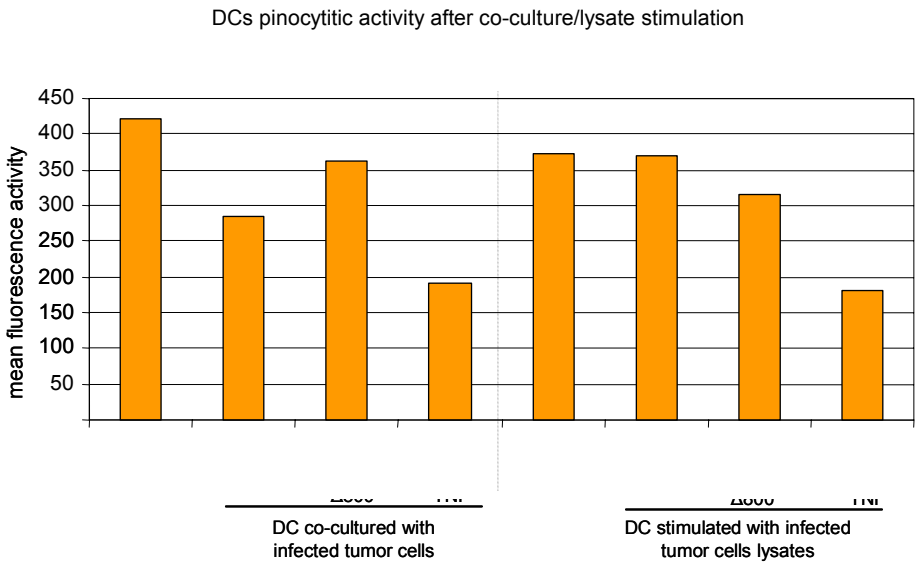


Figure 38 Changes in the pinocytotic activity of DCs after stimulation with infected tumor cells or their lysates
After stimulation $1,5x10^5$ DCs were tested for their pinocytotic activity (1 hour incubation with FITC-coupled dextran).

One of the parameters describing mature dendritic cells is the decrease of their pinocytotic activity. After stimulation with infected tumor cells or their lysates such an effect could be observed for DCs stimulated with Chi-MVMp/TNF-infected tumor cells. Co-culture was as effective as stimulation with the cell lysate. Non-infected Gl261 cells also decreased the pinocytotic activity of DCs, but this effect was not observed when DCs were stimulated with the lysate of non-infected cells. A slight effect was observed for Chi-MVMp/Δ800 infected tumor cells and their lysates (see Figure 38).

4.7.1.2 Changes in the expression profile of surface markers

Changes in the expression profile of surface markers indicate the status of DCs. A mature DC characteristic is characterized by an increase in the expression of CD80, CD86, DEC205 and MHCII. In a mature population more cells express these markers and an increased expression on the single cell level can be observed. For a phenotype characterisation DCs were stained with different antibodies against cell surface markers: anti CD86, CD80, DEC205 and MHCII ($3x10^5$ cells per sample). In order to distinguish DCs from Gl261 cells the CD11b marker was used. The samples were analysed by FACS.

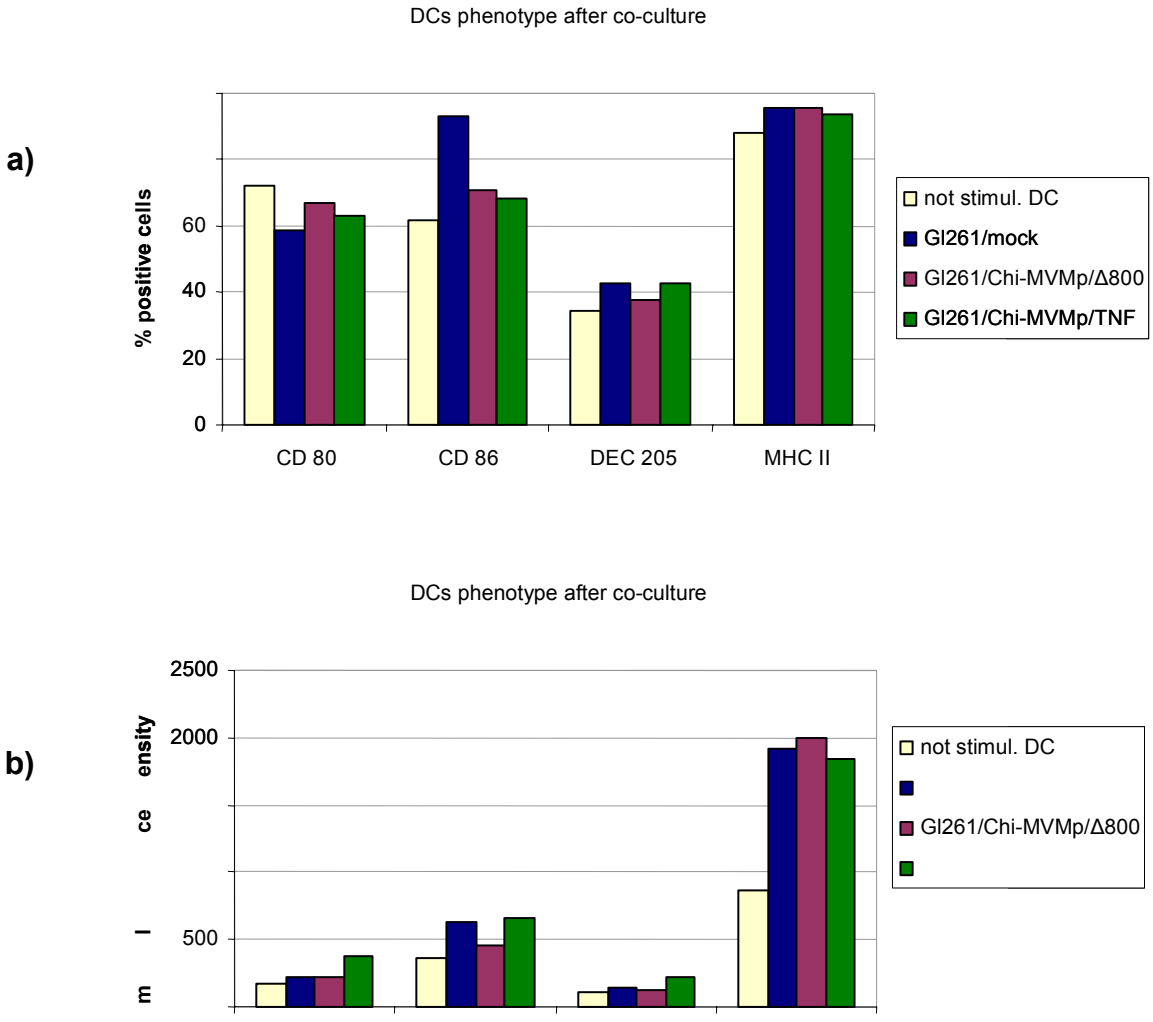


Figure 39 Changes in marker expression profile after stimulation of DCs with infected tumour cells
a) differences expressed as percentage of positive cells, b) differences expressed as mean fluorescence intensity

Stimulation with whole tumor cells moderately influenced the number of mature marker-positive cells. A slight expression increase was noticed for the DEC205 and MHCII markers, after stimulation with infected or non-infected tumor cells. The number of CD86 expressing cells notably increased upon stimulation with non-infected Gl261 cells (see Figure 39a). Co-culture promoted MHCII expression on the single cell level. Compared to non-stimulated DCs, the number of MHCII molecules on those cells cultured with Gl261 cells increased by a factor of 2 (see Figure 39b). Infection had no effect on the expression level. CD80, CD86 and DEC205 expression on single cells increased after stimulating the DCs with Gl261 cells infected with a TNFα-expressing vector. Yet, CD86 expression is also promoted by non-infected tumor cells as well as by cells infected with the control vector (Chi-MVMp/Δ800) (see Figure 39b).

Obtained results suggest that tumor cells infected with TNFα-expressing parvoviral vectors promote DC maturation more than non-infected cells. This finding may have a potential therapeutical implication.

The influence of lysates derived from infected tumor cells on the maturation of DCs was less pronounced than for whole tumor cells (see Figure 40).

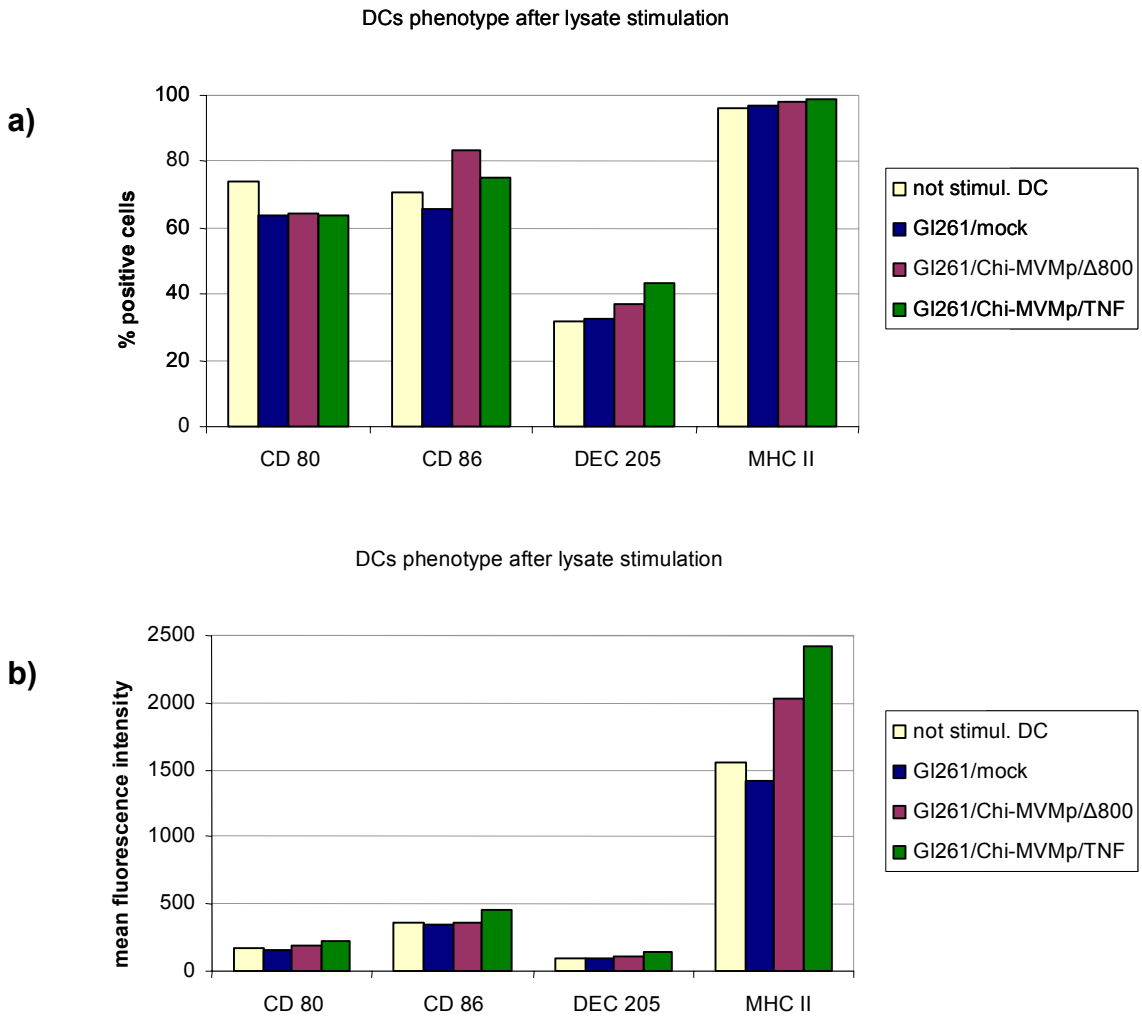


Figure 40 Changes in marker expression profile after stimulation of DCs with lysates derived from infected tumor cells
a) differences expressed as percentage of positive cells, b) differences expressed as mean fluorescence intensity

DC stimulation with Chi-MVMp/Δ800- or Chi-MVMp/TNFα-infected cell lysates increased the percentage of DCs positive for CD86 and DEC205 markers. DC stimulation with Chi-MVMp/Δ800- or Chi-MVMp/TNFα-infected cell lysates increased the mean fluorescence intensity for the MHCII marker (see Figure 40). Stimulation with lysates derived from non-infected tumor cells did not have any effect on the expression profile, whereas such an effect could be observed for intact cells (an increased percentage of CD86-expressing cells and an increased level of MHCII expression).

It may suggest that tumor cells alone release a factor that influences the maturation of DCs.

Viral infection may contribute to this process by transgene delivery (TNFα) and also by some other factors (infection with a control vector has promoted some maturation parameters as well).

4.8 Evaluation of the antitumoral mechanisms

Two approaches have been used in order to evaluate antitumoral mechanisms mediated by recombinant parvoviruses in Gl261 and U87 tumor models. One of them was a histological examination of tumor tissue isolated from experimental animals. The extent of tumor necrosis and the number of blood vessels was evaluated. Immunohistochemistry was done at the Department of Anatomy III, University of Heidelberg under the supervision of Dr. Ralf Kinscherf.

The other approach was a measurement of blood vessel density done by Magnetic Resonance Imaging. This work was performed in collaboration with Dr. M. Heilmann and Dr. F. Kießling at the Department of Radiology DKFZ Heidelberg.

4.8.1 The influence of recombinant parvoviruses on tumor necrosis

Solid tumors have several distinguishing characteristics from normal tissues, chief amongst them are an under-developed vasculature that delivers much of the oxygen and nutrients to those cells located in the periphery of the tumor. A nutrient-poor environment in the centre soon accompanies the growth of any solid tumor over a volume of 1 mm^3. This generally leads to a large number of necrotic (dead and dying) cells at the centre of any solid tumor. Simply measurement of tumor volume might not accurately assess the response to the treatment. The investigation of entire tumor tissue sections identifies regional changes after treatment with different viral vectors.

4.8.1.1 Evaluation of animal study with Gl261 cells infected *in vitro* at MOI1

Infected tumor cells (MOI1) were subcutaneously implanted into the fight flank of the animals. Tumor growth was monitored and when the longest tumor diameter reached 17 mm the animals were sacrificed, the tumors isolated and fixed. Histological examination of counterstained sections was done.

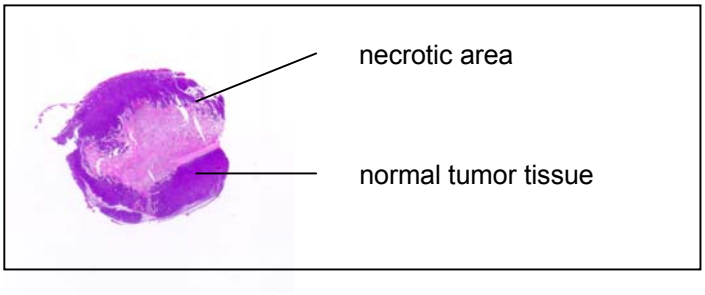


Figure 41 Representative hematoxylin-eosin stained

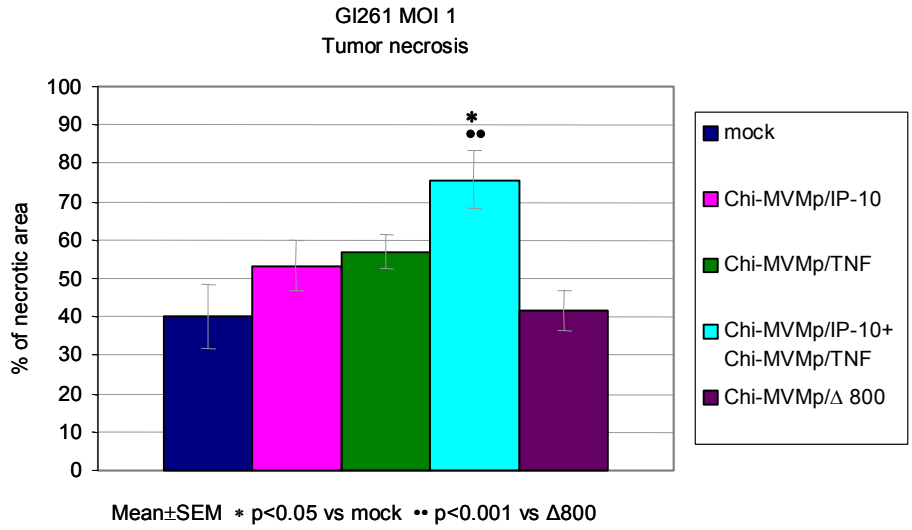


Figure 42 The percentage of necrotic area in tumor sections
The largest cross-sectional diameter of each tumor was evaluated. The percentage of necrotic tumor area was calculated as the ratio of the necrotic area to the total tumor area x 100.

Tumors were obtained from GI261 cells infected at MOI1.

The highest percentage of necrotic area was found in tumors of animals belonging to "Chi-MVMp/IP-10+Chi-MVMp/TNF" group. Compared to "mock" and "Chi-MVMp/Δ800" groups the differences were statistically significant (see Figure 42). In the animal experiment the most pronounced tumor growth delay was observed for "Chi-MVMp/IP-10" and "Chi-MVMp/IP-10+Chi-MVMp/TNF" groups (see Figure 22). For "Chi-MVMp/IP-10+Chi-MVMp/TNF", but not for "Chi-MVMp/IP-10" group, necrosis appearance is corresponding to the tumor growth delay.

4.8.1.2 Evaluation of *in vivo* treated GI261-derived tumors

Established GI261-derived subcutaneous tumors were treated *in vivo* with virus injections. In this study the effect of pseudotyped recombinant parvoviruses was

established (MVMp-based chimeric genome in H-1 capsid). 6 virus injections were given.

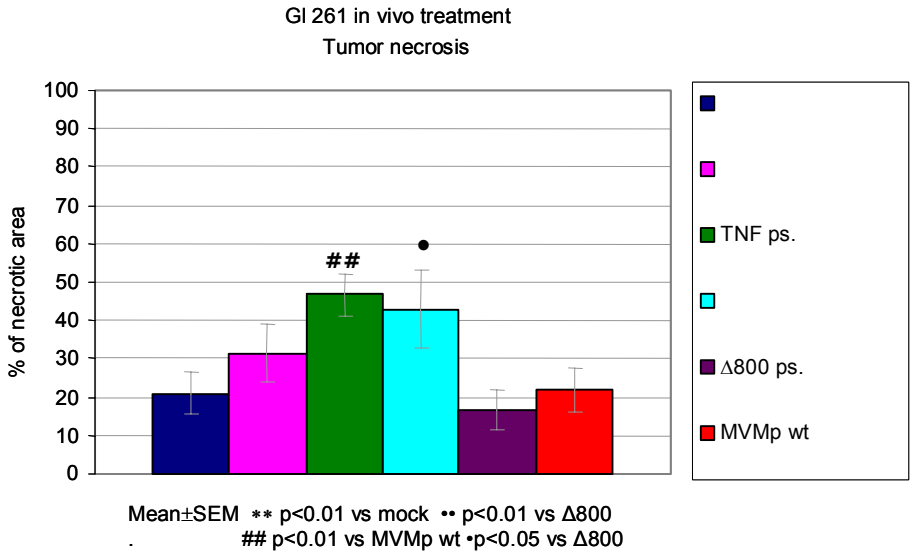


Figure 43 The percentage of necrotic area in tumor sections
The largest cross-sectional diameter of each tumor was evaluated. The percentage of necrotic tumor area was calculated as the ratio of the necrotic area to the total tumor area x 100.

Gl261-derived tumors were obtained from the animals treated *in vivo* with pseudotyped (ps.) vectors injections.

Increased necrosis incidence was observed for “TNF ps.” and “IP-10 ps.+TNF ps.” groups. In the “TNF ps.” group the the percentage of necrotic area was significantly higher when compared to “mock”, “Δ800 ps.” and “MVMpwt” groups, and for the “IP-10 ps.” group when compared to “Δ800ps.” (see Figure 43). Increased necrosis appearance corresponds with delayed tumor growth for “TNF ps.” and “IP-10 ps.+TNF ps.” groups. Notably, the same effect of tumor expansion delay was observed also for the “IP-10 ps.” group (see Figure 36). However, increased tumor necrosis was not observed in this group.

Concluding from both animal experiments it is possible to say that tumor necrosis appearance positively corresponds to the antitumoral effect (tumor growth delay) when the animals were treated with TNF – expressing virus or with the combination of TNF with IP-10 expressing vectors.

In both experiment the animals were sacrificed at several time points. This could influence the extent of tumor necrosis incidence due to the different age of tumors at the day of the isolation. In the experiment where pseudotyped vectors were used, the survival as well as antitumoral effect was very similar for 3 groups: “IP-10 ps.”,

"TNF ps." and "IP-10 ps. +TNF ps." Independently from that, increased tumor necrosis was present only in the two groups "TNF ps." and "IP-10 ps.+TNF ps." This means that the type of transgene plays a key role in this process. In the GI261 glioblastoma model TNFα seems to mainly contribute to tumor necrosis development.

4.8.1.3 Evaluation of animal study with U87 cells infected *in vitro* at MOI3

Infected at MOI3 tumor cells were subcutaneously implanted into the fight flank of the animals. Tumor growth was monitored and as the longest tumor diameter reached 17 mm the animals were sacrificed, the tumors isolated and fixed. Histological examination of counterstained sections was performed.

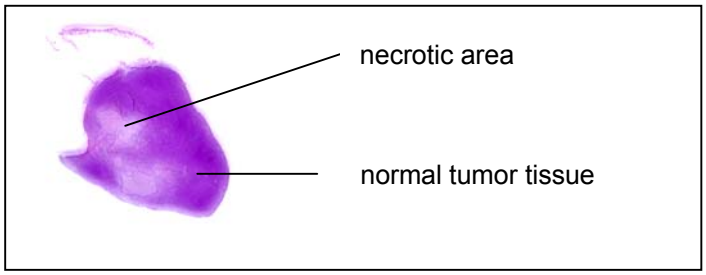


Figure 44 Representative hematoxylin-eosin stained U87 tumor section

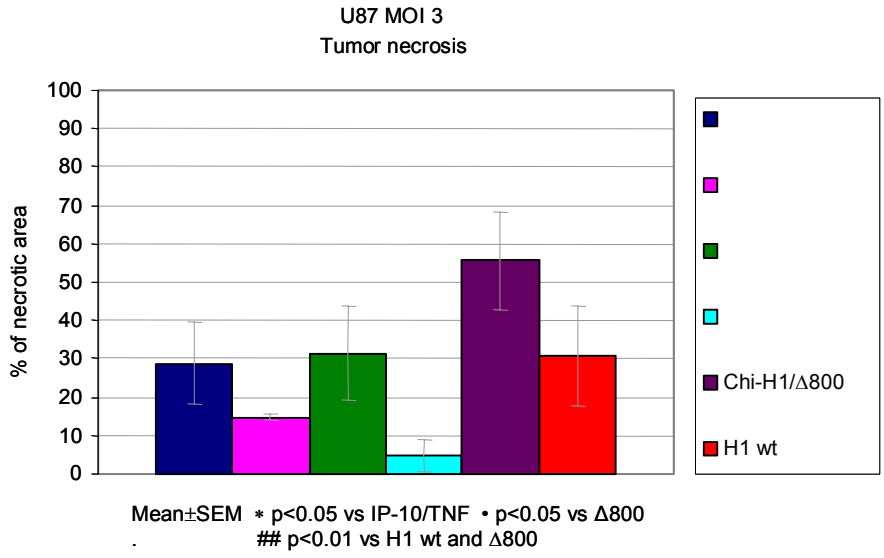


Figure 45 The percentage of necrotic area in tumor sections
The largest cross-sectional diameter of each tumor was evaluated. The percentage of necrotic tumor area was calculated as the ratio of the necrotic area to the total tumor area x 100.Tumors were obtained from U87 cells infected at MOI3.

Tumor tissue was isolated form all the animals at day 24 post implantation of tumor cells. This allows avoiding the inconvenience of analyzing the samples collected over longer period of time. At day 24 a significant antitumoral effect was observed for the group “Chi-H1/IP-10+Chi-H1/TNF” in comparison to other experimental groups (see Figure 18). The percentage of necrotic area was reduced in “Chi-H1/IP-10+Chi-H1/TNF” group and significantly smaller than in “mock”, “Chi-H1/Δ800” and “H1 wt” groups (see Figure 45). In “Chi-H1/IP-10” group necrosis incidence was significantly reduced compared to “Chi-H1/Δ800” group.

In the U87 system antitumoral effect (reduced tumor growth) negatively corresponds to the tumor necrosis development. It is possible, that in this model blood vessel network is not affected by the transgene expression and necrosis development simply corresponds to the tumor size.

4.8.2 The influence of recombinant parvoviruses on tumor vascularisation

The transgenes delivered by means of recombinant viruses have immunostimulatory and antiangiogenic properties. IP- 10 is well described as a chemoattractant for T cells and a potent antiangiogenic factor. TNFα is a pleiotropic activator of the immune response and, depending on the model, a stimulator or inhibitor of the blood vessels development. Direct inhibition of blood vessel formation or the influence on this process caused by the transgene proteins would be a critical procedure in the formation and growth of glioblastoma tumors, which are known to be especially highly vascularized among the tumors of different origin.

4.8.2.1 Evaluation of *in vivo* treated GI261-derived tumors

In this *in vivo* study the effect of pseudotyped recombinant parvoviruses was tested (MVMp-based chimeric genome in H-1 capsid). 6 virus injections were given.

Animals under test were divided into two cohorts. The first one contained 3 animals per group, which were sacrificed at day 10 of the study, i.e. three days after the third virus injection was applied. This experimental setting should allow to determine the influence of pseudotyped vectors on early tumor angiogenesis.

The second cohort contained 8 animals per group, which received 6 virus injections. Tumor growth was monitored until the animals were sacrificed. Tumor tissue was

isolated, fixed and analysed. This experimental setting should reveal the influence of pseudotyped vectors on late tumor angiogenesis.

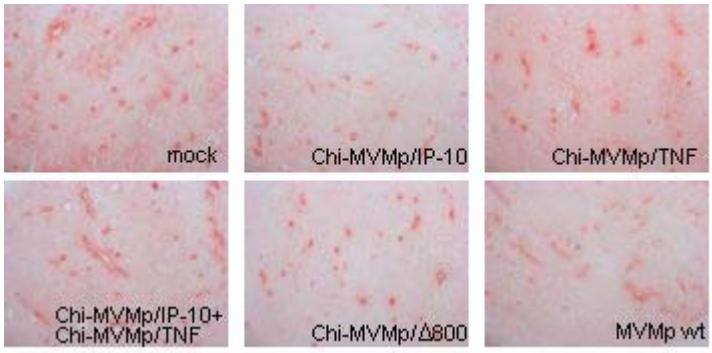


Figure 46 Anti-CD31-stained Gl261 tumor sections

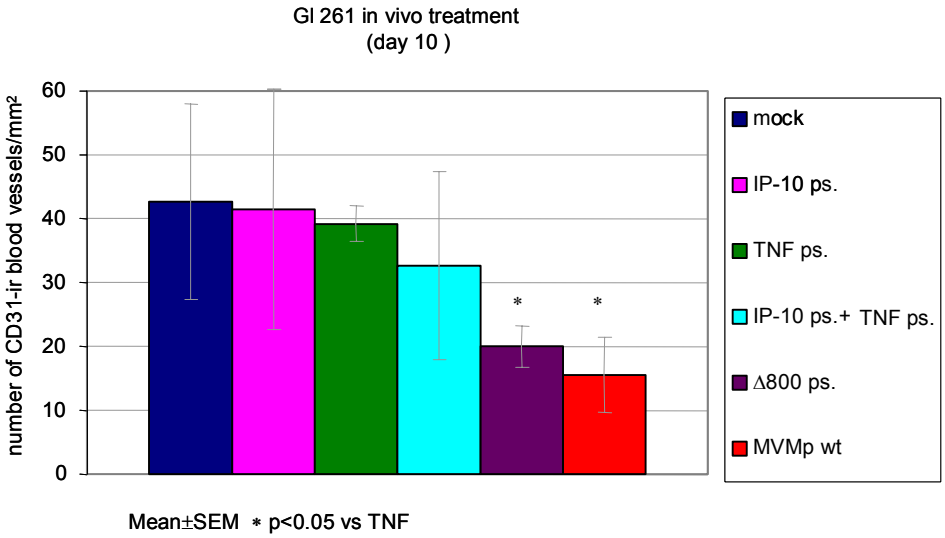


Figure 47 The density of blood vessels in tumor sections
Gl261-derived tumors were obtained from the animals treated *in vivo* with pseudotyped vectors injections. The cross-sectioned vessels were quantified counting CD31-positive vessels. The CD31 immunoreactive (ir) number of vessels was computer assisted morphometrically quantified and expressed as vessel number per mm^2.

10 days after tumor cell implantation the number of blood vessels was lower in "Δ800" and "MVMp" groups compared to "mock", "IP-10 ps.", "TNF ps." and "IP-10 ps.+TNF ps." groups. However, this difference is statistically significant only versus "TNF ps." group (see Figure 47). Comparing to the untreated "mock" group, transgenes do not show any antiangiogenetic properties at this stage of tumor development. There are no significant differences between the "mock", "IP-10 ps.", "TNF ps." and "IP-10 ps.+TNF ps." groups. The tumors treated with MVMp wt virus or with the control Δ800 vector revealed a decreased number of blood vessels.

The tumors from the second cohort were isolated at the end of the experiment. Tumor growth was delayed in “IP-10 ps.”, “TNF ps.” and “IP-10 ps.+TNF ps.” groups compared to “mock”, “MVMp” and “Δ800 ps.” groups (see Figure 36).

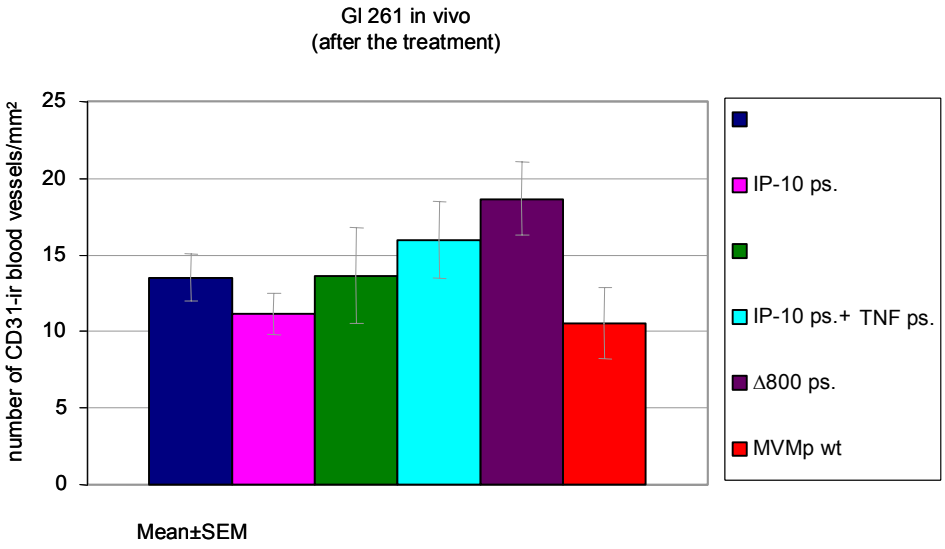


Figure 48 The density of blood vessels in tumor sections
Gl261-derived tumors were obtained from the animals treated *in vivo* with pseudotyped vectors injections. The cross-sectioned vessels were quantified counting CD31-postive vessels. The CD31 immunoreactive (ir) number of vessels was computer assisted morphometrically quantified and expressed as vessel number per mm^2.

The number of CD31 immunoreactive blood vessels was comparable among all groups under test.

Generally, at the early stage of tumor development (day 10) blood vessel density was higher and amounted to ~40 vessels per mm^2 in “mock”, “IP-10 ps.”, “TNF ps.” and “IP-10 ps.+TNF ps.” groups. At the end of the experiment the density was similar for all the groups and between 10 and 15 vessels per mm^2.

Although transgene induction suppresses, to some extend, tumor development, it does not seem to have any influence on the blood vessels formation. Because blood vessel density remains similar in all experimental groups, probably not angiogenesis, but other factors, induces the antitumoral effect in the Gl261 model

4.8.2.2 Evaluation of animal study with U87 cells infected *in vitro* at MOI3

Infected at MOI3 tumor cells were subcutaneously implanted into the fight flank of the animals. At the day 24 of the experiment the animals were sacrificed, the tumors isolated and fixed. Histological examination of anti-CD31-stained sections was performed.

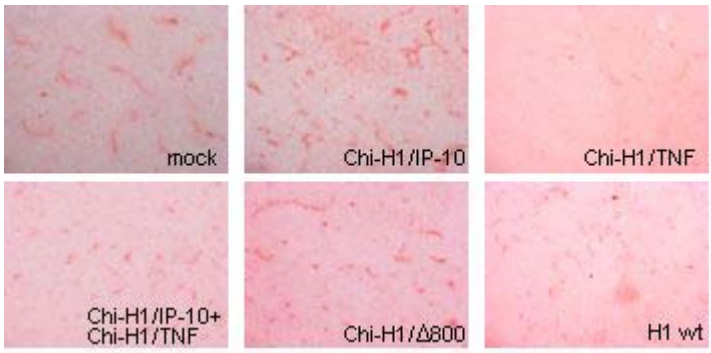


Figure 49 Anti-CD31-stained U87 tumor sections

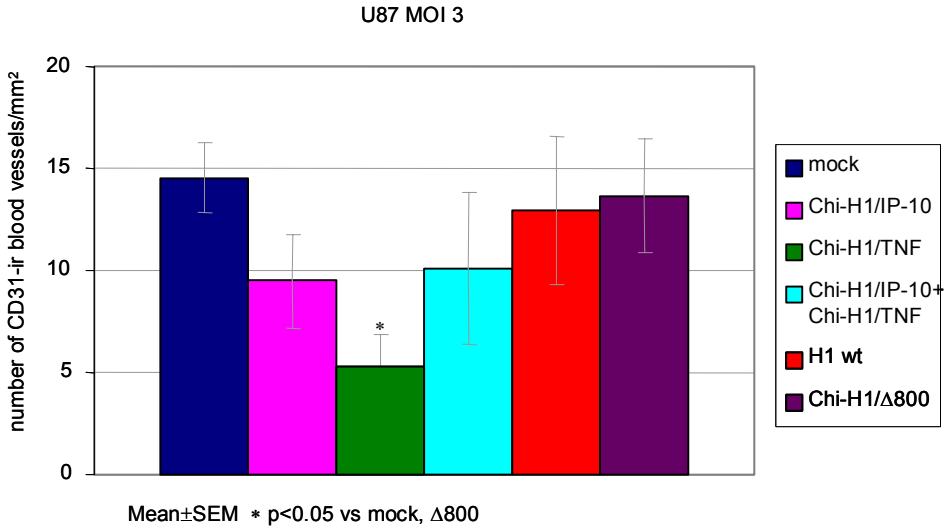


Figure 50 The density of blood vessels in tumor sections
The tumors were obtained from the cells infected *in vitro* with H1-based chimeric vectors at MOI3. The cross-sectioned vessels were quantified counting CD31-postive vessels. The CD31 immunoreactive (ir) number of vessels was computer assisted morphometrically quantified and expressed as vessel number per mm^2.

In the animal experiment the mean tumor volume was significantly smaller for the “Chi-H1/IP-10+Chi-H1/TNF” group compared to “mock” and “Chi-H1/Δ800” groups. Tumor volume for “Chi-H1/TNF” group was marginally lower compared to these two control

groups. For other groups no reduction of tumor growth at this time point was observed (see Figure 18).

Density of CD31-immunoreactive blood vessels was significantly reduced in the “Chi-H1/TNF” group in comparison to the “mock” and “Chi-H1/Δ800” groups. The number of CD31-immunoreactive blood vessels was also reduced in both IP-10-treated groups. In tumors of the “H1 wt” and “Chi-H1/Δ800” groups in comparison to the untreated “mock” group there was no reduction of CD31-immunoreactive vessels observed.

Combined IP-10 and TNFα expression revealed the best therapeutic effect in U87 model. This effect does not seem to appear exclusively due to the inhibition of angiogenesis, because combined expression of both transgenes only moderately decreased the density of the blood vessels. Inhibition of blood vessel development by TNFα expression did not significantly reduce tumor growth.

4.8.3 MRI analysis of Gl261-derived tumors vascularization

In order to investigate the influence of Chi-MVMp/IP-10 virus treatment on tumor angiogenesis an *in vivo* study was performed. Mice received 1x10^6 Gl261 cells in the right flank. After the tumors were visible (2-3 mm^3 at day 6 post implantation), 6 Chi-MVMp/IP-10 virus injections were given: at day 6, 7, 8, 10, 12, and 14 after tumor cell implantation. Each animal received 1x10^6 RU per injection. Control ("mock") animals were injected with 100 µl PBS. Tumor growth was monitored every 2-3 days.

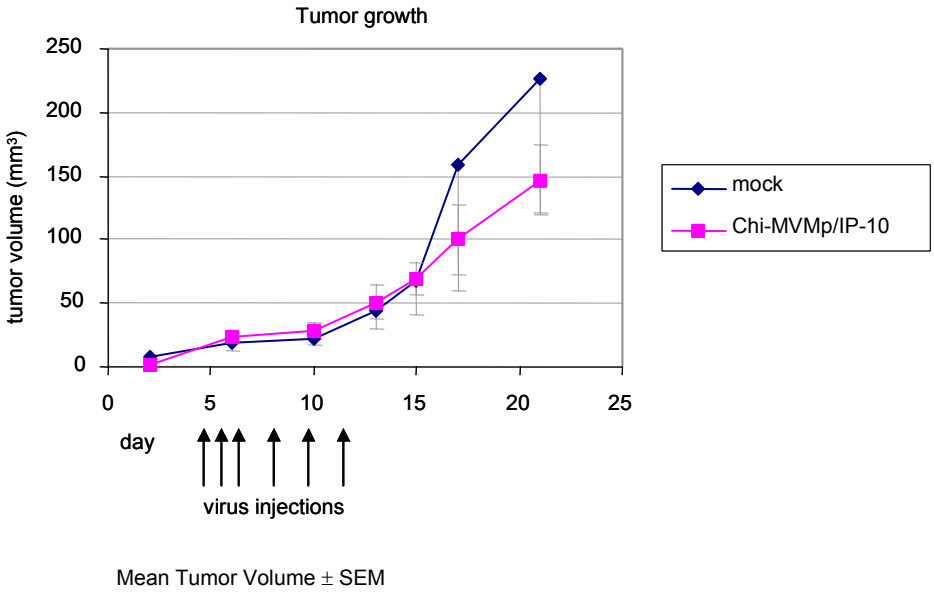


Figure 51 Subcutaneous established tumors were treated with repeated Chi-MVMp/IP-10 virus injections
Animals received subcutaneously 1x10^6 Gl261 cells. At day 6, 7, 8, 10, 12 and 14 virus injections were given. 5 animals per group were used.

Over the time period when the virus injections were done (up to day 15), tumor growth was similar for both groups. No difference between PBS-treated and virus-treated groups could be observed. After day 15 the mean tumor size became different for particular groups. The therapeutical effect of Chi-MVMp/IP-10 virus could be observed. There was a delay in tumor expansion and the growth rate for the virus-treated group compared to buffer-treated animals. However, at day 22 (the day of animal sacrifice) the difference in the mean tumor volume remained statistically insignificant.

The animals underwent MRI measurement 15 and 22 days post implantation of tumor cells. From day 16 onwards the difference in the mean tumor volume for particular

groups could be noted (tumors treated with Chi-MVMp/IP-10 virus remained smaller then control tumors) (see Figure 51).

Magnetic resonance imaging was performed using a clinical 1.5 T whole-body MRI-system and a custom-made radio-frequency-(rf) coil (“animal resonator”) for rf excitation and signal reception. After infusion of the contrast agent, an exchange of contrast agent between intra- and extravasal compartments was assumed, described by the rate constant $k_{ep.}$ Another functional parameter was the amplitude A, the relative change in signal intensity after the contrast agent injection relative to precontrast values. While amplitude A predominantly reflects the plasma volume, the rate constant k_{ep} is a compound parameter highly influenced by the vessel permeability but also by blood flow, and capillary exchange surface.

Amplitude and k_{ep} were calculated pixelwise, color-coded and overlaid on the morphological MR images to reveal parameter maps (see Figure 52).

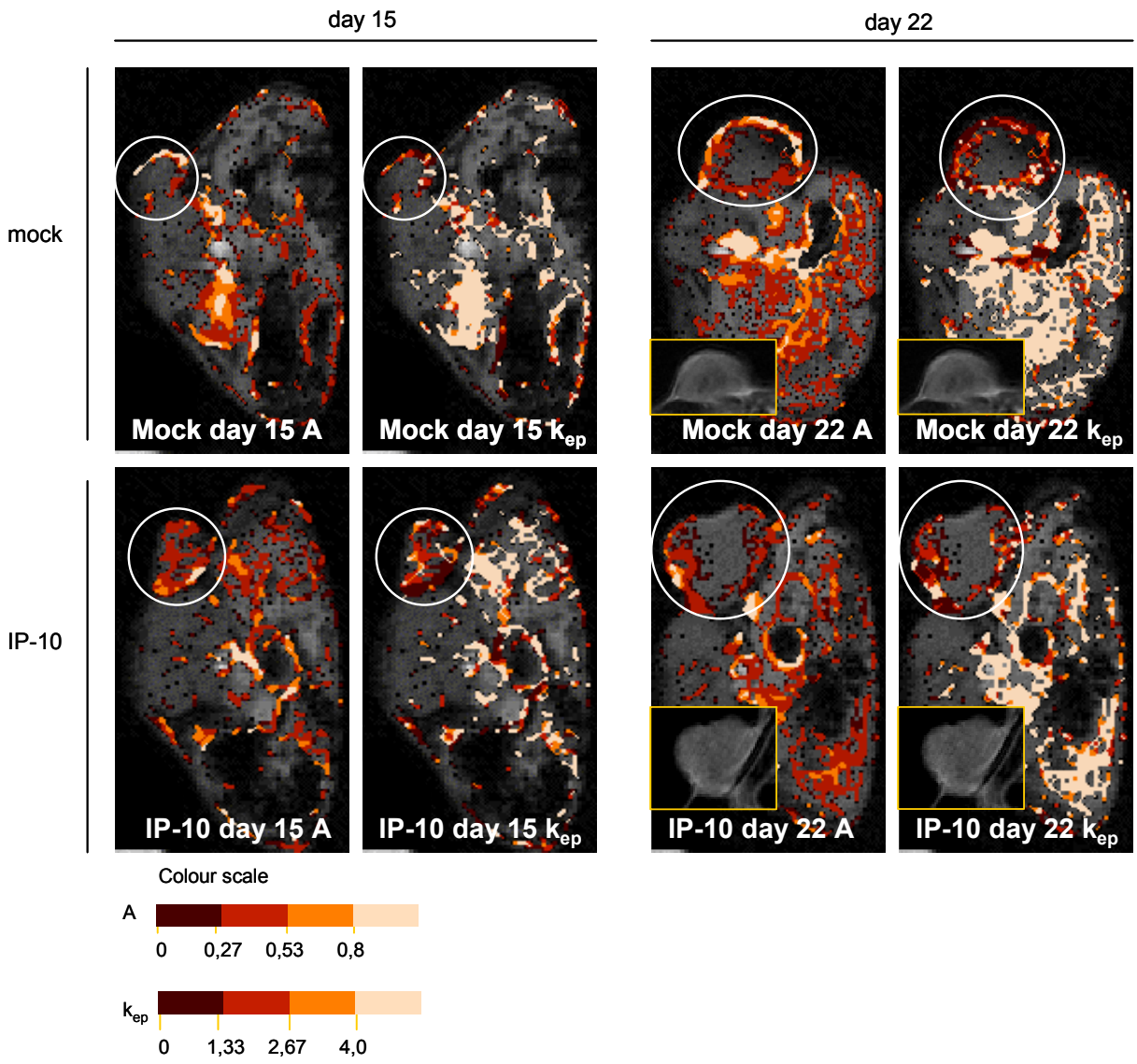


Figure 52 Amplitude (A) and exchange rate constant (k_{ep}) maps for GI261-derived tumors
The maps are shown for one representative animal from each group. Transversal sections of the whole mouse body including the tumor are shown. Tumor area is indicated.

Amplitude maps (A) correspond to blood volume and indicate the areas of different vascular density. The maps demonstrate the highest blood volume at the periphery of the tumors (very well visible for the “mock” animal at day 22). The centre of the tumor typically shows low vascular density (“mock” animal days 15 and 22). Changes in the tumor vascularization could be demonstrated with the example of Chi-MVMp/IP-10 – treated animal. At day 15 the signal was observed both on the periphery and in the centre of the tumor (see “IP-10” day 15). At day 22 there was no signal detected in the tumor centre (see “IP-10” day 22), what could illustrate tumor necrosis development.

Histological analysis showed central tumor necrosis for GI261-derived tumors (see Figure 41).

The exchange rate constant (k_{ep}) maps illustrate the ratio of contrast agent exchange between intra- and extravascular space and correspond to the vessel permeability. High k_{ep} values were calculated for the periphery of the tumors, confirming the presence of vascular network in these areas both for control and treated tumors.

Small pictures included for the day 22 show tumor morphology.

Following contrast agent injection, signal intensity-time curves were recorded. Signal intensity curves illustrate contrast agent uptake by tumor tissue within 11,3 minutes after its application (see Figure 53).

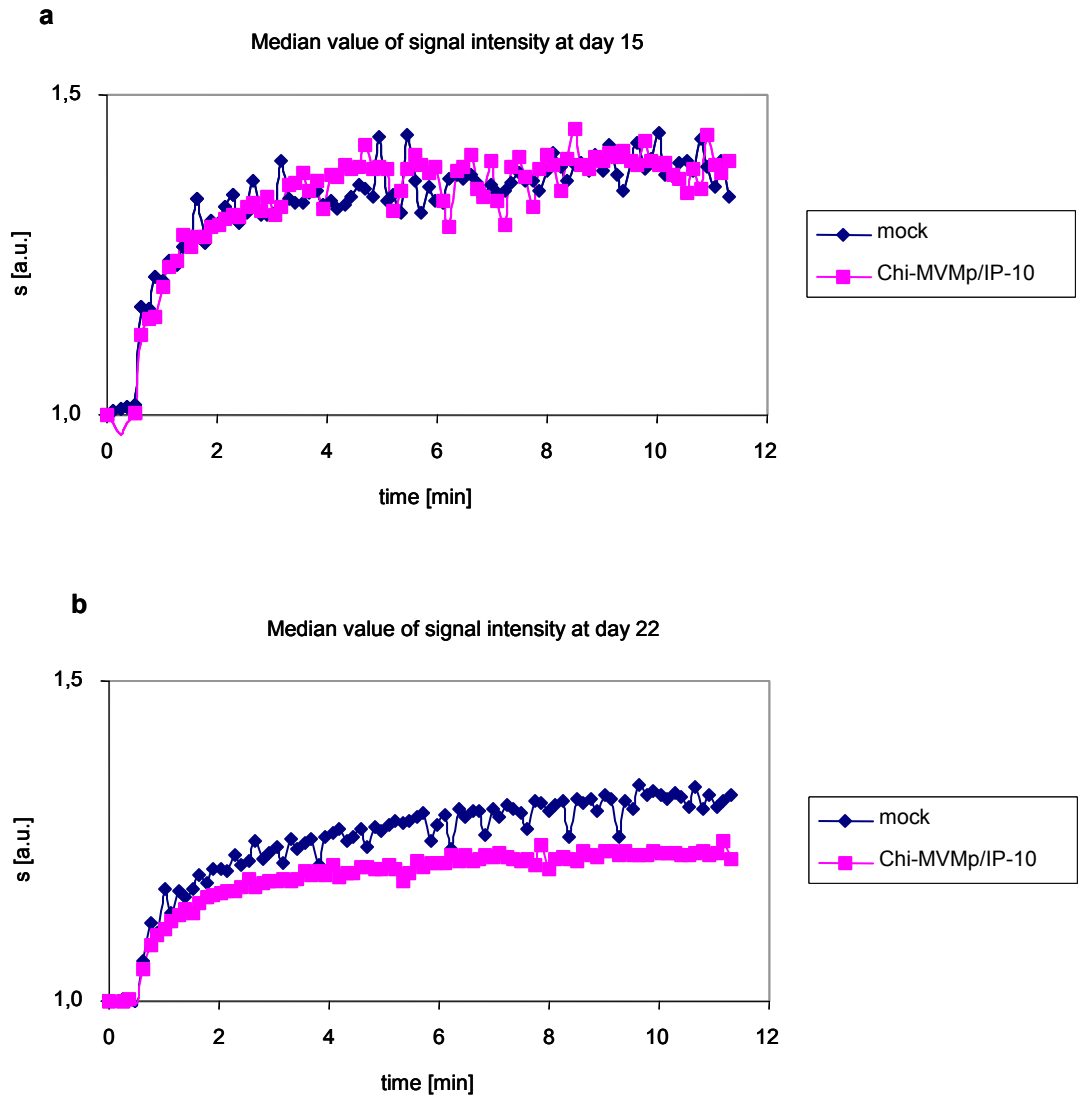


Figure 53 Signal intensity curves
MRI measurement was performed at day 15 (a) and 22 (b) post tumor cell implantation. Signal intensity curves illustrate contrast agent uptake by tumor tissue within 11,3 minutes after its application (s: signal, a.u.: arbitrary units)

At day 15 post tumor cell implantation there was no effect of Chi-MVMp/IP-10 virus on tumor growth observed. The signal intensity measured for both groups remained very similar (see Figure 53a). The uptake of the contract agent within the acquisition time did not demonstrate differences between control “mock” and treated “Chi-MVMp/IP-10” animals. This data suggest that at this time point the density of functional vessels in treated tumors did not decrease although potentially antiangiogenic factor has been applied.

At day 22 a therapeutic effect for “Chi-MVMp/IP-10” group was observed. Enhancement of signal intensity after injection of the contrast agent was for both groups lower compared to day 15. It might be due to developing tumor necrosis and decrease in the number of blood vessels. Contrary to day 15, a difference between experimental groups was observed. The values reached within the acquisition time were significantly lower for “Chi-MVMp/IP-10” animals ($p=0.05$) (see Figure 53b). Observed tendency suggests that the density of functional vessels in treated tumor might be lower compared to control tumors.

After the second measurement the animals were sacrificed, tumor tissue isolated, fixed and analysed. Histological examination evaluated the number of CD31-immunoreactive blood vessels in tumor slices and the percentage of necrotic areas. Necrosis development was similar for both groups. Percentage of necrotic area in control tumors amounted to 21% and in virus-treated tumors – to 24%. Density of CD31-immunoreactive blood vessels density was in “mock” group 21 vessels/mm^2 and for “Chi-MVMp/IP-10” group 18,4 vessels/mm^2. The differences between the values both in case of necrosis and vascular density evaluation remain statistically insignificant. However, histological data correspond to the results obtained with MRI measurement and show that there is a slight tendency for the decreased blood vessel density in Chi-MVMp/IP-10 treated tumors.

5 Discussion

5.1 The effects mediated by parvoviruses on glioblastoma cell cultures

Autonomous rodent parvoviruses and recombinant derivatives are promising candidate antitumor vectors (reviewed by Cornelis J.J. et al. 2004). Constructing parvoviral vectors encoding therapeutic products has been already reported. Such vectors were successfully used against different types of experimental tumors (Haag A. et al. 2000, Wetzel K. et al. 2001, Giese N.A. et al. 2002).

In this work, human IP-10 and mouse TNFα cDNA were cloned into basic chimeric vectors in order to evaluate their antitumoral potential against glioblastoma.

The effects mediated by the parvoviruses are strictly dependent on the cell line. Even cells from the same origin (e.g. melanoma) show different permissiveness to the virus, different transfection efficacy and different levels of transgene production (Kayser T. 2004). In this work 6 glioblastoma cell lines were examined: 4 human (A172, U87, U373 and U138) and 2 murine ones (Gl261 and MT539). Human cells were treated with H1 wild type virus or its derivatives and mouse cells – with MVMp wild type virus or its derivatives.

High infectability (many cells get infected) is desired for gene therapy with viral vectors. The number of infected cells usually correlates with the efficiency of transgene production by the cell population and increases the chances for effective cancer therapy. Infectability of glioma cells was determined by infecting the cells with a virus containing in its genome a gene coding for EGFP (Enhanced Green Fluorescing Protein). Cells of all tested lines expressed the reporter gene upon infection. When infected with the wild type viruses (as well as with different recombinant constructs) the major viral NS1 protein was detectable in cells of all lines. MVMp and H1 viruses produced cytotoxic effect in glioblastoma cell cultures even when cells were infected at low MOIs.

In other study, the effects of the parvovirus H1 on different established glioblastoma cell lines of rat and human origin and on short-term/low-passage cultures of human glioblastoma cells were evaluated (Herrero Y Calle M. et al. 2004). An efficient and dose-dependent killing of all glioma cell cultures at low MOI was observed.

In proposed treatment models parvoviral vectors should not only directly kill tumor cells but first of all serve as effective transgene transducers. IP-10 expression could reduce tumor angiogenesis and stimulate antitumoral immune response. TNFα expression could stimulate immune response against tumor cells and possibly contribute to the antiangiogenesis. ELISA tests done on supernatant collected from cell cultures infected with transgene-expressing vectors proved high effectiveness of transgene protein production in glioma cells. The IP-10 and TNFα concentrations were relatively high for all tested cell lines and accumulated up to 4-5 days post infection, indicating that the transduced proteins remained stable in the cell culture supernatants. The peak of transgene production was reached at day 2-3 post infection, what is a typical picture of transgene release after cell infection with parvoviral vectors (Wetzel K. 2001, Kayser T. 2004).

Infection with IP-10-expresing virus did not seem to affect cell survival. This was expected since IP-10 was not reported to have cytotoxic properties. Infection with TNFα-expressing vectors resulted in a dramatic decrease in the number of living cells and massive cell death in Gl261 cell culture. TNFα is known as cytotoxic agent for many different tumor cell lines. In order to verify if tested glioblastoma cells are sensitive to TNFα, cytotoxicity tests with recombinant cytokine were performed.

Murine cells but not human glioblastoma showed dose-dependent sensitivity to TNFα. It explains the observed cell death upon infection with TNF-expressing vector. Transgene-related toxicity contributes to cell death *in vitro* and shows in addition that virus-transduced TNFα is a fully biologic active protein.

Many cell types need a “sensitizer” to become susceptible to TNFα-mediated killing. A cycloheximide may be such a factor (Kaszubowska L. et al. 2001). Cytotoxicity tests on human glioma cells were repeated in the presence of different cycloheximide concentrations. No sensitizing to cytotoxic action of TNFα could be observed.

In mouse gliomas we could observe TNFα-induced toxicity at very low MOI (MOI1). Similarly, Moriuchi and co-workers could induce cytotoxicity in rat gliosarcoma 9L model at low MOIs. Replication-defective HSV vectors expressing herpes simplex virus thymidine kinase (HSV-TK) and *Escherichia coli* cytosine deaminase (CD) were used in the “sucide” gene therapy protocol. In cell culture experiments at MOI 0,1 and MOI3, combined expression of the two genes along with exposure to the matching prodrugs (ganciclovir and 5-fluorocytosine) showed increased cytotoxicity compared with exposure to either prodrug alone (Moriuchi S. et al. 2002).

Others demonstrated apoptosis induction in 9L and C6 rat glioma cells in culture upon infection with vaccinia virus vectors expressing p53 protein. A prolonged p53 protein production was measured in the supernatants of glioma cells infected with vaccinia virus expressing p53 (VV-TK-53) on day 5 to 7 after infection (Timiryasova T.M. et al. 2001). Analogous to our vectors, vaccinia virus used in this work was replication-deficient. Inactivation of viral DNA replication was obtained by treatment with psoralen and UV irradiation.

The same transgene induced by means of different viral vectors can produce contradictory effects. Apoptosis induced in human glioma cell culture (Gli36) was reported for herpes simplex-derived TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Within 24 hours of infection apoptotic cells could be detected (Shah K. et al. 2003). Infection of human malignant glioma cell lines with an adenoviral vector encoding full length human TRAIL (Ad/TRAIL) resulted in strong transgene expression and the release of full-length TRAIL into the cell culture medium. However, Ad/TRAIL was a poor inducer of cell death, even in the presence of inhibitors of protein synthesis. The adenovirally encoded full-length TRAIL is not a suitable molecule for glioma cancer gene therapy (Naumann U. et al. 2003).

Next to the studies performed in the established cell lines there are reports about studies completed in the short term cultures obtained from brain tumor biopsies or organotypic cultures. Maleniak and co-workers compared the results of gene therapy with the chemosensitivity in short-term human glioma cell cultures derived from surgical biopsies. Expression of herpes simplex virus type 1-thymidine kinase followed by gancyclovir treatment, induced apoptosis in all of the glioma cell cultures studied. Expression of murine Fas ligand also induced cell death in four of the five cell cultures studied. The results suggest also that sensitivity to chemotherapeutic agents does not necessarily correlate with the sensitivity to gene therapy treatments. Recombinant adenoviruses expressing therapeutic gene products in human glioma cell cultures are able to induce apoptosis even in some cells that are resistant to a commonly used chemotherapeutic agent (Maleniak T.C. et al. 2001).

Organotypic cultures are simulating *in vivo* conditions better then cell cultures. Vesicular stomatitis virus (VSV) has been used as direct oncolytic agent in the organotypic brain tissue slice-glioma coculture system. Both replication-competent and replication-restricted recombinant VSV vectors were evaluated. The tissue slices still showed signs of cytopathology when exposed to rVSV-wt. In contrast, pretreatment with

IFNβ and inoculation with a replication-restricted vector with its capsid glycoprotein gene deleted effectively destroyed rat C6 and human U87 glioma cells in the coculture, without causing detectable damage to the neuronal integrity and electrophysiological properties of the healthy tissue in the culture (Duntsch C.D. et al. 2004). It is an interesting observation and may have some implications also in the parvovirus-treated glioma models. Our vectors induce immunestimulatory molecules and are replication-deficient, similarly like those presented in the work of Duntsch and co-workers.

In the majority of research in gene therapy of glioblastoma modified viruses were employed. Due to the safety reasons the viruses are often deprived of the property to form progeny particles. As reviewed above cloning into the viral vectors a death ligand, cytokine or chemokine sequence or an enzyme sequence suitable for certain enzyme/prodrug protocol may produce vectors demonstrating ability to kill glioma cells under cell culture conditions.

The above-mentioned examples of the use of viruses as vectors in the gene therapy of glioblastoma clearly demonstrate that the choice of the vector and transgene plays a key role. Recombinant parvoviral vectors designed in this work seem to posses the most desired features for the gene therapy of glioblastoma. They could effectively infect different and human cells. The infection at low MOI leads either to cell population death or delayed growth (for wild type viruses). A high but transient therapeutic transgene production was reached for all tested cell lines upon infection with recombinant vectors at MOI1. One of the transgenes of choice (TNFα) exploited its cytotoxic activity against mouse glioma cells in culture.

5.2 Glioblastoma animal models

A wide variety of different animal models have been established in neurooncology over the last decades. Murine, canine and feline models have been described (Castro M.G. et al. 2003); however, the most widely used experimental animals are rats. It should be recognized that no currently available animal tumor model exactly reproduces human brain tumor growth and vascularization. The question whether a syngenic immunocompetent animal model (like Gl261 cells in the C57/Bl6 strain) or human xenografting into immunocompromized hosts (like U87 cells in the cd1 swiss nu/nu strain) is closer to the human *in situ* situation cannot be answered definitely. Nevertheless, animal models have several characteristics that make them good test

systems, including defined and reproducible location of tumor formation, rate of tumor growth and time to death.

In this work the growth of tumours derived from 4 human established cell lines in nude mice was tested.

Subcutaneous implantation of U373 cells did not lead to tumor growth. Injection of $1x10^7$ cells per animal caused an inflammatory reaction and tumor development only in a few animals. Palma et al. obtained tumor growth upon subcutaneous injection of $2x10^7$ U373 cells into the flanks of female nude mice (Palma C. et al. 2000). In another work mouse NK (natural killer) cells, monocytes and fetal thymocytes were inhibited by an intraperitoneal antibodies injection prior to subcutaneous tumor cell implantation. This potentiated tumor growth by inhibiting the rejection. $6x10^6$ U373 cells per animal were used (Li C. et al. 2000). It seems possible to obtain U373-derived tumors in nude mice at special conditions but in our hands it remained unsuccessful.

The U138 cell line is used for many *in vitro* but not *in vivo* studies. It is commonly used for experiments in cell cultures (for example apoptosis studies, Jiang Z. et al. 2004) but there is no data about the use of these cells in animal experiments. Under our conditions, although $1x10^7$ cells per animal were implanted, a tumor formation and growth was not obtained.

A172 cells are widely used in *in vitro* research. For example, with U373, U138 and U87 cell lines it was used for the investigation of the effect of the chemotherapeutic agent – temozolomide – in cultured glioblastoma cells in culture (Balzarotti M. et al. 2004). For *in vivo* experiments A172 cells were described as nontumorigenic and noninvasive in the subcutaneous and in the tracheal graft system in scid mice (Mercapide J. et al. 2003) as well as in the intracranial nude rat model (Finkelstein, S.D. et al. 1994). A172 cell implantation induced tumor growth, which, however, was very slow and did not correlate with the number of injected cells. For this reason this cell line was not considered for future experiments.

U87 is the most commonly used among human glioma-derived established cell lines, both *in vitro* and *in vivo* (Goldbrunner R.H. et al. 2000). These cells can be implanted subcutaneously in nude mice as a suspension (Wang H. et al. 2004) or as third generation xenografts (Huang P. et al. 1995). Intracranial models in nude or scid mice are also employed (Zhang Y. et al. 2004).

At our conditions subcutaneous implantation of these cells in cd1 swiss nu/nu mice lead to a desired, moderate tumor growth. The tumor enlargement ratio was

proportional to the number of implanted cells. Tumor development was similar for the animals belonging to one group. No inflammatory reaction was observed. $1x10^5$ and $5x10^5$ cells per animal were chosen as the optimal cell number for the animal studies.

5.3 Recombinant parvoviruses demonstrate antitumor effect in U87 glioblastoma model

5.3.1 Subcutaneous localisation of glioblastoma-derived tumors

The subcutaneous nude mice U87 model used in this work has several advantages as well as disadvantages. Animal glioma models poorly resemble the human glioma growth pattern. For that reason xenografting in the immunocompromized animals seems to be an optimal approach for studying the biology of human-derived tumors. In contrast to this big advantage, nude mice do not provide conditions to modulate and evaluate entire antitumor immune response.

Human brain tumor growth and vascularisation can only be stimulated in an orthotopic model. There is a general consensus that the invasive growth pattern of human glioma is a result of complex interactions of glioma cells with the brain cells and the brain-specific extracellular matrix (Goldbrunner R.H. et al. 2000). Therefore, the biggest inconvenience of using this model is the subcutaneous localisation of the tumor; however, it allows its easy monitoring (also with the available MRI technique) and isolation. The choice of the subcutaneous model is also a consequence of the moderate effectiveness of recombinant parvovirus production. Infecting adherent cells in culture can be performed with virus stocks of low concentration. Applying a reasonable number of viral vectors intracranially would require highly concentrated stocks, which are currently not available.

Using cell suspension allows also precisely defining the ratio between tumor cells and viral particles. It is required for the preliminary experiments. Such a control is not possible when tissue pieces are used; however, transplant physiology remains closer to natural tumor development and vascularisation.

5.3.2 Antitumoral effects

IP-10 and TNF are known for their immune stimulating and antiangiogenic properties (Angiolillo A.L. et al. 1995; Grell M. et al. 1995). Our study was based on the hypothesis that antitumoral effects of IP-10 and TNFα - transducing parvoviruses could be mediated both thorough the activation of the host immune response and antiangiogenesis. Taking advantage of these properties is a critical point in the use of recombinant parvoviruses expressing IP-10 and TNFα against glioblastoma. In the nude mice an antitumoral effect could be observed when simultaneous expression of these proteins was produced in U87 cells. None of them alone was demonstrating therapeutic effects. At the MOIs that were used for the animal studies (MOI 2 and MOI 3) neither VP-deleted basic vector Chi-H1/Δ800 nor H1 wild type virus was demonstrating a therapeutic effect. This means that the transgene expression achieved by cell infection with recombinant vectors is critical for the antitumoral response. Very low MOIs were used in order to distinguish between toxicity of the vector (mediated through NS1 protein) and the therapeutic effects of transgenes. Since low MOIs were shown to be effective without producing side effects *in vivo*, it is still possible to increase the doses of recombinant vectors.

These data suggest that parvoviral vectors are very suitable for IP-10 and TNFα delivery in the model described. In case of gene therapy with chemokines/cytokines transient expression that can be obtained after infection with parvoviral vectors is an advantage. Overdoses of such proteins could cause deleterious side effects. If necessary, prolonged expression of therapeutic transgene could be reached upon repeated administration of parvoviral vectors. Another advantage of parvoviral system is that high levels of therapeutic transgene can be reached upon infection at low MOI. Parvoviruses are weakly immunogenic what allows repeated administration without side effects and does not lead to a strong immune response. Finally, autonomous parvoviruses do not integrate into the host genome and do not belong to potentially oncogenic viruses.

In contrast to our data, Huang and co-workers observed a significant antitumoral effect of exclusive TNFα administration in subcutaneous U87 model (Huang P. et al. 1995). The animals received intraperitoneally 3,8 µg TFNα per animal for 7 days (approximate TNFα expression from parvovirus-infected cells reached approximately 1µg/day). A higher cytokine dose and its systemic administration could explain the

differences in tumor development observed between parvovirus- and recombinant-protein- treated animals.

Although in our system the production of two immunostimulating transgenes reproducibly resulted in significant antitumoral effects, there was no animal cure. Complete tumor regression upon induction of an immunomodulator was demonstrated for IL-4 – treated subcutaneous tumors. The mixture of U87 and stably transfected plasmocytoma cells (LT-1) expressing high levels of IL-4 was implanted subcutaneously or intracranially. In the intracranial model IL-4 expression resulted in a significant survival prolongation and increased eosinophil infiltration (Yu J.S. et al. 1993). Modulation of immune response seems to be effective in the treatment of gliomas.

Similarly to our data, no complete regression or inhibition of tumor growth was observed for Burkitt lymphoma subcutaneous tumors upon treatment with IP-10 alone. Purified human chemokine was administered intratumorally over a period of 30 to 35 days. Despite of continuous application, IP-10 alone did not demonstrate antitumor effect (Sgadari C. et al. 1996).

Our study hypothesizes that both immunostimulating and antiangiogenic properties of IP-10 and TNFα could generate antitumoral effects. There exist several studies showing that successful treatment of U87-derived tumors could be achieved by antiangiogenic therapy alone or by combining antiangiogenic factors with another one (Abdollahi A. et al. 2003, Kirsch M. et al. 1998, Bello L. et al. 2001, Lund E.L. et al. 2000).

A combination of a “direct” and an “indirect” antiangiogenic component – endostatin (affecting endothelial cells) and SU5416 (a VEGFR2 receptor kinase inhibitor) respectively – was used for the treatment of subcutaneous U87 tumors. Tumor growth was significantly delayed by each therapy alone; however, the combination demonstrated the best therapeutic effect (Abdollahi A. et al. 2003).

Human angiostatin produced from human plasma was used for the treatment of second generation xenografts derived from U87 cells. Applying 1 mg of angiostatin by intraperitoneal injections every 12 hours over a period of 21 days resulted in tumor weight reduction to 16% of the control tumor mass (evaluated at day 22 of the experiment) (Kirsch M. et al. 1998). We demonstrated with recombinant parvoviruses a significant tumor volume reduction at day 24 after tumor cell implantation. At our conditions a transient expression of the nanogram amounts of IP-10 and TNFα was sufficient to produce such an effect.

We combined an antiangiogenic and immunostimulatory molecule (IP-10) with an immunostimulator (TNFα) what resulted with the most pronounced therapeutical effect. In another approach recombinant human PEX (a fragment of matrix metalloproteinase-2) was used as an antiangiogenic factor that has been combined with low-dose chemotherapy. This treatment improved survival of the animals and reduced glioma growth *in vivo* (U87 cells implanted intracranially) compared to each therapy alone (Bello L. et al. 2001).

TNP-470 is an inhibitor of endothelial cell proliferation. Subcutaneous U87 xenografts were treated with irradiation, TNP-470 or their combination. Both irradiation and TNP-470 significantly inhibited growth of the tumors and a significantly enhanced effect was obtained by the combination of the treatments (Lund E.L. et al. 2000).

Taken together, different experimental data obtained in U87 animal model suggest that modulating immune response is a promising approach in the therapy of glioblastoma. Inhibiting angiogenesis and especially combining antiangiogenic factors with other approaches like immunostimulation, chemotherapy or radiation seems to be very accurate method in the treatment of glioblastoma tumors.

The results obtained with parvoviral vectors expressing IP-10 or TNFα are very promising, comparing to the literature data. Our approach reduced tumor growth and prolonged animal survival although very low amounts of the vectors were used, inducing nanogram levels of therapeutic proteins.

5.3.2.1 Antitumoral effect is independent from tumor necrosis

Tumor necrosis is a result of blood vessel damage, usually progressing from the tumor center. Such morphological changes could be a result of both IP-10 and TNFα action either directly on endothelial cells or indirectly by changing the cytokine milieu within the tumor and in its surroundings (Huang P. et al. 1995; Sgadari C. et al 1996). Because vascular changes were expected in the tumors derived from cells expressing these proteins, a histological examination was performed to evaluate the necrotic area and the density of blood vessels.

Analyzing tumor tissues collected at the same time point (U87 cells infected at MOI3; animals sacrificed at day 24) demonstrated differences in necrotic area. Surprisingly, expression of IP-10 and especially IP-10/TNF combination seems to reduce necrosis development. Although IP-10/TNFα expression significantly reduced

the tumor volume, it was not corresponding to necrosis development. The percentage of necrotic area of 5% was the smallest among the groups under test. This result was significantly lower - 30%, 55% and 30%, respectively - than in tumors of groups “mock”, “Chi-H1/Δ800” and “H1 wt”.

In contrast to our results, there are several reports in the literature describing TNFα and IP-10 and factors that promote tumor necrosis development however in other tumor models (Huang P. et al. 1995, Sgadari C. et al. 1996).

Animals bearing third generation U87 subcutaneous xenografts were treated with systemic TNFα administration. Tumors in TNFα-treated mice were significantly more necrotic compared to untreated animals. As hypothetical antitumoral mechanisms the authors proposed NK cells stimulation and blood vessel damage (Huang P. et al. 1995).

IP-10 may also participate in tumor necrosis development. In the work of Sgadari et al. established Burkitt lymphoma subcutaneous tumors (in nude mice) were treated with continuous (30 to 35 days) intratumoral injections of purified human IP-10. Chemokine - treated tumors demonstrated 20-70% necrosis whereas no necrosis was observed in control tumors. There was a tendency for vessel disruption (elastin fiber fragmentation) in IP-10 treated tumors but no clear antitumoral effect (Sgadari C. et al. 1996). This data suggests that depending on the system IP-10 can demonstrate different effects on tumor physiology, although they are not always connected with tumor regression or growth inhibition.

The influence of another immunomodulator on tumor necrosis was analysed by Yu and co-workers. IL-4 was induced in subcutaneously and intracranial U87 tumors. 3 or 4 days post tumor cell implantation the tissue was isolated and analyzed for necrosis development. The group reported that IL-4-expressing tumors developed necrosis in contrast to untreated tumors (both subcutaneously and intracranially). (Yu J.S. et al. 1993). Nevertheless, histological analysis of tumor samples 3 to 4 days post tumor cell implantation remains controversial.

TNFα can modulate the haemostatic properties of endothelial cells, antigen expression and is able to directly kill endothelial cells. It can lead to hemorrhages and blockage of tumor blood vessels and, consequently, to tumor necrosis (Robak T. 1995), also in the U87 subcutaneous model (Huang P. et al. 1995). It is possible that tumor cells infected at MOI3 do not provide cytokine concentration sufficient to induce necrosis in the examined system. Transient transgene expression might also be not adequate to cause such an effect.

IP-10 expression does not promote necrosis in the subcutaneous U87 model as well.

Reduced tumor necrosis observed in the TNFα/IP-10 group might simply correspond to the reduced tumor size and other physiological statuses of small tumors compared to big ones. Tumor necrosis induction does not seem to be the key mechanism of observed for a TNFα/IP-10 - combination antitumoral response.

5.3.2.2 Antitumoral effect is independent from angiogenesis inhibition

In the model we used TNFα expression by parvoviruses had only a moderate effect on tumor growth, but it significantly reduced blood vessel density. IP-10 expression (either alone or in combination with TNFα) only moderately decreased the blood vessel number (the differences are not statistically significant).

Only a combined IP-10 and TNFα expression has a therapeutic effect in the U87 model. This effect does not seem to be due exclusively to the inhibition of angiogenesis.

Nevertheless, antiangiogenic approaches can be effective in the glioma treatment, as was also shown with the U87 model. The use of antiangiogenic factors often succeeds with the decrease of the blood density in treated tumors. There exist several studies showing that the choice of the right antiangiogenic factor plays a pivotal role. Synergistic effects of combined methods might be observed.

A reduction of blood perfusion was observed after treatment either with endostatin or SU5416. The combination treatment resulted in a further reduction in tumor blood perfusion. It corresponded to the antitumoral effect. In the intracranial window model of the A549 lung carcinoma there was no difference in tumor growth, although, functional vessel density was significantly decreased for the combined therapy (Abdollahi A. et al. 2003). These data stay in agreement with our results, where reduced blood vessel density was found in TNFα - treated tumors. Nevertheless, this phenomenon was not corresponding to antitumoral effect.

The treatment with angiostatin significantly decreased the microvessel count in U87 subcutaneous tumors (Kirsch M. et al. 1998). Such an effect could be also observed when U87 intracranial tumors were treated with combined administration of low-dose chemotherapy plus antiangiogenic treatment (human PEX - a fragment of matrix metalloproteinase-2 was - used as an antiangiogenic factor). These tumors

showed the lowest microvessel count, similar to that observed for antiangiogenic treatment alone (Bello L. et al. 2001).

TNP-470 is an inhibitor of endothelial cell proliferation. Subcutaneous U87 xenografts were treated with irradiation, TNP-470 or their combination. After 1 week of TNP-470 treatment, no significant difference in vascular density was seen between treated and control tumors, despite a pronounced growth-retarding effect of TNP-470. Similar treatment schedules with TNP-470 had no effect on mice with intracranial tumors, given alone or in combination with irradiation. This discrepancy might reflect differences in tumor vessels due to differences in the tumor microenvironment (Lund E.L. et al. 2000).

In our work combining antiangiogenic and immunostimulating factor (IP-10) with an immunomodulator (TNFα) - that could in parallel demonstrate antiangiogenetic properties as well - did not result in decreased blood vessel density. TNFα appears to be the exclusive factor that inhibits blood vessel formation in subcutaneous U87 model.

The work of Lund et al. provides evidence that an antitumoral effect might be tightly linked to the tumor microenvironment. This thesis is strongly supported by the results of Roberts et al. who showed that vascular density was increased in U87-derived intracranial tumors compared to subcutaneously localized tumors (by a factor of 2). Intracranial tumors had drastically reduced the percentage of vessels with fenestrated endothelium and opened gaps compared with the subcutaneous tumors (Roberts W.G. et al. 1998).

Notably, antitumoral effects (tumor growth delay and prolonged survival) mediated by antiangiogenic factor (TNP-470) do not always correspond to the reduction of blood vessel density (Lund E.L. et al. 2000) and decreased blood vessel formation does not always result in therapeutic effects (Abdollahi A. et al. 2003; Sgadari C. et al. 1996, this work).

In this work significant antiangiogenetic effects could be demonstrated only when TNFα-expressing vector was used alone. In order to obtain combined IP-10/TNFα expression tumor cells were infected with both vectors at MOI 1,5 for each. This could result in a lower expression of TNFα compared to the expression reached upon infecting tumor cells with a single vector at MOI3. It might be the reason for the lack of antiangiogenic effects for an IP-10/TNFα combination. IP-10 is not known to modulate TNFα expression (Sgadari C. et al. 1996). Both factors show individual properties and their combination might produce some extraordinary effects *in vivo.*

TNFα is a major inflammatory cytokine and also regulates angiogenesis. However, studies on the antiangiogenetic properties of TNFα yield contradictory results. TNFα induces angiogenesis *in vivo* and stimulates endothelial cell migration *in vitro.* It can inhibit the action of factors such as VEGF and bFGF in endothelial cell growth *in vitro*. Angiogenetic properties of this cytokine might be mediated through a variety of secondary factors like PDGF, VEGF, IL-8 and bFGF. At a low concentration, TNFα can induce angiogenesis; at a high concentration TNFα promotes endothelial cell death (Chen J.X. et al. 2003). It is possible that infection with the recombinant vector at MOI3 produced TNFα levels that were high enough to switch on the antiangiogenic effects.

TNFα and IFNγ can individually inhibit MMP-2 expression in human glioblastoma cells (U251 and CRT) (Quin H. et al. 1998). Human glioma cells SNB19 are able to induce the formation of capillary-like structures by human endothelial cells. When tumor cells were infected with a recombinant adenovirus expressing antisense uPAR (urokinase plasminogen activator) Ad-uPAR at a MOI of 100 this process was inhibited by approximately 50%. Around 80% inhibition of capillary formation was observed with recombinant adenovirus expressing antisense MMP-9 (matrix metalloproteinase), Ad-MMP-9, at a MOI of 10. Treatment of established subcutaneous U87 tumors with intratumoral injections of the vector expressing simultaneously antisense uPAR and antisense MMP-9 resulted in 80% growth reduction compared to the control vector (Lakka S.S et al. 2003).

Taken together, these data suggest that TNFα expression with parvoviral vectors might inhibit angiogenesis by decreasing matrix metalloproteinases.

5.3.2.3 Antitumoral effect might be NK cell-dependent

NK cells are able to eliminate tumor cells without previous immunization. The cytotoxic effect of NK cells is not MHC-restricted (Jakobisiak M. et al. 2000). Nude mice have elevated levels of both macrophages and NK cells, which might play an important role in the antitumoral response in these animals (Research Animal Review 1(2) 1996).

The analysis of an antiangiogenic mechanism of IL-12 in the Burkitt lymphoma nude mice model showed that this process is NK cell-dependent. IL-12 stimulated IP-10 expression (upon the activation of IFNγ production). This might account for the presence of NK cells at the sites of inhibited vascularization, since IP-10 was also detected at the same sites (Yao L. et al. 1999).

In a work published by Ahmed and co-workers, 2001, nude mice bearing subcutaneous U87-derived tumors were treated with recombinant adenoviral vector expressing human interferon α2b. Intratumoral and intravenous administration significantly reduced tumor growth (Ahmed C.M. et al. 2001). IFNα and IL-12 are potent stimulators of NK cell activity. IL-12 was earlier described as an NK-cell stimulating factor (NKSF).

Both TNFα and IP-10 act chemotactically on monocytes and NK cells and increase the cytotoxic activity of NK cells (Jakobisiak M. et al. 2000). It is possible that mutual, transient expression of both proteins by recombinant parvoviruses provides optimal conditions for the development of an antitumoral response against U87-derived tumors in nude mice. Data from the literature suggest that this process could be NK cell-dependent and mediated both indirectly through angiogenesis inhibition (Yao L. et al. 1999) and directly through increased lysis of tumor cells. TNFα-treated (100 U/ml, 48 hours) U87 cells have increased susceptibility to lysis by NK cells and this effect is dependent on the increased expression of ICAM-1 molecules by target cells. TNFα at concentrations ranging between 1 and 100 U/ml was not cytotoxic to tumor cells; similar to our conditions (Kondo S. et al. 1994).

5.3.3 The effects of the vectors on the U87 cell culture

Infection of U87 cell culture provides a slightly different pattern of virus-mediated effects from those observed in the animals. At MOI2 Chi-H1/Δ800, Chi-H1/IP-10 and the combination Chi-H1/IP-10+TNFps. did not influence cell culture growth in comparison to the growth of non-infected culture. H1 wild type virus–infected cells were growing slower and their number decreased at day 6 post infection. H1 wild type virus has a cytotoxic effect on U87 cells in culture, but not on *in vivo* implanted cells.

Yamini and co-workers observed a relationship between *in vitro* and *in vivo* effects in U87 model. The cells were infected with adenoviral vector encoding for TNFα at MOI 100 (50 times higher compared to our vector) and subsequently treated with temozolamide. Compared to each treatment alone cell viability was significantly reduced. Combined treatment resulted *in vivo* with significant reduction of subcutaneous tumors volume. Induction of apoptosis contributed both to cell death *in vitro* and antitumoral effect *in vivo* (Yamini B. et al. 2004).

In our system cell death in culture did not correspond to the antitumor effect observed *in vivo* (H1 wild type virus). Combined IP-10/TNFα expression did not affect cell culture growth but produced antitumoral effect in the animals. Probably *in vivo* conditions provide special factors, which allow developing tumors to overcome virus-related toxicity. Secreted therapeutic proteins are obviously interacting with the host environment, since their expression inhibits tumor growth *in vivo* but does not affect cell growth *in vitro.*

5.4 Recombinant parvoviruses display antitumor effect in Gl261 glioblastoma model

5.4.1 Gl261 subcutaneous model

Seligman and Shear induced “glioma 261” in 1939 by chemical carcinogen implantation into brains of mice (Seligman A.M., Shear M.J. 1939). Since they were established, Gl261 cells are used for inducing both intracranial (Ausman J.I. et al. 1970; Ehtesham M. et al. 2002; Saito R. et al. 2004) and subcutaneous (Miyatake S. et al. 1997; Schueneman A.J. et al. 2003) tumors. It is an interesting intracranial model, because it recapitulates many of the histopathological and biological features of human high-grade glioma including both necrosis and invasion of the brain adjacent to the tumor (Newcomb E.W. et al. 2004). In this study it was shown that Gl261 cells injected subcutaneously induce tumor growth.

5.4.2 Antitumor effects in the animals

Our study was based on the hypothesis that antitumoral effects of IP-10 - and TNFα - transducing parvoviruses may be mediated both through the activation of the host immune response and antiangiogenesis. Direct parvovirus-mediated tumor cell killing is possible and since Gl261 glioma cells are very sensitive to parvoviral infection *in vitro*, this may also participate in the antitumoral effect *in vivo*.

In vitro infection of tumor cells prior their subcutaneous implantation in the animals resulted with the antitumoral effect that was dependent on the MOI and the transgene. Delayed tumor growth and prolonged survival was observed for animals that

received tumor cells infected with the vectors expressing therapeutic transgenes. Combined expression of IP-10 and TNFα upon infection with MOI 3 lead to complete tumor growth inhibition or tumor regression in 80% of animals under test.

If nude mice were used, no such effects could be observed, demonstrating that the antitumoral responses in Gl261 model require an intact immune system.

In *in vivo* experiments the effect of recombinant parvoviruses on established subcutaneous Gl261 tumors was also investigated. Repeated virus injections into the tumor surrounding resulted in delayed tumor growth and prolonged survival for animals that received the vectors expressing therapeutic transgenes while wild type MVMp virus did not produce any therapeutic effect. This suggests that the transgene expression achieved by cell infection with recombinant vectors is critical for the antitumoral response.

Our data show high effectiveness of parvoviral-transduced combined IP-10 and TNFα expression in subcutaneous Gl261 tumors. Ehtesham and co-workers demonstrated high efficacy of adenoviral-induced TNFα expression combined with expression of another immunestimulatory molecule (IFNγ) in the treatment of intracranial GL26 glioma. $5x10^8$ PFUs were administered intracranially 4 days post implantation of tumor cells. Comparing to the initially implanted amounts of tumor cells MOI 50 000 was used. In our studies the total amount of the virus used ranged between MOI 1 and 14. However, similar effects *in vivo* could be observed. The survival for IFNγ-TNFα- and IFNγ/TNFα - treated groups was prolonged but without differences among these groups (noted also for IP-10, TNFα and IP-10/TNFα combination) (Ehtesham M. et al. 2002). These results suggest that parvoviral transgene delivery system is very efficient compared to the adenoviral vectors.

GL261 intracranial tumors may be also successfully treated with another imunemodulator: adenoviral – induced IL-12, what was demonstrated by Liu et al. (Liu Y. et al. 2002). Antitumoral effect upon retroviral gene delivery could be established in another glioma models. Retroviral vectors stably transduced human GL15 and rodent 9L and C6 cells with murine angiostatin, endostatin and IFNγ what resulted in reduced volume of tumors derived from angiostatin- and IFNγ-transduced cells (De Boüard S. et al. 2003)

Lumniczky and co-workers used Gl261 intracranial model for evaluating the efficacy of therapeutic vaccination. Mice bearing Gl261-derived brain tumors were treated with subcutaneous therapeutic vaccines. These vaccines were produced by

infecting Gl261 cells with adenoviral vectors encoding IL-4, IL-6, IL-7, GM-CSF or TNFα. Vaccines expressing IL-6, IL-7 and TNFα were inefficient to prolong the survival of glioma-bearing mice when produced at MOI 10, 50, 100 or 200. GM-CSF-expressing vaccine was the most effective at MOI 10 (10 ng of GM-CSF/1x10^6 cells/24 h). IL-4 – expressing vaccine was effective when cells were transduced at MOI 100 with the adenoviral vector (50 ng of IL-4/1x10^6 cells/24 h). Combined vaccine (IL-4 MOI 100 with GM-CSF at MOI 10) had therapeutic effect similar to GM-CSF – producing vaccine alone (Lumniczky K. et al. 2002).

Compared to the above data, transgene induction in Gl261 model seems to be more effective using recombinant parvoviruses. At day 2 post infection done at MOI 1 Gl261 cells secreted 5000 ng IP-10/1x10^6 cells or 375 ng TNFα/1x10^6 cells.

Another gene therapy approaches that have been reported to be successful in Gl261 glioma model are DNA applications. A cancer treatment is described in which intramuscular injection of plasmid DNA encoding murine interferon α lead to potent antitumor effect demonstrated in subcutaneous B16F10 and Couldman melanoma and Gl261 glioma models (Horton H.M. et al. 1999). Kircheis and co-workers employed polycation-based DNA complexes encoding murine TNFα against subcutaneous Neuro2a tumors. The use of polycation-based DNA complexes resulted in high level tumor-specific TNFα expression without its systemic toxicity (Kircheis R. et al. 2002).
Applying molecules like TNFα, interleukins or chemokines may result with morphological changes in treated tumors. As a consequence necrosis development and vascular changes could be observed.

5.4.3 Antitumoral mechanisms

5.4.3.1 Morphological changes

5.4.3.1.1 Necrosis development corresponds to the antitumor effect

Animal study with *in vitro* infected Gl261 cells (MOI1) provided tumor tissue that has been analyzed for necrosis development. Histological examination showed that transgene expression corresponds to increased necrosis appearance. Combined IP-10/TNFα expression significantly supported necrotic areas development (compared to

mock- and control vector- treated groups). It was not observed for IP-10 expression alone although tumor growth delay and animal survival were similar for both groups.

Analyzing tumors collected after the *in vivo* study supported the observation that in Gl261 model tumor necrosis development corresponds to the antitumoral effect. Treatment with transgene-expressing vectors increased necrotic areas, particularly with the expression of TNFα alone or in combination with IP-10. Since IP-10 alone did not cause such pronounced effect it is possible that in this model TNFα is the main factor responsible for vascular damage and tumor necrosis development. Both transgenes alone as well as their combination had very similar therapeutical effects in animals but necrosis occurrence was strictly dependent on TNFα expression.

Others have also shown tumor necrosis development in subcutaneous murine glioma model produced by TNFα. Intravenous injection of the polycation-based DNA complexes encoding this murine cytokine inhibited tumor growth and induced hemorrhagic tumor necrosis. More than 80% of TNFα-treated animals developed tumor necrosis, whereas this was not found in control animals (Kircheis R. et al. 2002). This example illustrates that, independently of the transduction mode, TNFα might induce vascular damage and necrosis development of glioma tumors in mice.

It was shown that tumors treated with AdTNFα and a combination of AdTNFα and AdmIFNγ may develop necrotic areas in the tumor center in contrast to the tumors treated with AdmIFNγ alone or with control vector (AdLacZ) (Ehtesham M. et al. 2002). Similarly, in our studies (both implantation of infected cells and *in vivo* treatment) tumor necrosis was induced when TNFα-expressing vector were used (either alone or in the combination with IP-10-expressing viruses).

Although IP-10 is a well known inhibitor of angiogenesis, it may also promote vascular damage, and, as a consequence, tumor necrosis (Sgadari C. et al. 1996). Yet this was not observed in our experiments.

Another angiogenesis inhibitor, angiostatin, was reported as antitumor factor in human GL15 and rodent 9L and C6 cells. Angiostatin-expressing tumors were significantly smaller then the controls, however, without reduced vascularization or signs of necrosis (De Boüard et al. 2003). Taken together, these results suggest that the expression of these antingiogenic factors did not cause visible vascular damage and increased tumor necrosis.

5.4.3.1.2 Antitumoral effect does not depend on tumor vascularization

In the *in vivo* study the experimental settings allowed analysing tumor vascularisation at two time points. The animals were divided into two cohorts. The first one contained 3 animals per group and was sacrificed at day 10 of the study. The second cohort contained 8 animals per group and was monitored until the end of experiment.

At day 10 tumor volume remained similar for all the groups. Compared to the buffer-treated group, the expression of transgenes did not show any antiangiogenetic properties at this stage of tumor development. Surprisingly, tumors treated with MVMp wt virus or with the control Δ800 vector demonstrated decreased number of blood vessels. However, it did not yield a therapeutic effect in the later phase of tumor expansion.

The tumors from the second cohort were isolated at the end of experiment. Analyzed tissue samples did not demonstrate any differences in tumor vascularization between the groups.

Although IP-10 or TNFα transgene expression suppressed, to some extend, tumor development, it did not seem to have any influence on the blood vessels formation. Because blood vessel density remained similar in all experimental groups, probably not antiangiogenesis, but another mechanism, participates in the antitumoral effect observed in Gl261 tumor model.

De Boüard and co-workers also showed tumor growth suppression without reduced vascularity. They used retroviral vectors to stably transduce human GL15 and rodent 9L and C6 cells with murine angiostatin, endostatin or IFNα. Angiostatin-expressing tumors were significantly smaller then controls, however, without reduced vascularization. IFNα-expressing tumors were much smaller then controls and demonstrated drastically reduced density of blood vessels. Necrosis of tumor centre could be observed. Antiangiogenic effect of this cytokine seems to be the main antitumor mechanism (De Boüard S. et al. 2003).

Tumor growth suppression and tumor necrosis without decrease in tumor vascular density was observed upon treatment with the combination of murine IFNγ and human TNFα. There was no significant decrease in tumor vascular density in any of the treated mice compared to the control upon adenoviral vectors administration (Ehtesham M. et al. 2002). Similarly, we also did not notice antiangiogenic effect of our parvoviral vectors (apart from the decrease of blood vessel density measured at day 10 of the experiment for MVMp wt- and Δ800 ps. –treated tumors).

Both in the intracranial GL26 model treated with adenoviruses expressing IFNγ or TNFα (or with their combination) and in the subcutaneous Gl261 model treated with parvoviruses expressing IP-10 or TNFα (or with their combination) inhibition of angiogenesis did not contribute to an important antitumor effect.

Blouw et al. investigated in parallel the antitumor effects of HIF-1α (hypoxia-responsive transcription factor 1) deletion in subcutaneous and intracranial environment. The subcutaneous space has a number of intrinsic peculiarities that set it apart from sites elsewhere in the mammalian body. These include a lack of spatial constraints in the form of matrix or skeletal elements, relatively sparse vascularisation, and the interface of a number of different tissue types (fat, muscle, connective tissue) found immediately under the skin. Subcutaneous environment is poorly vascularized, in contrast with highly vascularized brain parenchyma.

The lack of HIF-1α impaired astrocytoma growth subcutaneously, but increased proliferative and invasive properties of astrocytomas in the brain. Intracranial tumors were not necrotic but well vascularized. Subcutaneously, these tumors were poorly vascularized and necrotic. They did not coopt existing vessels in subdermis like they did in the brain (Blouw B. et al. 2003). This study demonstrates that the same antiangiogenic approach used in different environments can either inhibit or support blood vessel formation. Nevertheless, TNFα transduced by different viral vectors either intracranialy (adenoviruses; Ehtesham M. et al. 2002) or subcutaneously (parvoviruses; this work) in the glioma tumors supports vascular damage.

5.4.3.1.3 IP-10 as an antiangiogenic agent in Gl261 subcutaneous model

Animals bearing established tumors treated either with parvovorus-delivered IP-10 or with PBS were submitted twice to MRI measurement. At the earlier time point (day 15) there were no differences in the tumor volume observed. During the second measurement (day 22) mean tumor volume for Chi-MVMp/IP-10 treated animals was smaller then for control animals. For the second measurement the signal intensity was found to be lower for Chi-MVMp/IP-10 - treated animals. It suggests that functional vessel density for this group could be lower compared to the untreated group. However, histological analysis showed similar CD31 – positive blood vessel density and necrosis development for both Chi-MVMp/IP-10 and PBS-treated group. Intravenous injection of the contrast agent allows its spreading exclusively in the functional (opened) blood

vessels. Anti-CD31 staining allows detection of all existing blood vessels independently on their functional status. Even collapsed (closed) vessels (typical for necrotic tumors) would be detectable in histological analysis. This may explain the discrepancy observed between *in vivo* measurement and tumor slice examination. MRI and histology data correspond for tumor necrosis. Necrotic areas were defined in the tumors centre both as signal-negative regions on the colour maps obtained for signal amplitude and exchange parameter and as hematoxilin-negative regions on the tumor slices. The influence of parvovirus-induced IP-10 and TNFα on functional vessel density requires further investigations.

The group of Cha and co-workers developed an MRI protocol to evaluate the growth and vascularity of intracerebrally implanted Gl261 cells. MRI measurements of relative cerebral blood volume (rCBV) were compared to histological assessments of microvascular density (MDV). In late tumors (3-4 weeks post implantation) both central tumor necrosis and angiogenesis could be demonstrated. Histological analysis confirmed these data (Cha S. et al. 2003). In this model angiogenesis is indeed a late event in tumor progression and first occurs close to the stage when the mouse dies of tumor mass (Zagzag D. et al. 2000).

Direct comparison of angiogenesis induced in subcutaneous and intracerebral models is not possible due to differences between these environments as mentioned above (Blouw B. et al. 2003). However, the studies done on intracerebral Gl261 model suggest that, also in the case of a subcutaneous model, angiogenesis could be a later event. Indeed, tumor necrosis, which is believed to accompany angiogenesis in this model, was observed in many animals 3-4 weeks after implantation of tumor cells. In our studies we delivered an antiangiogenic factor (IP-10) at the early stages of tumor development. The fact, that in the Gl261 model angiogenesis is a later event might explain why we were not able to observe any differences in histologically investigated tumor vascularization between experimental groups. TNFα expression alone seemed to promote vascular damage, but not inhibition of angiogenesis. Nevertheless, independently of angiogenesis, in our model, combined expression of parvovirus-transduced IP-10 and TNFα potentiated the antitumor effect observed with TNFα and IP-10 alone, and which could be mediated by immune system components.

5.4.4 The effects of recombinant parvoviruses delivering IP-10 and TNFα effects are different *in vivo* and *in vitro*

In vitro studies performed on the Gl261 cell cultures in parallel to the animal studies demonstrated that pronounced cytotoxic effect *in vitro* was mediated exclusively by TNFα-expressing vector.

Despite of mediating a strong cytotoxic effect *in vitro* infection of cells with TNFα - expressing vector prior implantation in the animals did not yield pronounced antitumoral effect both at MOI1 (no therapeutic effect) or MOI3 (tumor growth delay).

Under cell culture conditions tumor cells were submitted to the action of TNFα accumulated for 6 days in the medium. Under *in vivo* conditions the secreted protein was distributed in the tumor surrounding. Since a great variety of cell types are able to express TNF-receptors it is possible that the parvovirus-produced cytokine was captured by other cells and/or diluted in biological fluids. TNFα amounts remaining within the tumor mass could be not sufficient to mediate antitumor effect through a direct cytotoxic action on Gl261 cells.

Conversely, IP-10 expression *in vitro* did not affect cell culture growth but its expression *in vivo* produced an antitumor effect that was significantly increased by TNFα co-expression. This suggests that the co-expression of virus-delivered agents indeed modulate host antitumoral response through chemoattraction and activation of the immune system cells.

5.5 Antitumoral immune response

5.5.1 Experimental animals develop immune response against tumor cells

Viral vectors could modulate development of the immune response against tumors, first, by providing viral components, second, by providing the expression of immunomodulating proteins and/or inducing the expression of specific cellular genes.

Performed rechallenges proved that animals, which survived initial tumor implantation, were protected from the tumor formation upon second injection of Gl261 cells. This protection was not dependent on the presence of therapeutic transgene. ELISpot assays showed a specific recognition of tumor cells by host cells obtained from the spleen. Which cell subset contributed to this effect remains unknown. From the literature it is not clear which cells, $CD4^+$ or $CD8^+$, play the major role in antitumoral

immune response (Segal B. M. et al. 2002, Lumniczky K. et al. 2002, Horton H.M. et al. 1999, Yang S.Y. et al. 2004).

Segal and co-workers reported that protective immunity against Gl261 glioma subcutaneous tumors can be obtained by intraperitoneal injection of irradieted Gl261 cells. $CD4^+$T but not $CD8^+$ T cells were necessary to reject implanted tumor cells. In agreement with this, $CD8^+$T cells purified from the spleens of Gl261-vaccinated mice failed to lyse glioma targets in chromium release assay (Segal B. M. et al. 2002).
In a therapeutic vaccination approach against intracranial Gl261-derived tumors depletion of either $CD4^+$ or $CD8^+$ lymphocytes equally prevented the antitumor effect of the vaccine (Lumniczky K. et al. 2002).

On the contrary to the above-mentioned results other authors showed that the antitumor effect in experimental glioma models is rather $CD8^+$ cell – dependent.

A treatment is described in which intramuscular injection of plasmid DNA encoding murine interfernon α lead to potent antitumor effect demonstrated in subcutaneous B16F10 and Couldman melanoma and Gl261 glioma models. The same therapy performed in nude mice (T cell deficient) and beige-nude mice (NK- and T cell – deficient) showed that T cells are required for antitumor response. Depleting $CD8^+$ or $CD4^+$ cell subsets in immunocompetent animals showed that therapeutic effect was $CD8^+$ - dependent (Horton H.M. et al. 1999).

In the Gl26 intracranial model the treatment with adenoviral-induced IL-12 demonstrated therapeutic effect. IL-12 – treated animals with a long-time survival were rechallenged intracranially. They did not develop the tumors suggesting that long-lasting protection was obtained. Tumor infiltrating leukocytes (TILs) isolated from IL-12 - treated animals displayed increased cytotoxicity against Gl26 cells (Liu Y. et al. 2002).

Functional analyses demonstrate that $CD8^+$ cells seem to play the major role in the antitumoral response in glioma models. Histological data may support this hypothesis. A strong infiltration of rat brain tumors with $CD4^+$ and especially $CD8^+$ cells could be observed when human embryonal neural stem cells engineered to release IL-12 were implanted intratumorally (Yang S.Y. et al. 2004).

Additional studies must be performed to answer the question which lymphocyte subset plays a major role in the antitumoral response in the subcutaneous Gl261 model infected with parvoviruses expressing IP-10 and/or TNFα. Yet, we performed an experiment that proved the key role of T cells in tumor growth suppression in our system. A study with *in vitro* infected Gl261 cells was done in parallel in

immunocompetent and nude mice. The cells were infected with the combination of IP-10- and TNFα- expressing vectors at MOI3. The results showed that intact immune system suppress tumor growth (mock-treated tumors were growing slower in immunocompetent animals) and is necessary for transgene-induced antitumoral response (IP-10/TNFα co-expression induced stronger antitumor effects in the imunocompetent animals). In the immunocompetent animals subcutaneous tumors derived from cells infected with recombinant vectors (IP-10/TNFα combined expression) could be completely eradicated, whereas in the nude mice only a tumor growth delay can be observed. Since nude mice are deprived of T cells, the effect induced by IP-10/TNFα combination is likely to be T cell – dependent. Further experiments are necessary in order to investigate if the main cellular subsets participating in antitumor response are $CD8^+$ T cells, as suggested by literature data. Challenge experiments in immunocompetent animals also suggest a key role of $CD8^+$ cells. Tumor regression was a long process, taking part over 70 days, arguing also for a role of $CD8^+$ cytotoxic memory cells.

The $CD8^+$ cytotoxic memory cells (CTL) are major components in sustaining immunological memory. Memory $CD8^+$ cells are defined by their ability to persist for a long time in larger numbers and respond more rapidly to antigen than naive $CD8^+$ T cells. Memory CTL are generated from effector CTL and are maintained by different cytokines (Fernando G.J.P. et al. 2002).

5.5.2 TNFα-expressing vector promotes maturation of dendritic cells *in vitro*

Dendritic cells (DCs) are one of the most potent cells of the immune system. They collect and process antigens for presentation to T cells, directing them to different types of immune response or to tolerance (Banchereau J. et al. 2000).

It would be of great potential advantage if DCs could be stimulated by virus-infected tumor cells rather than by non-infected cells. Such a situation would also be promising for the development of a therapeutic vaccine. DCs co-cultured with infected tumor cells would stimulate T cells to act against infected tumor cells. Direct infection of DCs with parvoviral vectors could lead to the presentation of viral antigens and, again, direct T cells to the response targeted against the virus-infected tumor cells.

In our studies, changes in DCs pinocytotic activity and expression profile of surface markers upon infection were monitored. Tumor cells infected with TNFα-expressing

vector significantly promoted dendritic cell maturation compared to non-infected cells. Viral infection itself could also contribute to this process, since infection with a control vector, devoid of any transgene, partially supported maturation as well.

Dendritic cells are professional APC (antigen presenting cells) that have unique capability for activating T cells. DCs express high levels of MHC, adhesion and costimulatory molecules (Saito R. et al. 2004). Application of dendritic cells as immunostimulatory agent is a widely studied and promising approach, also in the Gl261 glioma model (reviewed below).

It was demonstrated that dendritic cells pulsed with Gl261 cell extracts and administrated intraperitoneally to the mice bearing intracranial Gl261 tumors prolonged animals survival. Cured animals showed an increased delayed-type hypersensitive response to Gl261 cells and survived when rechallenged with intracranial tumor cells implantation (Ni H.T. et al. 2001).

In order to increase effectiveness of the pulsation with Gl261 tumor cell extracts the cationic liposomes were used (Aoki H. et al. 2001). In the animals treated with DCs pulsed by tumor extracts, tumor progression was inhibited. The group treated with DCs pulsed by tumor extract and liposomes showed substantial tumor volume reduction in all the mice. $CD8^+$ - positive cytotoxic T cells were recognized among tumor cells. The CTLs showed a specific antitumor activity for Gl261 cells (Aoki H. et al. 2001). The same group used gene therapy combined with immunotherapy against intracranial Gl261 tumors. IFNβ gene entrapped in cationic liposomes was applied intracranially. This treatment was followed by subcutaneous administration of dendritic cells that have been pulsed with Gl261 cell lysates. Compared with each treatment alone combined therapy, when repeated, inhibited tumor growth and prolonged animal survival. Strong infiltration of $CD8^+$ positive cells into the tumors treated with IFNβ was detected (Saito R. et al. 2004).

It was shown that dendritic cells injected intracranially are able to migrate to the lymph nodes. Inoculation of both DCs and irradiated Gl261 cells prolonged the survival of intracranial Gl261 tumor-bearing mice compared to the animals treated with either dendritic cells or irradiated tumor cells alone. Depletion of NK cells and especially $CD8^+$ T cells resulted in the reduction of the antitumor effect (Kikuchi T. et al. 2002).

Glioma cells suppress maturation of dendritic cells (Kikuchi T. et al. 2002). Engineering or stimulating DCs with certain agents like TNFα or IFNγ may be needed to overcome the negative effects of tumor cells on DCs. Therefore, it is possible that

infecting tumor cells with viruses expressing immunostimulating factors like TNFα and IP-10 could contribute to develop antitumor immunity. Morover, maturation of DCs can be induced by phagocytosis of necrotic or apoptotic cells. Gl261 are sensitive to the cytotoxic action of TNFα and viral NS1 protein. It is possible that parvoviral-mediated TNFα expression would both induce tumor cell death and DCs maturation.

Virally-delivered TNFα may support development of the antitumoral immune response, what could be shown in several different models. Kianmanesh and co-workers demonstrated that intratumoral administration of low doses (10^7 PFU) of an adenovirus encoding TNFα (AdTNFα) together with syngenic dendritic cells acts synergistically to suppress preexisting tumors without systemic toxicity. Administration of AdTNF and dendritic cells into tumors elicited tumor-specific cytotoxic T cells and protected animals against subsequent challenge with the same tumor, suggesting that adaptive host immune response was obtained. Such effect could be observed in colon, melanoma and prostate tumor models. Experiments evaluated in nude mice, SCID mice and SCID-beige mice supported the concept that a fully intact immune system is necessary to mediate the low-dose AdTNFα and dendritc cell - mediated suppression of tumor growth. In the colon tumor model $CD8^+$ T cells mediate the antitumor effect in this therapy (Kianmanesh A. 2001).

Recent advances in immunology have led the possibility and efficacy of immunotherapy using peripheral dendritic cells against the tumors of central nervous system (Aoki H et al. 2001). A phase I clinical study evaluated safety and bioactivity of tumor lysate – pulsed dendritic cell vaccination to treat patients with glioblastoma multiforme and anaplastic astrocytoma. This approach was safe and no evidence of autoimmune disease was detected. A significant $CD8^+$ T cell infiltrate was noted intratumorally. Tumor lysates – pulsed vaccination was associated with a 133-week median survival as compared with a 30-week median survival for control patients (Yu J.S. et al. 2004).

The data obtained in this work suggest that it may be especially efficient to use parvoviral vectors in order to stimulate dendritic cell maturation. Preliminary data are promising; however, further investigations are necessary.

The infection of tumor cells with recombinant parvoviruses encoding IP-10 or TNFα as well as treating established tumors with these vectors provides safe conditions for the development of an antitumor effect. The best therapeutic effect – complete tumor

eradication - was reached when both types of virus were administered simultaneously. Histological analysis showed that this effect was not dependent on the inhibition of angiogenesis.

We also showed that intact immune system is necessary to obtain strong antitumor effect. Rechallenged animals are protected from tumor growth. GI261 glioma cells can be specifically recognized by host spleenocytes. From the literature it is suggested that the main effectors in the antitumoral response could be $CD8^+$ T cells. TNFα - expressing vector demonstrated the ability to support dendritic cell maturation.

Taken together, the data obtained during this work are promising and suggest that recombinant parvoviruses are good candidates for gene therapy of glioma.

5.6 Possible influence of parvovirus-induced IP-10/TNFα expression on brain immunology

Although the CNS has been characterized as an immune privileged site, it is also a site of inflammation, either in response to exogenous antigens (infection) or as a result of disrupted peripheral tolerance to self-antigens (autoimmunity). The CNS-endogenous cells may initiate, regulate and sustain an immune response (Becher B. 2000). Brain immunity must be considered when viral vectors expressing immunostimulating molecules like IP-10 and TNFα are thought to be used in glioma gene therapy.

Microglia are well described as potent immunocompetent cells, recognizing pathogens and initiating an inflammatory cascade. Early response cytokines produced by microglia include proinflammatory cytokines (IL-1β, TNFα, IL-6), that can permeabilize the BBB (blood-brain-barrier) as well as chemokines (MIP-1α, IP-10) (Becher B. 2000). Local TNFα administration achieved by parvoviral vectors may support immune response against tumor cells. The presence of viral components in infected tumor cells could additionally stimulate microglia activity. However, in the CNS TNFα may demonstrate dual effects, leading either to escalation of the brain inflammation or inhibiting the development of the disease (Lampson L.A. 2003).

In vitro study performed by Kimura et al. investigated the cytotoxic effect of TNFα, IL-1β, IL-6 and IL-8 on cultured cerebral microvascular endothelial cells. TNFα induced apoptosis in these cells through the caspase activation (Kimura H. 2003). This

effect could be an advantage when affecting the tumor vasculature only, while keeping the normal endothelial cells intact.

TNFα and IFNγ can also kill tumor cells directly. Intracerebral injection of IFNγ or TNFα can safely increase extravasation of activated T cells and monocytes from the blood, presumably by increasing expression of relevant adhesion molecules on cerebral endothelial cells (Lampson L.A. 2003).

In vitro study on the regulation of human IP-10 gene expression in astrocytoma cells by inflammatory cytokines showed that, when present together, IFNγ and TNFα induced robust accumulation of hIP-10 mRNA. Synergistic effect of the cytokines resulted from an increased rate of IP-10 transcriptional initiation (Majumder S. 1998).

During the last years IP-10 has been an intensively studied chemokine due to its possible role in autoimmune brain inflammation. IP-10 and its receptor CXCR3 are expressed by the CNS and by CNS infiltrating lymphocytes in patients with ongoing CNS inflammation, suggesting an important role for these molecules in the pathogenic process. Different studies identified IP-10 as a potential therapeutic target for the treatment of the lymphocyte recruitment into the CNS during inflammation. The reports about its role (increasing or decreasing inflammatory process) remain contradictory. However, it seems that IP-10 expression alone is not sufficient to direct lymphocytes cross the intact BBB and to induce pathology (Klein R.S. 2004).

IP-10 participates both in innate and adaptive immune response by contributing to cell migration and activation. Trifilo and co-workers demonstrated that IP-10 played a pivotal role in inducing innate immune response within CNS against mouse hepatitis virus (MHV). IP-10 mediated its protective effect (reduced animal mortality, reduced viral replication) by coordinating the infiltration and activation of NK cells into the brain. No chemokine yet has been shown to exert a clearly defined role in coordinating an innate immune response following viral infection of the CNS. In addition, it was shown that NK cells can exert an antiviral protective effect within the CNS (Trifilo M.J. 2004).

Intracerebral administration of IP-10-expressing adenoviral vector resulted in rapid and prolonged infiltration of both $CD4^+$ and $CD8^+$ T cells. Despite increased T cell infiltration, production of proinflammatory chemokines was moderate and mice developed only a limited neuropathology (Trifilo M.J. 2003).

Side effects linked to the inflammatory response in brain may be decreased by developing a vector encoding truncated version of IP-10. IP-10 is processed by a specific membrane-bound protease CD26. It results in reduced CXCR3-binding

properties, loss of calcium signaling capacity through CXCR3, and more than 10-fold reduced chemotactic potency. Truncated IP-10 retained its ability to inhibit the angiogenic activity of IL-8 in the rabbit cornea micropocket model (Proost P. 2001). Such modified chemokine may still demonstrate antiangiogenic activity in the brain but without inducing adverse immune response. This would be an advantage in the glioblastoma treatment. However, in the subcutaneous glioma system investigated in this work, IP-10 did not demonstrate any antiangiogenetic properties. Antitumor effect observed upon parallel expression of IP-10 and TNFα seem to be mediated through activation of the immune system. While similar mechanism would be observed intracerebrally, immunostimulating properties of IP-10 should be retained. Truncated chemokine, deprived of its chemotactic potential, would not be able to induce antitumoral response.

Up to now the influence of parvoviruses or parvovirus-based vectors on brain immunology was not investigated. The use of viruses and viral vectors in the therapy of glioma is considered as potentially dangerous due to possible occurrence of severe side effects like brain tissue damage or uncontrolled inflammation.

The immune response imposes limitations on viral-based gene transfer into the brain. It was demonstrated, that viral vectors injected into the brain's ventricular system elicit innate and adaptive immune responses. However, when injected directly into brain parenchyma, they elicit only transient inflammation owning to the absence of dendritic cells. If viruses are delivered carefully into the brain parenchyma, and care is taken not to inject the ventricular compartment, viruses may avoid priming the adaptive immune response, such both the gene therapy and the brain could remain unharmed. The absence of immune priming suggests that gene therapy using viruses might turn out safer than predicted (Lowenstein P.R. 2002).

High efficacy of IP-10 and TNFα-encoding parvoviral vectors could be demonstrated in two subcutaneous glioblastoma models. In the future antitumoral effects of these vectors should be investigated in the intracranial system such as the well-described GI261 model. Intensive studies should be performed in order to identify the processes taking part in treated brain (immune response, antiangiogenesis, necrosis development) and to define potential side effects (possible toxicity on the brain cells, healthy brain tissue damage). However, literature data reviewed in this chapter suggests that intracranial application of IP-10 and TNFα-encoding parvoviral vectors could result in significant antitumoral response.

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Acknowledgments

The following dissertation was done at the German Cancer Research Center's Department of Applied Tumorvirology within Prof. Dr. Jean Rommelaere's division. I would like to thank him for offering me the opportunity to complete my PhD at such an attractive place of work.

I owe special thanks to Dr. Christiane Dinsart for supervising the project, scientific discussions and insightful reading of the dissertation. I am grateful to Dr. Jan Cornelis for his support and help.

I appreciate as well the support provided by Prof. Buselmaier and Prof. Gissmann who kindly agreed to supervise my PhD. I owe much to Prof. Lutz Gissmann, my adviser from beginning to end. I want to thank him very much for his scientific and personal support.

I, also, wish to thank other members of the Dissertation Committee: Prof. Dr. Petersen and Prof. Dr. Hell for their contribution.

Members of the lab 2.206 deserve a special mention. I thank all of them for their help and for creating a friendly atmosphere at work. More specifically: Alex, for her excellent technical problem solving skills, Claudia for her professional advice, Susanne for introducing me to the animal studies and to Tim for experience exchange and constructive suggestions. Two special people helped me to enjoy the internationality of our division. I thank here my friends Dessi and Ziling for being on my side, their help and for the fun times we had together.

I am grateful to other members of our institute who were always ready to answer the most complicated questions and share their experiences. I would like to thank the people working at the animal station, especially Ute and Martin, for their excellent assistance.

The following dissertation, while an individual work, benefited from the engagement and direction of several people.

I would first like to thank Silke Vorwald and Dr. Ralf Kinscherf who so willingly participated in this study. They gave not only their time, but they also gave me support and encouragement. Ralf exemplifies the high level of learning to which I aspire.

I am grateful to Dr. Melanie Heilmann and to Dr. Fabian Kießling for their fruitful cooperation and enthusiastic introduction to a new field of research.

I thank Dr. A. Vecchi and Dr. S. Sozzani for common work on the project that opened up for me new scientific opportunities.

I appreciate the support and help of my Family, especially Kieran for proof reading the dissertation. I would like to thank all my Friends who supported me, especially Bernhard and Wolfgang for being on my side and for their words of encouragement.

I am very grateful to my loving husband Bernhard who always supports me. I thank him for his help during my PhD and for wonderfully completing the non-scientific part of my life.