



# **Dissertation**

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Oral examination:

**Role of Parvovirus MVMP in generation of antitumor  
immune responses**

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## Summary

Rhodent autonomous parvoviruses display a pronounced tropism for tumors and their infection may ultimately result in lysis of established tumors. This work evaluated the efficacy of Minute Virus of Mice prototype (MVMp) as a stimulus of specific antitumor immune response against mouse melanoma and glioblastoma tumors.

MVMp was shown to effectively infect both melanoma B78/H1 and glioma GL261 cell lines and induce cell death. MVMp was more effective inducing cell death in glioma GL261 cultures where 10% live cells were detected 96h after virus infection at multiplicity of infection (MOI) 10. In comparison only 40% cell death was observed in the melanoma B78/H1 cells after virus infection at high MOI 100. MVMp infected glioma GL261 cells displayed characteristics of apoptosis, such as caspase-3 activation and DNA fragmentation, which were not detected in melanoma.

Activation of antigen presenting cells (APC) provides the initial cue for an innate and adaptive immune response. Therefore, we generated tumor cell lysates after MVMp-infection and investigated their capacity to induce activation of dendritic cells (DCs) and microglia, specific subtypes of APC. Whereas MVMp infected B78/H1 melanoma cells were unable to activate DCs, virus infected glioma cells efficiently induced DC activation. Two different DC subpopulations (myeloid and lymphoid) were activated after co-culture with MVMp-infected glioma GL261 cells, and activation was monitored by upregulation of specific activation markers such as CD80, CD86, and MHC class II. In addition, the release of proinflammatory cytokines such as TNF- $\alpha$  and IL-6 was measured. Similarly, microglia were activated, when co-cultured with MVMp-infected glioma GL261 cells, measured by upregulation of activation markers and cytokine production. Taken together these results demonstrate that glioma GL261 cell lysates generated after MVMp infection can substantially activate DCs as well as microglia.

Toll-like receptors (TLRs) expressed in DCs and microglia mediate crucial signaling pathways initiating effective innate and adaptive immune responses. To assess the contribution of TLR signaling in the context of PV-based virotherapy, stable cell lines expressing single murine TLRs were established. These cell lines were used as a model to correlate specific TLR activation with the exposure to virus induced tumor cell lysates. A direct downstream target of the TLR signaling cascade is the transcription factor NF- $\kappa$ B and thus a reporter approach was utilized to measure NF- $\kappa$ B activation. Upon culture of single TLR expressing stable cell lines with MVMp-mediated tumor cell lysates, the cell line stably expressing TLR3 had an eight fold higher activation of NF- $\kappa$ B reporter gene expression compared to control. This finding called for a role of TLR3 mediated signaling in the activation NF- $\kappa$ B and downstream target genes such as pro- and inflammatory cytokines.

The *in vitro* findings were extended to *in vivo* studies and antitumoral effects mediated by MVMp were assessed in immunodeficient RAG2<sup>-/-</sup> and immunocompetent C57BL/6 mice.



Immunocompetent mice completely (100%) rejected MVMP-infected glioma GL261 at MOI 3 and MOI 30 with greater efficiency than control tumor cells (17%). Eradication of tumor cells mediated by the oncolytic effect of MVMP at MOI 30 amounted to only 20% in immunodeficient RAG2<sup>-/-</sup> animals. In addition, the release of IFN- $\gamma$  from splenocytes of mice, which were injected with MVM-infected glioma cells, in response to uninfected glioma cells was assessed *ex vivo* and was significantly higher (2.3 times more IFN- $\gamma$  producing cells) compared to control splenocytes. These findings provide evidence of a specific immune response towards glioma GL261 cells and highlight the importance of a T cell mediated immune response to obtain a strong antitumor effect. Furthermore, it was shown in re-challenge experiments that mice were protected from tumor growth. This highlights the induction of a tumor specific memory response.

In conclusion, MVMP induced GL261 tumor cell lysates stimulate activation of APC, initiate cytokine production via NF- $\kappa$ B signaling and *in vivo* initiate primary and secondary tumor specific immune responses. These findings could place PV as promising candidates to break tumor tolerance and treat glioma.

## Zusammenfassung

Autonome Parvoviren zeichnen sich aus durch einen ausgeprägten *in vivo* Tumortropismus, der zur Lyse bereits etablierter Tumore führen kann. Ziel der vorliegenden Arbeit war, am Beispiel des autonomen Parvovirus „Minute Virus of Mice prototyp“ (MVMp) zu untersuchen, ob spezifische Immunreaktionen an dessen antitumoraler Wirkung beteiligt sind.

Es zeigte sich, dass MVMp *in vitro* sowohl Maus-Melanomzellen der Linie B78/H1 wie auch Gliomzellen der Linie GL261 infizieren und in diesen Kulturen Zelltod induzieren kann, wobei die Effizienz in GL261 deutlich höher war. In GL261 wurden im Gegensatz zu B78/H1 nach MVMp Infektion zudem Apoptose-typische Veränderungen beobachtet.

Die Aktivierung Antigenpräsentierender Zellen (APC) ist das erste Signal für die Einleitung einer angeborenen oder adaptiven Immunantwort. Aus diesem Grund wurde überprüft, ob myeloische Dendritische Zellen (DC), lymphoide DC oder Mikroglia als wichtigste Vertreter peripherer bzw. zentraler APC sich durch Tumorzelllysate aktivieren lassen, die nach MVMp Infektion generiert wurden. Es zeigte sich, dass Lysate aus MVMp infizierten GL261 Gliomzellen im Gegensatz zu infizierten B78/H1 Melanomzellen sehr effizient DC aktivierten gemessen an der Zunahme der spezifischen Aktivierungsmarker CD80, CD86 und MHC II. Darüberhinaus wurde eine erhöhte Menge der proinflammatorischen Zytokine TNF- $\alpha$  und IL-6 freigesetzt. Auch Mikroglia zeigten nach Inkubation mit Lysaten MVMp-infizierter Gliomzellen verstärkte Expression der Aktivierungsmarker CD80 und CD86 sowie TNF- $\alpha$  und IL-6 Sekretion. Zusammengefasst zeigen die Ergebnisse, dass sowohl DC als auch Mikroglia durch Lysate aus MVMp infizierten Gliomzellen aktiviert werden können.

“Toll-like” Rezeptoren (TLR) sind wichtige Erkennungsstrukturen für pathogene Mikroorganismen, die von DC oder Mikroglia exprimiert werden und wichtige Signale übermitteln, die angeborene oder adaptive Immunantworten auslösen. Im Rahmen dieser Arbeit wurde untersucht, ob Lysate MVMp-infizierter GL261 Zellen auch TLR aktivieren können. Dazu wurden Zelllinien erzeugt, die einzelne Maus TLR stabil exprimieren. Die Aktivierung des Transkriptionsfaktors NF- $\kappa$ B, die *in vivo* eine unmittelbare Reaktion auf eine TLR-Aktivierung darstellt wurde in diesem Modell indirekt über eine Reporter-genaktivität gemessen. Nachdem die verschiedenen stabilen TLR-exprimierenden Zelllinien mit MVMp-induzierten Tumorzelllysaten inkubiert wurden, ergab sich nur in der Zelllinie, die TLR 3 exprimierte eine - verglichen mit der der Kontrolle - achtfach erhöhte Reporter-genaktivität.

Die *in vitro* gewonnenen Befunde wurden in *in vivo* Mausmodellen weiter untersucht. Dabei wurden die Tumor-inhibierenden Eigenschaften von MVMp in immundefizienten RAG2-/- und in immunkompetenten C57BL/6 Mäusen verglichen. Immunkompetente Mäuse bildeten keine Tumoren, nach Injektion von GL261 Gliomzellen, die mit MVMp bei einer MOI von 3 oder 30 infiziert worden waren während 83% der Mäuse nach Gabe nicht infizierter Tumorzellen Tumore bildeten. In 80% der RAG2-/- Mäuse traten Tumore trotz vorheriger MVMp Infektion mit MOI 30

auf. Darüber hinaus zeigten Milzzellen aus Tieren von MVMp-infizierten Gliomzellen eine 2,3-fach höheren Anteil an IFN- $\gamma$  produzierenden Leukozyten gegenüber solchen aus Tieren mit nicht-infizierten Tumorzellen. Die erneute Injektion nicht-infizierter Gliomzellen führte zu keinem Tumorwachstum in immunen C57BL/6 Mäusen. Zusammen deuten diese Ergebnisse auf einen MVMp-induzierten spezifischen antitumoralen Effekt unter Beteiligung von T-Zellen hin.

### **1. Introduction.**

#### **1.1. The Clinical Role of Glioma and Melanoma Tumors.**

##### **1.1.1. Gliomas.**

Gliomas are primary central nervous system (CNS) tumors that arise from astrocytes, oligodendrocytes or their precursors (reviewed by Buckner J.C. et al., 2007). As the normal development of the CNS ceases, neurons become post-mitotic with the exception of stem cells in a small compartment. However, glial cells (astrocytes, microglia, oligodendrocytes) keep their ability to proliferate throughout life. Thus, it might be not too surprising that most neurological tumours in adults are of glial origin.

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; Louis D.N., 2006). Invasion of the tumor might be accomplished by various proteases, such as serine, cysteine and metalloproteinases (Mohanam S. et al. 1999; Forsyth P.A. et al. 1999; Sawaya R.E. et al. 1996). These proteases might enable tumour cells to digest the extracellular matrix (ECM), to penetrate connective-tissue barriers, to induce vascular remodeling and to destroy normal brain tissue.

The World Health Organization (WHO) established a grading system (Grade I through IV) to classify gliomas, based on the degree of their malignancy: grade I gliomas being the least aggressive tumour mostly with a benign phenotype and grade IV are the most malignant ones. These criteria were determined by histopathological analysis. Grade IV gliomas are usually

infiltrating astrocytic neoplasms, and are known as the glioblastoma multiforme (GBM) one of the most aggressive human cancers.

Gliomas are refractory to most standard therapies, including surgical resection, radiation therapy and chemotherapy (Fischer U., Meese E., 2007). From the time of diagnosis with glioblastoma, the mean survival time ranges from 17 weeks without treatment to 30 weeks with surgery and radiation. The median survival time of less than one year has not changed a lot over the past two decades (Maher E.A. et. al., 2001; Holland E.C., 2001). Despite established treatments these tumors will recur and cause neurological deterioration and death (Dai C. et. al., 2001). Glioma patients are often immunosuppressed. Molecules like transforming growth factor- $\beta$  (TGF- $\beta$ ), 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 MHC class II expression and by inhibition of the development cytotoxic T lymphocytes (Castro M.G. et. al. 2003).

Traditional therapeutic modalities have been shown to be very ineffective in curing tumors of the CNS, due to the radioresistance of glioma cells and the difficulty of achieving complete tumor resection as gliomas usually have diffuse, infiltrative growth patterns (Aoki T. et. al., 2007). The tremendous complexity of the brain as a highly seclusive organ makes it even more difficult to treat these tumors with chemotherapy. Thus, alternative therapies have become more and more attractive in the treatment of gliomas.

### **1.1.2. Melanoma.**

There are several types of skin cancer, which include melanoma, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC develops from basal cells, and SCC develops from keratinocytes. BCC and SCC usually occur

in older people. Most cases of non-melanoma skin cancer are easily treated and cured.

Melanoma is a malignant tumor of melanocytes which are found predominantly in the skin but also in the intestine and the eye. Around 160,000 new cases of melanoma are diagnosed worldwide each year, and it is more frequent in males and caucasians. According to the WHO Report about 48,000 melanoma related deaths occur worldwide per annum (Lucas, R. 2006). Mortality rates for cutaneous malignant melanoma depend on stage at diagnosis; thus, efforts are aimed at early detection and identification of risk factors for melanoma to distinguish those individuals requiring close surveillance (Gerstenblith MR, et. al. 2007).

There are five stages of melanoma (Balch C. et. al., 2001):

- Stage 0: Melanoma in Situ, 100% Survival,
- Stage I/II: Invasive Melanoma, 85-95% Survival,
- Stage II: High Risk Melanoma, 40-85% Survival,
- Stage III: Regional Metastasis, 25-60% Survival
- Stage IV: Distant Metastasis, 9-15% Survival.

Features that affect prognosis are tumor thickness in millimeters, type of melanoma, presence of lymphatic/perineural invasion, presence of tumor infiltrating lymphocytes (if present, prognosis is better), location of lesion, presence of satellite lesions, and presence of regional or distant metastasis (Homsí J., et.al., 2005). When melanomas have spread to the lymph nodes, one of the most important factors is the number of nodes with malignancy. Extent of malignancy within a node is also important; micrometastases in which malignancy is only microscopic have a more favorable prognosis than macrometastases. Once distant metastasis have occurred, the cancer is generally considered incurable. The five year survival rate is less than 10% (Balch C. et. al., 2001). The median survival is 6 to 12 months. Metastases to skin and lungs

have a better prognosis. Metastases to the brain, bone and liver are associated with a worse prognosis.

Despite many years of intensive laboratory and clinical research, surgery is the first choice therapy for localized cutaneous melanoma. Treatment of advanced malignant melanoma is performed from a multidisciplinary approach (adjuvant treatment; chemo- and immunotherapy, or radiation therapy).

### **1.2. Glioma- and Melanoma- directed Therapy.**

#### **1.2.1. Standart Therapy.**

Currently, the standard of care for the treatment of patients with high-grade malignant glioma and melanoma is resection followed by focal radiotherapy. 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 (for gliomas). The biopsies of inoperable tumors provide information for possible implementation of other therapies (Castro M.G. et. al., 2003).

Adjuvant therapy (radio- and chemotherapy) increases survival in patients with high-grade malignant glioma and melanoma. It has become the standard adjuvant treatment for these patients (Brandes A.A., 2003; Aoki T. et. al., 2007). Patients treated with radiotherapy plus adjuvant chemotherapy had a survival advantage. 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).

### 1.2.2. Alternative Therapy.

Other strategies have been pursued to efficiently treat tumors. New alternative approaches are used as adjuvant strategies complementing current strategies (surgery, radiation, chemotherapy). Although considerable preclinical data have been accumulated, promising results for therapy of human glioma have only recently appeared (Cavaliere R. et. al., 2007). The resistance of brain tumors and melanomas to the attempts at conventional therapy have made them particularly attractive for gene therapy trials (King G.D. et. al., 2005 ; de Groot J.F., Gilbert M.R., 2007; Lassman A.B., Holland E.C., 2007). “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. 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.

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). The 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.

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



These attempts have been actually utilized mouse, rat, human *in vitro* models, and clinical trials. They include:

- induction of increased immunogenicity of tumor cells by enhancement of the immune response using cytokines, for example by inducing the production of IL-2, IL-4, IL-13 or GM-CSF within the tumor (Okada H. et al., 1999; Glick R.P. et al., 2000; Shimamura T., 2006),

- reversal of tumor derived immunosuppression by enhancement of T cell activation (by upregulation MHC I on the tumor cell surface, upregulation of tumor antigens, upregulation of B7 costimulatory antigens) (Parney I.F. et al., 1997; Okada H. et al., 2004; Mitchell D.A, et al. 2003; Abad J.D., Wrzensinski C., et al., 2008),

- *ex vivo* manipulation of effector cells, for example, manipulation of dendritic cells as tool for cancer immunotherapy by stimulating them with specific antigens or promoting their maturation (Aoki H. et al., 2001; Hiasa A., Hirayama M., et al., 2008),

- 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 (Ishikawa E. et al., 2004).

Viral vectors can be used as effective carriers 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 tumors has proven to be a promising novel treatment modality (Aghi M., Chiocca E.A., 2006), for example such as a murine melanoma model B16F10 using Sendai virus vectors with IFN-beta pulsed DC (Yonemitsu Y. et al., 2008). 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 even synergize with existing standard therapies (Shah A.C. et al. 2003).

The most studied viruses proposed for the use in the tumor gene therapy include Herpes simplex virus-1 (HSV-1) (Markert J.M. et. al., 2006), Newcastle disease virus (NDV), Reovirus (Errington F., White C.L., et. al., 2008), Poliovirus-derived viruses, and Vaccinia virus.

A considerable number of clinical trials have been initiated based on these approaches. Although most therapeutic strategies proved safe, clinical responses fell short of expectations raised by preclinical results. This, to a large extent, has to be attributed to a lag in the development of efficient vector systems. Although much effort has been put into this area of research, oncologists are still in await of a vector system allowing for selective and efficient tumor cell transduction. This has led to increased interest in distinct but related strategies, e.g. oncolytic viruses or direct intra-tumoral delivery of anti-sense oligonucleotides.

Especially the approach using oncolytic viruses has gained much attention (HSV for glioma model - Todo T., 2008; adenovirus for glioma model - Sonabend A.M. et. al., 2006). On one hand, viruses can serve as vectors delivering genes exerting anti-tumoral effects. On the other hand, these viruses themselves have oncolytic or even oncosuppressive properties.

A well defined oncolytic as well as oncosuppressive virus is the family of autonomous parvoviruses (PV). Some of these autonomous PV, for example the murine minute virus of mice prototype (MVMp) and rat H1 PV, have been shown to infect and lyse tumor cells with great efficiency (Wollmann G., 2005; Moehler M., 2001).

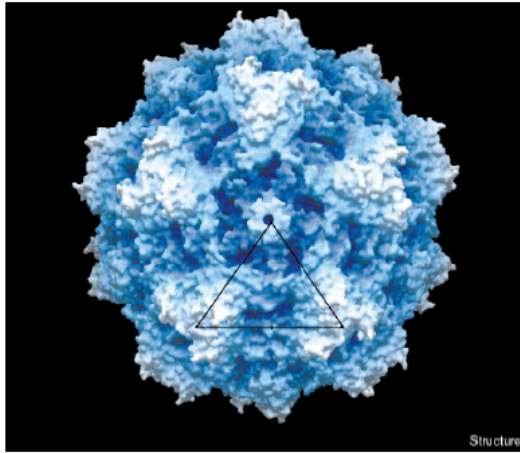
### **1.3. Autonomous Parvovirus Therapy as an Alternative Strategy.**

The autonomous PV is a small (18 to 26 nm), non-enveloped, nuclear-replicating DNA virus, which belongs to the Parvoviridae family. It infects a broad variety of species from insects to humans. Members of the Parvoviridae family, which are able to infect vertebrates, can be placed into one of three genera: the genus Dependoviruses comprises the adeno-associated parvoviruses (AAV) which cannot replicate efficiently without the help of some tumor viruses, while the erythrovirus as well as the parvovirus are self-sufficient in regard to their replication capacities (Cornelis J.J. 2004).

The first parvoviral isolates were derived from tumors and tumor cell lines (Kilham L. et al., 1959). These facts initially brought PV into the class of oncogenic viruses, but this was not confirmed since PV infections did not correlate with higher tumor incidence. On the contrary, later investigations revealed that PVs could prevent the formation of tumors in animal models (Dupressoir T. et al. 1989; Rommelaere J. et al. 1991).

Only one member of the PV family - 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 (Cassinotti P. et al. 1994). Autonomous PVs cause infectious pathology in some animal species but none of them has been associated with human diseases (Rommelaere J. et al. 2001).

PVs do not possess an envelope and consist of an icosahedral capsid in which a single-stranded DNA genome of around 5000 nucleotides is packaged. The three-dimensional structures of several PVs have been determined using X-ray crystallography (Fig. 1).



**Fig. 1. The Capsid of MVM.** Depth-cued representation of a whole MVM particle. The particle surface shading is based on distance from the viral center: the lighter regions represent residues that are at the greatest radial distance and the darkest regions represent residues at the shortest radial distance. The icosahedral fivefold, threefold and twofold axes for one viral asymmetric unit are indicated. Agbandje-McKenna (1998), *Structure* 6(11): 1369-81.

The 5'- and 3'-end of the parvoviral genome consists of palindromic sequences which can form hairpin structures. These hairpin structures serve as self-priming origins of DNA replication. The genome of PV usually includes two large open reading frames (ORFs) and two promoters. The ORFs code for the two non-structural (NS) polypeptides and viral capsid proteins (VPs) (Cornelis J.J. 2004).

NS1 is an 83 kDa multifunctional nuclear protein. It is relatively stable with a half-life of approximately 6 hours (Miller C.L. et al., 1995). Minute virus of mice (MVM) NS1 protein is a multifunctional phosphoprotein endowed with a variety of enzymatic and regulatory activities necessary for progeny virus particle production. NS1 is the only viral DNA protein being essential for DNA replication in all cell types tested.

A productive PV infection may be associated with cell death, for which NS1 is considered to play a major role (Vanacker J.M. et al. 1995). H1 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 NS proteins (Rayet B. et al. 1998).

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 protein kinase C (PKC) lambda phosphorylation *in vivo* and that this modification is essential for the helicase activity (Nuesch J.P. et al. 2003).

NS2 is a small protein with a molecular weight of 23-25kDa. In contrast to 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). 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).

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). The VP1/VP2 ratio is about 1:5, and this is the proportion in which they appear in the capsid. The role of the VP proteins is first to assemble into empty capsids (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 MVM but fails to initiate a productive infection (Tullis G.E. et al. 1993).

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).

**The Life Cycle of Parvoviruses.** The life cycle of MVM starts with the binding of the virus to N-acetylneuraminic acid containing glycoproteins, which are located on the cell surface. The PV enters the cell via endocytosis. It was shown for the canine PV that the virus enters the cells by dynamin-promoted, clathrin-coated vesicles (Suikkanen S. et. al., 2002). The viral particles are

transported via late endosomes to the perinuclear vesicular compartments. It is very probable that the DNA of PV is released from the lysosomal compartment to the cytoplasm to be then transported to the nucleus. The microtubulus network might play an important role in the whole transport process of PV in the host cell as it is actively protected during MVM infection in contrast to other cytoskeleton elements such as actin filaments (Neusch J.P. et.al., 2005).

The single-stranded genome of PV is converted into a duplex replication form in the nucleus. This conversion reaction is strictly S-phase dependent as early S-phase specific cyclin A and/or its kinases are required for this reaction (Cornelis J.J. 2004). Thus, PV cannot replicate in cells, which do not enter S-phase. The duplex intermediate form is amplified via multimeric forms and is also used as a template for the synthesis of mRNA. The amplification is performed by a rolling circle-like mechanism, which is extraordinary for viruses. Progeny single-stranded negative DNA is packaged into empty capsids (Cotmore S.F. et. al. 2005).

PV replication in permissive cells leads to the release of newly synthesized virions, which is usually associated with cell death. Cell death can either occur by apoptosis or by necrosis and might be dependent on the infected cell system (Moehler M. et. al. 2001; Ran Z. et. al. 1999). The NS1 protein, as it is accumulated in infected cells upon viral replication, seems to be the main cytopathic effector of autonomous PV although the mechanism, by which the cell killing is induced, is not fully understood. Studies have shown that NS1 protein blocks cellular DNA replication, that it causes nicks in cell chromatin, arrests cells in S-phase and that it is related to changes in the synthesis and phosphorylation of cell proteins (Op de Beeck A. et. al., 1997; Anouja F. et. al., 1997).

Among the oncolytic viruses that have been proposed as vectors for the gene therapy of cancer are the autonomous PVs H1 and MVMp (prototype) (Blechacz, B., 2004). Vectors derived from MVMp are well expressed in

oncogene-transformed cells of different types but not in equivalent non-transformed cells (Cornelis J.J. 2004). The mechanisms underlying oncospecific gene expression of MVMp are still not known. Clearly, PV replication and gene expression take advantage of perturbations of the cell cycle, in particular the S-phase, a hallmark of tumor cells (Cornelis J. J. 2004).

**Oncosuppressive and oncolytic properties of PV.** Although PVs infect a wide variety of species, their replication is restricted to tissues with high proliferation index. Therefore, rodent PV infection can have severe pathogenic effects in fetuses and neonates. For instance, in neonate hamsters, infection with the rat PV H1 led to high incidence of dwarfed and deformed animals with mongoloid traits (Toolan, H. W. 1960). Interestingly, these animals were found to have a 20-fold lower incidence of spontaneous tumors than non-infected animals, indicating that PV infection led to an oncosuppressive phenotype of these animals (Toolan, H. W. 1967). Persistent or latent infections with PVs may significantly protect against spontaneous tumorigenesis. When PVs 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).

Besides their oncosuppressive impacts, PV was reported in a number of research papers to have oncolytic properties. 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 PVs (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 and rodent cell lines established from various tumors (fibrosarcoma, epidermoid and mammary carcinomas, gastric cancer, hepatoma and lymphoma) are more susceptible to killing by MVMp or H1 virus than the corresponding normal cells (Cornelis J.J. et al. 2001; Ohshima T. et al. 1998; Herrero Y.C.M. et. al. 2004). Humans are hosts for a number of PVs such as 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 PV-infected organisms (as shown, for instance by the isolation of PVs 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.

H1 was already tested in clinical trials in patients with advanced disseminated cancer as feasibility studies (Le Cesne A. et. al., 1993). No pathological side effects were observed although it was not yet possible to investigate the spreading of injected H-1 virus into the central nervous system, nor was it possible to rule out possible long-term side effects.

It seems that PV does not have any severe side effects in adult organisms and so far, there is no evidence that PV integrates into the host genome. As a consequence, PV MVMp and H1 might be an effective, non-dangerous therapeutic tool which could be of great use in future cancer treatments.



## **1.4. Anti-tumor immune response.**

### **1.4.1. Innate immune response.**

The immune system has been shown to play a crucial role in the recognition and elimination of tumor cells. As highlighted above, gene therapy focuses mainly on the activation of cell-mediated activity of the immune system. Oncolytic viruses are able to initiate the two major components of the immune system – innate as well as adaptive – efficiently.

The innate immune system comprises the cells and mechanisms that defend the host from foreign antigen, in a non-specific manner. One of its main features is the pro-inflammatory response, which is induced by antigen-presenting cells (APC), such as macrophages, dendritic cells (DC) and microglial (MG) cells, which are particularly located in the brain.

The major functions of the innate immune system include:

- 1) recruiting immune cells to sites of infection and inflammation, through the production of cytokines and chemokines.
- 2) activation of the complement cascade to identify bacteria, activate cells and to promote clearance of dead cells (phagocytosis) or antibody complexes.
- 3) activation of the adaptive immune system through a process known as antigen presentation.

Increasing experimental evidence suggests that organisms distinguish invading microorganisms by recognizing pathogen-associated molecular patterns (PAMPs). They include molecules such as lipopolysaccharide (LPS) associated with the outer membrane of Gram-negative bacteria, mannose, fucose, and other sugar residues; teichoic acid (associated with the peptidoglycan cell wall of Gram-positive bacteria), N-formyl peptides, unmethylated CpG motives in bacterial DNA, double-stranded RNA in RNA viruses and mannans

in yeast cell walls (Ulevitch R.J. et al. 1995; Medzhitov, R. 1997). Specific protein families have been identified to recognize PAMPs: C-type lectin, the scavenger receptors, the pentraxins, which are components of the acute phase reaction that are synthesized in the liver and released into the plasma in response to pathogens (Emsley J. et al. 1994), lipid transferases, which can bind LPS (Schumann R.R. et al. 1990), integrins, complement control proteins and the most prominent of PAMP receptors, the so-called Toll-like receptors (TLRs), which play a central role in pattern recognition and induction of immune responses.

Binding of PAMPs to their receptor on the host cells usually results mainly in the activation of transcription factors such as NF- $\kappa$ B and interferon regulatory factor 3 (IRF3), which provide the inflammatory context for the rapid activation of host defenses. The cells which appear first in the site of inflammation and recognize foreign antigens are monocytes. As a consequence of the inflammatory microenvironment, monocytes that infiltrate this site can differentiate into macrophages and dendritic cells.

**Dendritic cells** (DCs) are recognized as one of the most important cell types for initiating the priming of naive CD4<sup>+</sup> T helper cells and for inducing CD8<sup>+</sup> T cell differentiation into killer cells. Immature DCs are found at strategic anatomical sites throughout the body, thereby allowing them to respond rapidly to microbial invasion. Immature DCs, on sensing potentially dangerous microbial signals through their pattern recognition receptors (PRRs), undergo maturation and subsequent migration to secondary lymphoid organs to prime naive T cells. At least three different subsets of DCs (myeloid, lymphoid and Langerhans cells) have been described based on their origins and phenotypic characteristics (including expression of distinct TLR repertoires). Hence, activation of different DCs has the potential to induce qualitatively distinct immune responses. Activation of lymphoid DCs, because of their preponderance

to secrete IL-12, may be important for priming Th1-like responses, whereas early activation of myeloid dendritic cells may lead to Th2- or Th0-like responses. TLR signaling also has an important role in determining the quality of these helper T cell responses. DCs may also exert indirect effects through, for example, the blockade of negative regulators of immune activation. It has been suggested that the production of IL-6 by DCs may be responsible for inhibiting the suppressor activity of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. (reviewed by Pashine A. et. al., 2005).

**Macrophages** are cells within the tissues that originate from monocytes. Monocytes and macrophages are phagocytes, acting in both innate immunity as well as cell-mediated immunity. Their role is to engulf and then digest cellular debris and pathogens, and to stimulate lymphocytes and other immune cells to respond to these pathogens. Macrophages provide yet another line of defense against tumor cells and body cells infected with fungus or parasites.

**Microglia** belongs to the mononuclear phagocyte lineage related to other organ specific macrophage populations (Vilhardt F., 2005). The old dogma states, that the brain is an immuno-privileged site. This might be true in regard of adaptive immunity, since hardly any or only weak adaptive immunity is exhibited in response to injury, infection, or tumor formation. However, the brain does exhibit a robust innate immune response carried out by microglia, which represent the major resident immuno-competent cell in the CNS and are capable of mounting macrophage-typical innate defence actions. Their functions include cell proliferation, migration, phagocytosis, enhanced antigen-presenting capabilities upon activation, up-regulation of innate immune cell surface receptors, secretion of pro-inflammatory mediators and reactive oxygen species (ROS). Infectious agents, such as bacteria, viruses, molecules in abnormal concentrations and other unidentified signals and conditions, which are related

to disturbed CNS functions and tissue destruction, trigger the transformation of resting microglia to an alerted, activated and fully reactive states.

In almost every CNS disease, accumulation of microglia can be observed, which could be a result of migration mediated by chemokines. Such accumulation of microglia is also the case in brain tumors although the exact role of these glia cells in the biology of glioma is still unknown. There is evidence for anti-tumor as well as pro-tumor activity of microglia in brain tumors (Badie B. et. al., 2001).

Activation of macrophages, as well as microglia, can occur by different stimuli and by recognition through different receptors, and results mainly in the activation of transcription factors such as NF- $\kappa$ B and interferon regulatory factor 3 (IRF3). The NF- $\kappa$ B pathway controls the expression of proinflammatory cytokines such as IL-1 $\beta$  and tumor necrosis factor (TNF), whereas the IRF3 pathway leads to the production of antiviral type I interferons (IFN- $\alpha$ , IFN- $\beta$ ).

Microbial stimuli, which are mainly recognized by PRRs, mediate the innate activation, which is followed by the production of type I IFNs, ROS and nitric oxide (NO). Along with these cytokines, various chemokines (such as IL-8, monocyte chemoattractant proteins, macrophage inflammatory proteins and RANTES) are released and their receptors are expressed on the surface of activated cells. As a result, vascular endothelial cells may alter surface expression of selectins and intercellular cell adhesion molecules, leading to the extravasation and selective retention of some leukocytes at the inflamed site. This cellular infiltrate consists of activated monocytes, neutrophils, basophils, eosinophils and NK cells, many of which also express TLRs and may subsequently become activated by the presence of their respective ligands.

Enhanced expression of co-stimulatory surface molecules (for example CD80, CD86) favors antigen presentation. Scavenger receptor-A and mannose receptor promote phagocytosis and endocytosis of host, as well as exogenous

ligands. The classical activation, on the other hand, is mediated by the priming stimulus IFN $\gamma$ , followed by microbial trigger, such as LPS. Activation results in secretion of pro-inflammatory cytokines, NO, oxidative burst, leading to tissue damage and cellular immunity. Activation of macrophages and microglia is quite complicated since they express further cytokine receptors (besides IFN $\gamma$  receptors) for TNF, IL-1, IL-4, IL-10, IL-12, IL-13, IL-15, IL-18 and TGF- $\beta$ ; as well as complement receptors, prostaglandin receptors, chemokine receptors, Fc-receptors and Toll-like receptors (Aloisi F. 2001).

**Toll-like Receptors (TLRs)** are mammalian homologues of the Toll protein, which was first discovered as an essential molecule for the establishment of the dorsoventral axis in the *Drosophila* embryo (Akira S., 2004). So far, 11 TLRs have been discovered and described for mice and altogether 12 members of the TLR-family were identified in mammals. Members of the TLR-family share characteristic extracellular and cytoplasmic domains. They belong to a larger superfamily, which also includes the IL-1 receptors (IL-1R). The extracellular domain contains multiple leucine-rich repeats, whereas the cytoplasmic domain is strikingly similar to the IL-1R, commonly known as the Toll/IL-1R homologous regions (TIR domains) (Slack J.L. et.al., 2000). TLRs detect multiple PAMPs, a brief summary is given in Table 1.

**Table 1. Toll-like receptors and their ligands.**

Pattern-recognition receptors	Ligands
TLR1	Bacterial lipoproteins from Mycobacteria, Neisseria
TLR2	Zymosan yeast particles, peptidoglycan, lipoproteins, glycolipids, lipopolysaccharide
TLR3	Viral double-stranded RNA, poly:IC
TLR4	Bacterial LPS, plant product taxol, Hsp60
TLR5	Bacterial flagellins
TLR6	Yeast zymosan particles, lipotechoic acid, lipopeptides from mycoplasma
TLR7	Single-stranded RNA
TLR8	Single-stranded RNA
TLR9	CpG oligonucleotides
TLR10	Unknown
TLR11	Bacterial components from uropathogenic bacteria

Recent research indicates that, under certain conditions, such as deficient clearance of apoptotic cells, the host-derived nucleic acids (often in complex with DNA-or RNA-binding proteins) may become available for activating TLRs, which may break tolerance and lead to autoimmunity (Leadbetter E.A. et al., 2002). Since TLRs play crucial role in the recognition of foreign antigens, their activation and modulation might enhance specific immune response, particularly anti-tumor immune response.

Most of the TLRs are well described. TLR4 was the first characterized mammalian TLR. It recognizes LPS, and requires several accessory molecules. LPS is first bound to a serum protein, which transfers LPS to CD14. CD14 is a high-affinity LPS receptor, which can be expressed as a glycoposphoinositol

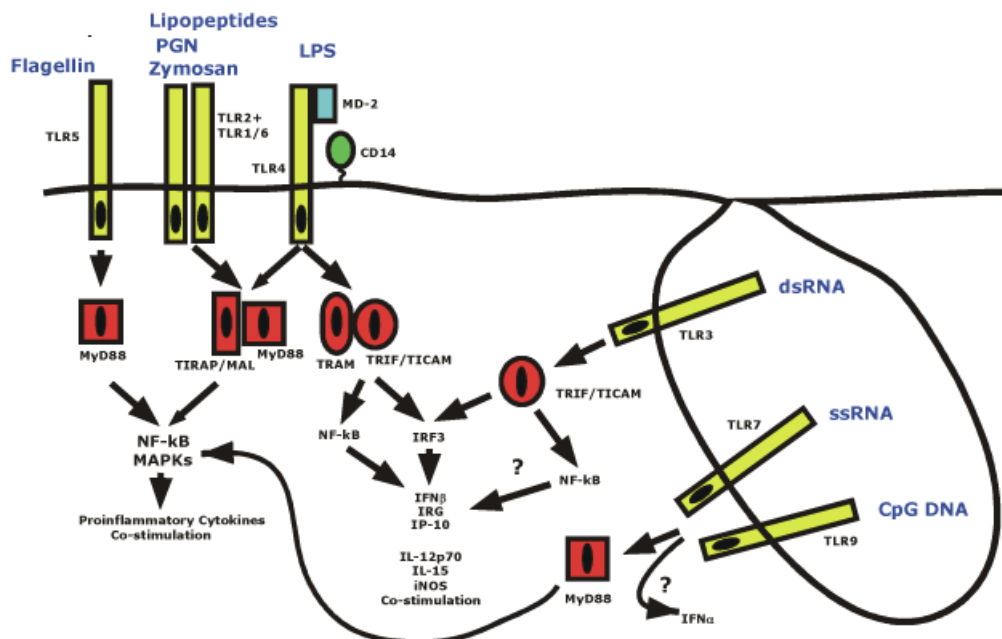
(GPI)-linked protein on the surface of macrophages, besides the possibility to be secreted in the serum. Another protein, which is associated with the recognition of LPS by TLR4, is MD-2. MD-2 is a small protein without a transmembrane domain and is expressed on the cell surface in association with the extra-cellular portion of TLR4. Despite the fact, that MD-2 function is not well understood, it increases the efficiency of LPS interaction with TLR4 as CD14 does.

TLR2 can recognize a broad range of microbial products, including peptidoglycan from Gram-positive bacteria, bacterial lipoproteins, mycobacterial cell-wall lipoarabinomannan, glycosylphosphatidylinositol lipid from *Trypanosoma Cruzi*, cell walls from yeast and LPS, which is structurally different from Gram-negative LPS. The fact that TLR2 can cooperate with TLR1 and TLR6 by formation of heterodimers, might be an explanation for the broad range of ligands recognized. In such a way, hetero-dimerization between TLR2 and either TLR1 or TLR6 dictates the specificity of ligand recognition (Ozinsky A. 2000). The full repertoire of possible TLR heterodimers is not yet known. Some TLRs, such as TLR4, TLR5 as well as TLR2 are likely to function as homodimers.

TLR3 functions as a receptor for double-stranded RNA (dsRNA), which is a molecular pattern produced by most viruses at some point of their infection cycle. Cells, which were deficient for TLR3 were shown to have a profound defect in their responsiveness to polyinosine-polycytosine (polyIC), the synthetic analogue to dsRNA (Alexopoulou L. et al. 2001). Therefore, TLR3 contributes to the antiviral defense of the host as its specific ability to recognize dsRNA broadens the range of pathogens that can be detected by the TLRs.

The TLRs dimerize and undergo conformational changes after ligand binding, which are required for the recruitment of a number of downstream factors in the TLR signaling. The signaling pathways of TLRs can be divided in two groups: the MyD88 dependent pathway and the MyD88 independent pathway. All TLRs can activate a common MyD88 dependent pathway that

culminates in the activation of NF- $\kappa$ B transcription factors, as well as the mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). NF- $\kappa$ B is released and translocated into the nucleus, where it activates NF- $\kappa$ B-dependent genes (Fig. 2).



**Fig. 2. Activation of NF- $\kappa$ B, MAPKs, and IRF3 upon TLRs-TLRs ligand recognition.** Modified from Re F., Strominger J., 2004.

A vast amount of proteins is regulated by NF- $\kappa$ B, including TNF, IL-1, IL-2, IL-6, IL-8, IL-12, various chemokines, such as monocyte chemoattractant protein-1, RANTES, and many other factors and proteins (Varney, M.L. et. al. 2002).

#### 1.4.2. Adaptive immune response.

TLRs activation in APCs can lead to abundant production of pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-6, IL-12, TNF and type-I IFN,



expression of co-stimulatory molecules and chemokines (Iwasaki A. 2004). The secretion of cytokines in turn can either activate APCs themselves by autocrine and paracrine mechanisms or attract and stimulate other cells (for instance leukocytes) for surveillance, attack and containment of invading pathogens. The products of TLR signaling can shape the adaptive immune response (Akira S. et al. 2001). TLR induced pro-inflammatory cytokines are important mediators for the activation and regulation of the adaptive immune response.

**Tumour necrosis factor (TNF)** is a major proinflammatory 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 TNF (Nagata S. 1997). TNF mediates both physiological and pathological effects. It plays a role in normal physiological processes like: embryogenesis, haematopoiesis, inflammation, and protection against infections. Pathological effects mediated by TNF appear upon stress, which generally increases TNF production. Well known is TNF contribution to the induction of sepsis, arthritis, pancreas inflammation and ischemia (Reimold A.M. 2003). TNF affects cellular growth, differentiation and metabolism. TNF 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 TNF can induce apoptotic or necrotic cell death. Due to the potential use in the cancer therapy, antitumor properties (tumor growth suppression) of TNF are being intensively studied now. TNF $\alpha$ -mediated antitumor effect is often depending on the dose, however, high doses of this cytokine result with systemic toxicity. For many cell lines TNF is only cytostatic, but not cytolytic and many lines are totally resistant to its cytotoxic activity. Some tumors undergo regression upon combined treatment (TNF applied with IFN $\gamma$  or with cytostatic drugs).

TNF 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).

**Interleukin 6 (IL-6)** is a pleiotropic cytokine with many different functions. It is involved in the acute phase response, B cell maturation, and macrophage differentiation. IL-6 is an essential molecule for antiviral humoral responses, as well as for the induction of the acute phase reaction (Kopf M. et al. 1994). Secreted IL-6 from fibroblasts in contact with monocytes, up-regulates M-CSF receptors on monocytes. Up-regulation of M-CSF receptors allows monocytes to be activated by their own M-CSF, which in turn promotes monocyte differentiation to macrophages rather than DCs (Chomarat, P. 2000).

The **type I interferon** family consists of IFN- $\alpha$ , IFN- $\beta$ , IFN- $\omega$  and IFN- $\lambda$ , whereas IFN $\gamma$  is predominant in the type II IFN family. The IFN- $\alpha$  gene family consists of many members. In contrast, there is a single gene encoding IFN- $\beta$ . Stimulation of certain cell types (mainly DCs, macrophages and other cells of hemotopoietic origin) with viruses, bacteria or TLR ligands, such as polyIC, LPS, CpG and imiquimod, resulted in the production of IFN- $\beta$  and IFN- $\alpha$ 4. Thus, it was shown that type I IFNs are produced in response to TLR3, TLR4, TLR7 and TLR9 activation, which makes these IFNs to important TLR response genes (Hertzog P. J. et al. 2003). IFNs were first discovered because of their ability to confer an antiviral state on cells. Any stage in the replication cycle of viruses appears to offer the possibility for inhibition by IFNs, including entry and/or uncoating, transcription, initiation of translation, maturation and assembly and release (Stark G.R. et. al. 1998; Muller U. et al. 1994). Besides their antiviral properties, type I IFNs were reported to promote the proliferation of memory T cells and prevent T cell apoptosis, by activating STAT4 directly

and inducing IFN- $\gamma$  secretion from CD8<sup>+</sup> T cells in mice and CD4<sup>+</sup> T cells in humans (Nguyen K.B. et. al. 2002; Rogge L. et al. 1998).

The innate immune response is usually followed by an adaptive immune response, which is mediated by the clonal selection of lymphocytes and leads to long-term immune protection. The exquisite specificity of antigen recognition by the T cell arm of the immune response provides an important basis for cancer immunotherapy. The ability to discriminate tumor cells from normal tissues is critical for enabling effective tumor destruction while minimizing toxicity. Indeed, the isolation of tumor-specific T cells from cancer patients has fueled the search for tumor-associated antigens. Given that most non-hematopoietic tumors express MHC class I molecules, which serve as the restricting element for CD8<sup>+</sup> T cell recognition, but do not express MHC class II molecules, which are required for CD4<sup>+</sup> T cell recognition, it has been assumed that the predominant tumoricidal effectors mechanism is killing by CD8<sup>+</sup> CTL. The requirement for CD4<sup>+</sup> T cells in these responses has been attributed to providing help during priming to achieve full activation and effectors function of tumor-specific CTL. However, several lines of evidence suggest a broader role for CD4<sup>+</sup> T cells in mediating other significant antitumor effectors functions. Depletion of either CD4<sup>+</sup> T cells or NK cells, but not CD8<sup>+</sup> T cells, resulted in the inability to reject the MHC class I negative variant, suggesting that CD4<sup>+</sup> T cells may provide help as well as a measure of antigen specificity to effector cells of the immune response that do not themselves have the capacity for antigen-specific recognition (El Andaloussi, A., et. al. 2006; Sonabend, A.M., 2008).

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 therefore is likely to be the cytokine 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). TGF $\beta$  is also well established as a multifunctional immunosuppressive cytokine. Produced by tumors, significantly reduces the potency of DC/tumor fusion vaccines. TGF beta stimulates in an autocrine as well as 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).

It is known that the anticancer activity of certain oncolytic viruses, notably the Newcastle disease virus (NDV), is not only mediated by their direct killing effect on cancer cells, but also involves an adjuvant effect of the virus on the immune system (Schirmacher V. et al. 2000; Schirmacher V. et al. 2005). Besides ‘exposing’ tumor-associated antigens, oncolytic viruses can provide danger signals and/or modulate the production of immunoregulating molecules, resulting in the activation of the immune system and the breakage of tolerance towards the tumor. Therefore, this work was focused on the investigation of immunostimulating properties of MVMp-mediated tumor lysates.

### **1.5. Aim of work.**

Malignant gliomas and melanomas are refractory to most standard therapies, including resection, radiation therapy and chemotherapy. A variety of different alternative approaches were performed within the last decades. PV represent one of the promising approaches due to their oncolytic and oncosuppressive properties.

In this study, we hypothesized that MVMp-infected glioma GL261 and B78/H1 melanoma tumor cells possess the ability to induce a specific immune response leading to tumor eradication.

*In vitro* experiments have been performed to evaluate the efficacy of MVMp to infect and lyse tumor cell lines; to investigate the activation of DC and microglia upon co-culture with PV-infected tumor cells. Additionally, the possible role of TLRs in induction of immune response was investigated.

*In vivo* studies consisted of the evaluation of a tumor specific immune response induced by MVMp in murine models.

## 2. Materials

### 2.1. Materials for Tissue Culture.

**Plastic.** All plastic materials for tissue culture (tubes, dishes, plates, pipets, tips etc.) were purchased from Greiner Bio-One, Sarstedt, NUNC and FALCON.

#### Mediums, buffers and solutions

**1M HEPES** – 238.31g of HEPES were dissolved in ddH<sub>2</sub>O to a volume 1L. pH was titrated to 7.0 - 7.2 with 1M NaOH. Solution was filtered through a 0.2µm nitrocellulose filter.

**2.5M CaCl<sub>2</sub>** – 183.7g CaCl<sub>2</sub>\*2H<sub>2</sub>O were dissolved in ddH<sub>2</sub>O to a volume 500mL. Solution was filtered through a 0.2µm nitrocellulose filter and stored in working aliquots.

**2xHBSS** - buffer saline pH 7.05 – 0.267g of Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>O, 0.746g of KCl, 16.36g of NaCl, 2.162g of (D)-Glucose and 11.91g Hepes were solved in 800mL ddH<sub>2</sub>O. pH was titrated to 7.05 with 10M NaOH. H<sub>2</sub>O was added to 1L. 2xHBSS was sterilized by filtering through a 0.2µm nitrocellulose filter.

**cDMEM** – DMEM plus 10% FCS, L-glutamine (100x), non-essential aminoacids (100x), 25mM HEPES, Na-pyruvate (100x), β-mercaptoethanol (1000x).

**cDMEM with antibiotics** – cDMEM plus Penicillin/Streptomycin (100x)

**Gey's solution** - 20 parts Stock A; 5 parts Stock B, 5 parts Stock C; 70 parts sterile ddH<sub>2</sub>O.

Stock A: 35.0 g NH<sub>4</sub>Cl; 1.85 g KCl; 1.13 g Na<sub>2</sub>HPO<sub>4</sub>• 7H<sub>2</sub>O; 0.12 g KH<sub>2</sub>PO<sub>4</sub>; 5.0 g glucose bring to 1 L with ddH<sub>2</sub>O and autoclave.

Stock B: 0.42 g MgCl<sub>2</sub>•6H<sub>2</sub>O; 0.14 g MgSO<sub>4</sub>•7H<sub>2</sub>O; 0.34 g CaCl<sub>2</sub>

Bring to 100 ml with ddH<sub>2</sub>O and autoclave.

Stock C: 2.25 g NaHCO<sub>3</sub> bring to 100 ml with ddH<sub>2</sub>O and autoclave.

**Magic Medium (MM)** – Iscove's medium plus 10% FCS, L-glutamine (100x), non-essential aminoacids (100x), 25mM HEPES, Na-pyruvate (100x), β-mercaptoethanol (1000x), Penicillin/Streptomycin (100x), Gentamicin (1000x)

**RIPA buffer** - 10mM Tris pH7.5, 150mM NaCl, 1mM EDTA pH 8.0, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS

**SM (staining medium)** – HBSS plus 2% FCS, 25mM HEPES

Na-pyruvate (Gibco 11360-039), Non-essential aminoacids (Gibco, 11140-035), PBS-EDTA 1.5 mM (0,22µm sterile filtered), HBSS (Invitrogen 14175-053), Iscove's medium (Sigma I3390), L-glutamine (Gibco 25030-24), Tissue culture (TC) double distilled H<sub>2</sub>O, DMEM (Sigma D5796), FCS (Biochrom AG S0115), Trypsin-EDTA 1x (Gibco 25300-054), β-Mercaptoethanol (Gibco, 31350-010)

### **Chemicals**

0.4 % Trypan Blue (Sigma, T-8154)

Collagenase A (Roche, 10103578001)

DMSO

DNase I (Roche, 11284932001)

Propidium Iodide (Sigma, P4170)

Poly-L-Lysine

MTT (Sigma, M5655)

### **Antibiotics**

Blasticidin S (InvivoGen, ant-bl)

Geneticin G418 (Sigma, A1720, Gibco, 11811)

Gentamycin (Invitrogen 04390311 A)

Penicillin/Streptomycin (Gibco 15140-122)

**Cytokines and growth factors**

Recombinant human TNF (Peprotech, 300-01)

Recombinant murine FLT-3 Ligand (Peprotech, 250-31)

Recombinant murine GM-CSF (Peprotech, 315-03)

Recombinant murine IL-4 (Peprotech, 214-14)

Recombinant murine Stem Cell Factor (Peprotech, 250-03)

Recombinant murine TNF (Peprotech, 315-01)

**TLRs ligands, mitogens**

Concanavalin A (Sigma, C5275)

Lectin from Phaseolus vulgaris, Phytohemagglutinin (Sigma, L8902)

Lectin from Phytolacca Americana (pokeweed) (Sigma, L8777)

LPS from Escherihia coli 011:B4 (InvivoGen, tlr1-elps))

LPS from Escherihia coli 026:B6 (Sigma-Aldrich L2654)

ODN1826 (InvivoGen, tlr1-modn)

ODN1826 Control (InvivoGen, tlr1-modnc)

Peptidoglycan from Staphylococcus aureus (InvivoGen, tlr1-pgnsa)

Zymozan (InvivoGen, tlr1-zyn)



## 2.2. Materials for Molecular Biology Methods

### List of primers for PCR.

Gene	Primer type	Sequence
$\beta$ -actin	<b>Sense 5'-3'</b>	TGG GTC AGA AGG ACT CCT ATG
	<b>Antisense 3'-5'</b>	CAG GCA GCT CAT AGC TCT TCT
IFN- $\beta$	<b>Sense 5'-3'</b>	CCC TAT GGA GAT GAC GGA GA
	<b>Antisense 3'-5'</b>	CTG TCT GCT GGT GGA GTT CA
IL-1 $\beta$	<b>Sense 5'-3'</b>	CAG GCA GGC AGT ATC ACT CA
	<b>Antisense 3'-5'</b>	TCC ATT GAG GTG GAG AGC TT
IL-6	<b>Sense 5'-3'</b>	AAT TTC CTC TGG TCT TCT GGA
	<b>Antisense 3'-5'</b>	GCC ACT CCT TCT GTG ACT CC
NS-1	<b>Sense 5'-3'</b>	GTT TTG GGA GCA ACC AAC TG
	<b>Antisense 3'-5'</b>	TCC AGC CAG AGT CAC TGC TA
mTLR1	<b>Sense 5'-3'</b>	CAA TGT GGA AAC AAC GTG GA
	<b>Antisense 3'-5'</b>	TGT AAC TTT GGG GGA AGC TG
mTLR2	<b>Sense 5'-3'</b>	CAG ACG TAG TGA GCG AGC TG
	<b>Antisense 3'-5'</b>	GGC ATC GGA TGA AAA GTG TT
mTLR3	<b>Sense 5'-3'</b>	GAG GGC TGG AGG ATC TCT TT
	<b>Antisense 3'-5'</b>	TGC CTC AAT AGC TTG CTG AA
mTLR4	<b>Sense 5'-3'</b>	GCT TTC ACC TCT GCC TTC AC
	<b>Antisense 3'-5'</b>	CGA GGC TTT TCC ATC CAA TA
mTLR5	<b>Sense 5'-3'</b>	GCT TTG TTT TCT TCG CTT CG
	<b>Antisense 3'-5'</b>	ACA CCA GCT TCT GGA TGG TC
mTLR6	<b>Sense 5'-3'</b>	GCA ACA TGA GCC AAG ACA GA
	<b>Antisense 3'-5'</b>	GTT TTG CAA CCG ATT GTG TG
mTLR7	<b>Sense 5'-3'</b>	ATT CAG AGG CTC CTG GAT GA
	<b>Antisense 3'-5'</b>	AGG GAT GTC CTA GGT GGT GA
mTLR8	<b>Sense 5'-3'</b>	TCC TGG GGA TCA AAA ATC AA
	<b>Antisense 3'-5'</b>	AAG GTC GTA GCG CAG TTC AT
mTLR9	<b>Sense 5'-3'</b>	ACC CTG GTG TGG AAC ATC AT
	<b>Antisense 3'-5'</b>	GTT GGA CAG GTG GAC GAA GT

Chloroform, Ethanol, Isopropanol and Methanol were purchased from Fluka.

Luciferase Assay Reagent (Promega, E1483)  
 Luciferase Cell Culture Lysis Reagent (Promega, E1531)  
 Roti-Aqua-Phenol (Carl Roth GmbH A9803)  
 Trizol (Invitrogen, 15596-018)  
 UltraPure DNase/RNase-Free Distilled Water (Invitrogen 10977-015)  
 Luciferase Assay Reagent (Promega F1483)  
 Luciferase Cell Culture Lysis Reagent (Promega E1531)  
 LIA-Plate 96 well (Greiner Bio-One 764075)

### 2.3. Materials for FACS.

#### List of antibodies for FACS.

specificity	clone	Conjugates	Source
B220	RA3-6B2	FITC, PE, biotin	Pharmingen
CD117 (c-kit)		APC	eBioscience
CD11b	M1/70	PE, biotin	Pharmingen
CD11b	M1/70	FITC, biotin	eBioscience
CD11c	HL3	FITC, PE, biotin	Pharmingen
CD11c	N418	APC	Caltag
CD25	7D4	FITC, biotin	Pharmingen
CD4	RM4-5	FITC, PE, biotin	Pharmingen
CD40	HM40-3	Biotin	eBioscience
CD40	HM40-3	FITC	Pharmingen
CD69	H1.2F3	FITC, biotin	Pharmingen
CD69	H1.2F3	Biotin	eBioscience
CD8	53-6.7	FITC, PE	Pharmingen
CD80 (B7-1)	16-10A1	FITC, biotin	Pharmingen

CD86	GL1	Biotin	Pharmingen
CD86	GL1	Biotin	eBioscience
Goat-anti Rat IgG		PE	Caltag
MHC II	25-9-17	FITC, biotin	Pharmingen
NK1.1	PK136	Biotin	Pharmingen
Sca-1 (Ly-6A/E)	D7	FITC, biotin	Caltag
Sca-1 (Ly-6A/E)	D7	PE.Cy5, biotin	Caltag
Sheep anti-Rat IgG	polyclonal		DynalBiotech
TCR	H57-597	FITC, biotin	eBioscience
TCR	H57-597	FITC	Pharmingen

Monensin (NatuTec, 00-4505-51)

#### **2.4. Materials for ELISA/ELISPOT**

TMB Substrate Reagent Set (BD, BD OptEIA 555214)

Mouse IL-6 ELISA set (BD, BD OptEIA 555240)

Mouse TNF ELISA set (BD, BD OptEIA 558874)

#### **2.5. Additional Reagents and Equipment**

Freezing box (Nalgene)

Kimwipes Lite 200 (Kimberla Clark)

Neubauer Zählkammer

Nitex (Sefar, 03-85/34)

Pasteur pipets

## **2.6. Cell Lines**

### **293 FT**

The 293FT cell line was originally generated from human epithelial kidney cells. The cells were originally purchased from Invitrogen and were obtained from Dr. Matteo Di Piazza (ATV) in the forth passages. The 293FT cells were maintained in cDMEM and were selected periodically in Geneticin (500 mg/mL).

### **HEK293**

The HEK293 cells were also generated from human epithelial kidney cells. The cell line was obtained from Dr. Cerwenka (DKFZ). The cells were maintained in cDMEM.

### **GL261**

The GL261 mouse glioma tumor was originally induced intracerebral injection of the chemical carcinogen methylcholantrene into C57/BL6 mice and maintained in its syngenic host by alternating intracranial and subcutaneous implantation of small tumor pieces. Safrany et. al. (Szatmari T. et. al., 2006) have established the permanent GL261 cell line from the tumor. The cells were maintained in cDMEM.

### **B78/H1**

The mouse B16 melanoma subclone B78/H1 was obtained from Dr. Jan Cornelis (ATV). The cells were maintained in cDMEM.

### **B16F10/GM-CSF and B16F10/FLT3L**

Both of the cell lines were originally obtained in lab. Dr. G. Dranoff (Dranoff G. et. al., 2003 ; Mach N. et. al. 2000). The cells were maintained in cDMEM.

### **Stably transfected cells /TLRs**

Stably transfected HEK293 and 293FT cell lines with single mouse TLR2 or mouse TLR4 were generated in the laboratory by Dr. Ute Koch and H. Hong.

Stable clones were selected with 25µg/mL Blasticidin. The cells were maintained in cDMEM.

Cell lines 293 stably expressed mouse TLR3, mouse TLR2/TLR6, mouse TLR4/MD2/CD14 were purchased from Invivogen. The cells were maintained in cDMEM with 10µg/mL of Blasticidin.

The 293.2 mTLR9 NFκB Promoter cells were obtained from the lab of Dr. S. Bauer (Bauer S. et. al., 2001). Stable clones expressing mTLR9 with a 6-fold NF-κB luciferase reporter plasmid were selected with 0.7 mg/ml G418.

### **2.7. Experimental Animals**

Experimental mice were obtained from Charles River Wiga company (Sulzburg, Germany). Mice represented strains C57/B16. 5-6 week old male were grouped up to 5 animals per cage. The cages were placed in the isolator at 21-24<sup>0</sup>C, 40-60% humidity. All animals were housed under the same pathogen-free conditions (food and water, 12h dark-light). Animals were killed by cervical dislocation. The Referat Veterinärwesen at the Regierungspräsidium, Karlsruhe, Germany approved the animal studies.

### **3. METHODS.**

#### **3.1. Cell Culture Methods.**

##### **3.1.1. Maintenance of Cell Lines.**

The cells were maintained as monolayer cultured - all used cell lines were adherent. Cells were usually cultured in cDMEM without antibiotics and propagated in T75 flasks at 37<sup>0</sup>C, 5% CO<sub>2</sub> and 90% humidity. Dependently on the cell growth rate the cells were splited one or two times a week. Confluent flasks were washed once with HBSS. Cells were trypsinated with Trypsin-EDTA for 5-8 min. When a single cell suspension has been obtained, the cDMEM was added to the cells. An aliquot of cell suspension was diluted with Trypan-Blue. Cells were counted with the Neubauer Zahlkammer and split as desired. Maximum passage number of cells was ten.

Microglia was cultured in cDMEM with antibiotics, as the danger of contamination was significantly higher when working with cells derived from primary culture. Dendritic cells and splenocytes were cultured in MM.

##### **3.1.2. Freezing and Thawing Mammalian Cells.**

For freezing, the cells from the confluent culture were collected using trypsin, centrifuged and resuspended in FCS containing 10% w/v DMSO. The cell suspension was transferred into cryovials and frozen in a cell freezing box at -80<sup>0</sup>C.

For thawing, a criovial was kept at 37<sup>0</sup>C until the content was melt. The cell suspension was transferred into a 15mL tube containing 10mL of proper medium. Cells were centrifuged at 1500rpm for 10 min. The supernatant was removed, cells resuspended in 10mL fresh medium and placed in a cell cultured flask.

### 3.1.3. Cells Viability Methods.

**MTT activity assay.** For the determination of cell viability, the metabolic activity of mitochondrial dehydrogenases was measured through the ability of these enzymes to produce a formazan dye through reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).

Glioma GL261 and melanoma B78/H1 cells were plated in 96-well plates ( $2 \times 10^3$  cells/well) in cDMEM. After 18h medium was removed and cells were infected in 50 $\mu$ L of DMEM without supplements with MVMp at different MOIs. Plates were incubated at 37 $^{\circ}$ C for 1.5 hours. During infection, plates were rocked every 15min. 150  $\mu$ L of cDMEM was added to the cells after 1.5h. After infection time (24h, 48h, 72h, 96h p.i.) 50 $\mu$ l of medium was removed, and MTT was added (final concentration 0.5mg/mL) to the cultures, and incubation was continued for 4h at 37 $^{\circ}$ C. After centrifugation of the plate, the supernatant was removed, and the cells were dried for 30min at 37 $^{\circ}$ C before lysis by addition of 100 $\mu$ l isopropanol. The absorbance of the formazan dye was measured at 595nm using an ELISA plate reader. The viability of infected cells was expressed as the ratio of the corresponding absorbance to that of noninfected cells taken arbitrarily as 100%.

**Trypan Blue Dye Exclusion Method.** Trypan blue is the most common stain used to distinguish viable cells from non-viable cells; only non-viable cells absorb the dye and appear blue and may 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 10  $\mu$ L aliquot of cell suspension was placed in appropriate tube and 90 $\mu$ L of 0.4% Trypan blue was added, and gently mixed. 10 $\mu$ l of stained cells were placed in a hemocytometer and the number of viable (unstained) and dead (stained) cells

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was counted. The total number of viable cells was determined according to the following calculation:

$$\begin{aligned} \text{cells/mL} &= \# \text{ of cells counted in a square} \times 10^4 \times \text{dilution factor} \\ \text{total cells} &= \text{cells/mL} \times \text{volume of original cell suspension} \end{aligned}$$

### 3.1.4. Generation of Microglia.

Brains from the newborn C57/BL6 mice were used for the preparation of Microglia. The heads were cut off from the mice using scissors. The skullcap was opened medially with a spiky pair of tweezers. The brain was carefully removed and transferred into a tube with 1x HBSS. Meninges were removed under a stereoscope. Mice brains were then transferred on ice into 50mL polypropylene tubes with 10mL cDMEM. The brains were trypsinised with trypsin-EDTA in the presence of DNase (0.05%) for 20 min. The cells were spun at 1000 rpm for 10 min and supernatant sucked off. The cell pellet was resuspended in cDMEM with antibiotics and plated in T75 flasks precoated with poly-L-Lysine (20 $\mu$ g/mL) in a final 10mL volume and then incubated at 37 $^{\circ}$ C, 5%CO $_2$ . The medium was changed once per day following 2 days, and on day 7 post-plating. At day 14, adherent cells were confluent and consisted of a mixture of astrocytes and microglia. At this time, the medium was sucked off, and Trypsin-EDTA was added for 5 min at 37 $^{\circ}$ C. The Trypsin-EDTA was sucked off and cDMEM plus DNase (0.05%) was added. The cells were easily detached using Pasteur pipette, spun as before, and the pellet replated on 6 cm dishes at 37 $^{\circ}$ C, 5%CO $_2$  to enable microglia to adhere while astrocytes remained in suspension. After 1h of incubation, the supernatant was transferred to cell culture graded dishes to develop astrocyte cultures. Subsequently, medium was changed once every week and astrocytes used at 14 days whereas microglia was ready for use at 21 days following replating.



### 3.1.5. Generation of Dendritic Cells from Bone Marrow

To enrich the HSC population in BM and deplete actively cycling cells, 5-fluorouracil was given intravenously as a single dose of 150 mg/kg body weight 5 days prior the cells isolation. BM was extracted from the tibia and femur with SM using a 2mL syringe and 25-gauge needle. This single cell suspension was centrifuged at 1200 rpm 5min at 4<sup>0</sup>C and the pellet resuspended in a 5mL of Gey's solution for 5min to lyse erythrocytes. The single cell suspension was collected, washed two times with SM, and counted. Desired amount of cells were resuspended in proper amount of SM. To deplete non-HSC the pellet was incubated with supernatant from the following cell culture (see Table.1), for 45min on ice.

**Table 1. Staining for lineage markers.**

Antibody	Source	Number	Number of hybridoma	Dilution
Anti-CD4	TCSN	YTS 191.1	A170	1:100
Anti-CD8	TCSN	YTS 169.4	A150	1:100
AntiCD19	TCSN	Id 3	A200	1:100
Anti CD3	TCSN	YCD 3 $\epsilon$	A110	1:8
Anti CD3	TCSN	Thy1.2/3OH10	A690	1:50
Anti TER 119	TCSN	TER 119	A330	1:100

The cells were collected and washed in SM. Afterwards, the pellet was labelled with Sheep anti-Rat IgG coupled to the magnetic beads for 20min at 4<sup>0</sup>C on the rotor. Beads-coupled cells were eliminated on the Magnetic column and HSC-containing suspension was spun down at 1200rpm, 5min, 4<sup>0</sup>C. The procedure with magnetic beads was repeated twice more.

The remaining cells were cultured in MM in 6 well plates (0.5x10<sup>6</sup>cells/well) with supplement of SCF for the first 24h, and GM-CSF, FLT3L, combination of GM-CSF and FLT3L for 9 days. Medium with fresh

cytokine cocktail was added every 3 days. The morphological changes of cells were observed under the microscope. After 9 days DCs were used in follow experimental settings (see 3.1.8).

### 3.1.6. Isolation of Dendritic Cells from Spleen and Lymph Nodes.

C57/BL6 mice were injected s.c. with B16F10/GM-CSF or B16F10/FLT3L ( $1 \times 10^6$  cells/mouse in 100 $\mu$ L PBS) 14 days prior DCs isolation. LN and Spleen were isolated from each single mouse. The organs were chopped with scissors and were gently pressed through the metal sieve using a rubber plunger in a syringe, all the while gently applying fresh media on the spleen to keep the cells moist and add transfer through the sieve. The pellet was resuspended in a 5mL of Gey's solution for 5min to lyse erythrocytes. The single cell suspension was collected, washed two times with SM and counted. Desired amount of cells were resuspended in proper amount of SM. To deplete non-CDs the pellet was incubated with supernatant from the following cell culture (see Table 2.), for 45min on ice.

**Table 2. Staining for lineage markers.**

Antibody	Source	Number	Number of hybridoma	Dilution
Anti-CD4	TCSN	YTS 191.1	A170	1:100
Anti-CD8	TCSN	YTS 169.4	A150	1:100
Anti B220	TCSN	RA3 6B2	A120	1:100
AntiCD19	TCSN	Id 3	A200	1:100
Anti CD3	TCSN	YCD 3 $\epsilon$	A110	1:8
Anti CD3	TCSN	Thy 1.2 3OH10	A690	1:50
Anti MAC1	TCSN	M1/70	A100	1:100
Anti GR1	TCSN	RB6-8C5	A130	1:100
Anti TER 119	TCSN	TER 119	A330	1:100

The cells were collected and washed with SM. Aftrewards, the pellet was labelled with Sheep anti-Rat IgG coupled to the magnetic beads for 20min at 4<sup>0</sup>C on the rotor. Beads-coupled cells were eliminated on the Magnetic column and cells-containing suspension was spun down at 1200rpm, 5min, 4<sup>0</sup>C. The procedure with magnetic beads was repeated twice more. After that DCs were used in follow experimental settings (see 3.1.8).

### **3.1.7. Isolation of Splenocytes**

Spleens were isolated from each single mouse. The organs were chopped with scissors and were gently pressed through the metal sieve using a rubber plunger in a syringe, all the while gently applying fresh media on the spleen to keep the cells moist and add transfer through the sieve. The pellet resuspended in a 5mL of Gey's solution for 5min to lyse erythrocytes. The single cell suspension was collected and washed with SM. Afterthat splenocytes were used in follow experimental settings.

### **3.1.8. Co-culture of Dendritic Cells and Tumor Cells**

On day 0 glioma GL261 or melanoma B78/H1 cells were plated in 6-well plates, at  $5 \times 10^5$  cells per well. On day 1 the cells were washed once with DMEM and were infected in 0.5mL of DMEM without supplement using MVMp (see 3.5.) at MOI10 for glioma GL261 and MOI100 for melanoma B78/H1. Plates were incubated at 37<sup>0</sup>C for 1.5hours. During infection, plates were rocked every 15min. 2.5mL of cDMEM was added to the cells after 1.5hours.

On day 3, after 48h p.i., when a fraction of infected tumor cells was lysed (see 4.1.2.), DCs ( $1 \times 10^6$ ) were added into the wells with tumor lysates. In addition, DCs were added to MOCK-infected tumor cells and to lysates from tumour cells after 4 cycles of freezing and thawing. As a positive control, DCs

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were stimulated with 5µg/mL of LPS. In addition, DCs were incubated alone or were infected with MVMp at MOI100.

On day 5, after 48h of co-culture, DCs were harvested from the culture wells. Supernatants were collected and frozen at -20<sup>0</sup>C for ELISA assay (see Methods 2.4.1.). Cells of the 6 well plates were processed for 2 different procedures:

- for FACS analysis of expression activation markers (see 3.4.2.1),
- for RNA extraction (see 3.3.3.).

### **3.1.9. Co-culture of Microglia and Glioma GL261 cells**

On day 0 glioma GL261 cells were plated in 10cm dishes, at 5x10<sup>5</sup> cells per well. On day 1, tumor cells were washed once with DMEM and were infected in 1mL of DMEM without supplement using MVMp (see 3.5.) at MOI10. Plates were incubated at 37<sup>0</sup>C for 1.5 hours. During infection, plates were rocked every 15 min. 7mL of cDMEM was added to the cells after 1.5 hours.

On day 2 microglia was harvested and plated. Briefly, the supernatant from the microglia (see 3.1.4.) was discarded. To detach microglia from the dishes, the cells were incubated with 10mL of PBS/EDTA for 15min at RT. Cells were harvested using Pasteur pipette while carefully sprinkling the plate in order to detach cells which were still adherent. Microglia was transferred into sterile 50mL FALCON tubes, already containing 10mL cDMEM. Cells were collected and washes with SM. Pellet was resuspended in appropriate amount of cDMEM and counted. 1x10<sup>6</sup> microglia were plated into 10cm dishes.

At day 3, supernatant from microglia was sucked off and discarded. After 48h p.i., when a fraction of infected tumor cells was lysed (see 4.1.2.), glioma GL261 cells were scraped and cell suspension/lysate was transferred to the dishes with microglia. In addition, microglia was co-cultured with MOCK-infected glioma GL261 cells and lysates from tumour cells after 4 cycles of

freezing and thawing. As a positive control, microglia was stimulated with 5µg/mL of LPS/ 30 ng/mL IFN $\gamma$ . In addition, microglia was incubated alone or was infected with MVMp at MOI100.

On day 5, 48h after co-culture, microglia was harvested from the culture dishes. Supernatants were collected and frozen at -20°C for ELISA assay (see 3.4.1.). Cells from the 10cm dishes were processed for 2 different procedures:

- for FACS analysis of expression activation markers (see 3.4.2.1),
- for RNA extraction (see 3.3.3.).

### **3.1.10. Transient Transfection for Luciferase Assay**

The 293FT or HEK293 cells stably transfected with single TLRs ( $0.6 \times 10^5$  cells/well) were plated into 6-well tissue culture plates 3mL cDMEM and were incubated at 37°C 5%CO<sub>2</sub> overnight. Cells should be at 80-90% of confluence next day.

After 16h, 14µg of DNA (Translucent Reporter Vector pNFkB-Luc (Panomics LR0051) or Translucent Control Vector (see Appendix); (stock concentration 1µg/mL) were diluted in 616µl of sterile ddH<sub>2</sub>O to a final volume 630µl. Then 70µl of sterile filtered 2.5M CaCl<sub>2</sub> was added to the DNA mixture. Afterwards, 700µl of 2xHBSS (pH7.05) was added. Immediately, a mechanical pipettor attached to a plugged pipette was used to mix the suspension of 2xHBSS and DNA/CaCl<sub>2</sub> by feeding in air bubbles for 15sec. Mixture was vortexed for 5sec right away and incubated at RT for 10 min so that precipitates could form.

During the incubation time, medium from the dishes with adherent cells was removed carefully. Cells were washed once with 1xHBSS (3mL per well) and 3mL cDMEM was added to the tissue culture dish. 200µl of the precipitate were distributed over each well of the 6-well tissue culture plate. Cells were incubated for 48h at 37°C with 5%CO<sub>2</sub>.

Highest expression of Luciferase was detected between 24h and 48 hours. Thus, around 40 hours post transfection, cells were stimulated with human TNF (50 ng/mL), LPS (5 µg/mL), and specific ligands for TLRs, were infected with MVMp, or were co-cultured with MVMp-infected glioma GL261 or melanoma B78/H1 cells.

8h later, growth medium from the cells was removed. 400µl Trypsin-EDTA per well was added and incubated at 37<sup>0</sup>C for 10min. Then 3ml of ice-cold cDMEM was added. From this time on, cells were kept on ice. Cells were suspended and transferred into 4.5 mL tubes for centrifugation at 4<sup>0</sup>C, 1200rpm for 5min. After removal of the supernatant, the cells were lysed by resuspending the pellet in Luciferase Cell Culture Lysis Reagent. The lysates were centrifugated once more at 4<sup>0</sup>C, 1200rpm for 10min and the supernatants were transferred into 1.5mL tubes and stored at -80<sup>0</sup>C, until luciferase assays were performed (see 2.3.1).

## **3.2. Animal Techniques.**

### **3.2.1. Injection of In Vitro Infected Tumor Cells**

The glioma GL261 cells were seeded in 10cm in cDMEM dishes one day before infection (1x10<sup>6</sup> cells/dish). On the day of infection medium was removed and cells were infected with indicated MOIs in 1mL of DMEM without supplements. The dishes were placed at 5% CO<sub>2</sub>, 37<sup>0</sup>C, for 1.5h and gently rocked every 15min. After 1.5h the cells were supplied with cDMEM and placed in the incubator. Four hours after infection the cells were trypsinized, collected, washed two times with PBS and counted. Desired amount of cells were resuspended in proper amount of PBS. 100µl of cell suspension (1x10<sup>6</sup> cells) were injected subcutaneously in the right flank of each animal. Growing tumors were examined and measured every 2-3 days. For the evaluation of tumor's volume length, breadth and height were measured every 3 days with the

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assistance of a calliper. Tumor volume was calculated for an ellipsoids according to the formula  $V=\pi/6*L*B*H$ .

### **3.2.2. Isolation of In Vitro Infected Tumor Cells**

Tumors were isolated from each single mouse and were digested in the presence of DNase (0.05%) and collagenase for 20 min. Afterthat, the tumors were gently pressed through the metal sieve using a rubber plunger in a syringe, all the while gently applying fresh media on the tissue to keep the cells moist and add transfer through the sieve. The single cell suspension was collected, resuspended in a 5mL of Gey's solution for 5min to lyse erythrocytes, and washed with SM. After that single cell suspension was used in follow experimental settings.

## **3.3. Molecular Biological Methods**

### **3.3.1. Luciferase assay**

Luciferase Assay Reagent was thawed and maintained into a water bath at room temperature for at least 30min to ensure temperature equilibrium. Samples, which had been lysed in the Luciferase Cell Culture Lysis Reagent, were thawed on ice.

The luminometer was programmed for 2sec delay time and 10sec reading time. 10 $\mu$ L of cell lysate were applied per well in a LIA-plate. Plate with samples was plated into luminometer. The injector of the luminometer added 50 $\mu$ L of Luciferase Assay Reagent per well, the well was then read immediatly.

### **3.3.2. Westernblot analysis**

**Protein isolation from the Mammalian Cells.** For the detection of NS1, LC3, and caspase-3 active form proteins upon MVMp infection a protein extract

from  $1 \times 10^6$  cells cultures in 10cm dishes was prepared. The medium was removed, cells were washed with PBS and subsequently lysed with RIPA buffer. The lysates were collected, transferred into a 1.5mL tubes and placed on ice for 20 min. After that the samples were centrifuged for 10 min at 14000rpm at  $4^{\circ}\text{C}$ . The supernatant was transferred into a fresh tube and stored at  $-20^{\circ}\text{C}$ .

**Western Blot analysis.** The proteins were separated of using SDS-polyacrylamide gel electrophoresis. A desired amount of protein extract (containing 20  $\mu\text{g}$ , 40  $\mu\text{g}$  of total proteins) was mixed 1:1 with loading buffer, denaturated 5 min. at  $95^{\circ}\text{C}$  and placed on ice. After loading on a gel, electrophoresis was performed over night at 70V, 100mA. A nitrocellulose membrane (Protran; Schleicher & Schuell, Relliehausen, Germany) was placed on the gel and, by using of a semidry blotting apparatus (Bio-Rad Laboratories, Munich, Germany) 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 primary antibodies used for immunostaining were directed against LC3, caspase-3 active form, cathepsin B,  $\beta$ -tubulin (Sigma), NS1 (rabbit antiserum  $\alpha\text{SP8}$ ). Incubation with primary Abs was overnight at  $4^{\circ}\text{C}$ . 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. Peroxidase-conjugated secondary antibodies were all purchased from Santa Cruz. Immunodetection was performed with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany). The membrane was exposed of to an x-ray film (Kodak).



### 3.3.3. Total RNA Extraction from Cells

For the isolation of total RNA cells were transferred into the Eppendorf 1.5mL, treated with 1 mL of Trizol, and frozen at  $-80^{\circ}\text{C}$ . Subsequently, per 1 mL Trizol, 0.2mL of chlorophorm was added, mixed and the sample was placed at RT for 2-3 min. Afterwards, the sample was centrifugated for 15 min at 14000 rpm at  $4^{\circ}\text{C}$ . The upper aqueous phase, containing RNA, was collected in another tube, the same amount of acid phenol and 1/10 of chlorophorm was added in order to remove remaining DNA/protein contamination and purified RNA. The sample was centrifugated at 14000 rpm for 5 min at  $4^{\circ}\text{C}$ . Afterwards, RNA from aqueous phase was precipitated by mixing with equal volume of cold isopropyl alcohol. The sample was incubated overnight at  $-20^{\circ}\text{C}$ . RNA formed a gel-like pellet and was spun down by centrifugation at 14000 rpm for 15 min at  $4^{\circ}\text{C}$ . The pellet was washed once with 1mL of cold 80% ethanol, spun down by centrifugation at 9600 rpm for 10 min at  $4^{\circ}\text{C}$ , air-dried for 10 min, resuspended in DNA, RNA-free water and stored frozen at  $-80^{\circ}\text{C}$ .

### 3.3.4. RT-PCR (Reverse Transcription-Polymerase Reaction) of RNA

RT-PCR (Reverse Transcription-Polymerase 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 is first transcribed into complementary cDNA and then DNA is multiplied.

As a first step 1  $\mu\text{g}$  RNA was mixed with 1 $\mu\text{L}$  Random Primers (Invitrogen). Total sample volume was 10  $\mu\text{l}$  (adjusted with sterile  $\text{dH}_2\text{O}$ ). The probe was incubated 10min. at  $70^{\circ}\text{C}$ , 10 min at  $25^{\circ}\text{C}$ , and placed on ice. After that MasterMix was added

Reaction mix 5,0  $\mu\text{l}$  10x RT enzyme buffer (Promega)

1,0  $\mu\text{l}$  RT enzyme M-MLV (Promega)

1,0  $\mu\text{l}$  40U Rnasin (Promega)

2,0  $\mu\text{l}$  5 mM dNTP mix (Sigma)

Reaction conditions: 50 min. at 42°C, 15 min. at 70°C, kept at 4°C.

During the step of reverse transcription RNA was transcribed into cDNA. Ready cDNA sample was diluted 1: 10 with H<sub>2</sub>O and stored at -20°C.

### **3.3.5. PCR**

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

The PCR product was amplified from cDNA templates using a heat-stable DNA polymerase from *Thermus aquaticus* (Taq DNA polymerase) in an automated thermal cycler through repeated cycles of denaturing, annealing of primers, and polymerization steps. After amplification, the DNA products were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide.

### **3.3.6. Southern Blot**

For analyses of the viral DNA intermediates produced after infection, cells were infected at various MOI, trypsinised, washed in PBS, processed for low-molecular weight DNA in a mixture (1:1) of vTE buffer and 2x Hirt buffer (20 mM Tris [pH 7.4], 20 mM EDTA, 1.2% SDS) and then digested with proteinase K (400 µg/ml) for 18 h at 46°C. After cellular genomic DNA was sheared by several passages through 0.5- and 0.4-µm needles, DNA samples (2 µg) were fractionated through 0.8% agarose gel electrophoresis. After DNA denaturation and transfer onto Hybond-N nylon membranes (Amersham Pharmacia Biotech), the viral intermediates were identified by hybridization with a <sup>32</sup>P-labeled DNA probe corresponding to the EcoRV (nt 385)-EcoRI (nt

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1084) fragment of MVMp NS gene. Membranes were exposed to BioMax MS Film (Kodak, 8294985) for 18hrs at -20°C.

### **3.4. Immunochemical methods**

#### **3.4.1. Cytokine ELISA**

Level of TNF and IL-6 was measured in the supernatants from stimulated DCs and microglia (see 3.1.7 and 3.1.8) by using Mouse IL-6 ELISA set and Mouse TNF ELISA set.

#### **3.4.2. FACS analysis**

##### **3.4.2.1. Phenotypic characteristic of Dendritic Cells and Microglia**

All cellular preparations were performed at 4°C, except where indicated. Cells from various preparations (see above) to be analysed were first blocked from non-specific binding for 30min. Then, they were washed once with 3mL of SM, and labelled with Abs against surface markers. After that, the cells were washed with 3mL of SM and spun down at 1200rpm, 5min. If it was necessary cells were labelled with secondary Abs for 35 min on ice and were washed once again with 3mL of SM. 10-50x10<sup>6</sup> cells per sample were analysed. Prior to analysis, propidium iodide (PI) was added to the sample (1µg/mL) to label dead cells for subsequent dead cell exclusion. To exclude non-DCs and non-Microglia contamination during analysis, double staining CD11c/CD11b was performed for each single sample.

FACS was performed at FACSCalibur (Beckton Dickinson) using a CellQuest computer software. Data were processed by using the CellQuest or FlowJo software packages.

### 3.4.2.2. Intracellular detection of NS1

Glioma GL261 or melanoma B78/H1 cells were plated in 6-well plates, at  $5 \times 10^5$  cells/well. Next day the cells were washed once with DMEM and were infected in 0.5mL of DMEM without supplement using MVMp (see 3.5.) at MOI10 for glioma GL261 and MOI100 for melanoma B78/H1. Plates were incubated at 37°C for 1.5hours. During infection, plates were rocked every 15min. 2.5mL of cDMEM was added to the cells after 1.5hours, and cells were incubated at 37°C, 5% CO<sub>2</sub>. After indicated time of infection, cells were collected by trypsinisation, fixed in 1% cold PFA for 15 min at 4°C, washed in ice-cold PBS-BSA (0.1%), resuspended in 80% ice-cold methanol and incubated overnight at -20°C ( $1 \times 10^6$  cells for each sample). Following removal of fixative by washes in ice-cold PBS, cells were washed once with ice-cold PBS-BSA (0.1%) and permeabilised by two washes with PBAT at 4°C. Blocking was performed for 30min at 4°C in PBS-40% rat serum followed by incubation with the NS1-specific antibodies (clones 2C9 and 3D9 mixed at a 1:10 final dilution each) for 45 min (4°C), three washes in PBAT and incubation with the secondary Cy2-conjugated rat anti-mouse antibody for 60min at 4°C. After two washes in PBAT, cells were filtered through Nitex and analysed by flow cytometry.

### 3.4.2.3. TUNEL assay.

One of the later steps in apoptosis is DNA fragmentation, a process which results from the activation of endonucleases during the apoptotic program. These nucleases degrade the higher order chromatin structure into fragments of ~300kb and subsequently into smaller DNA pises of about bp length. A method which is often used to detect fragmented DNA utilizes a reaction catalyzed by exogeneous TdT, often referred to TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labelling). The DNA fragments

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were labelled with APO-BRDU Kit (BD Pharmingen, 556405) and measured by FACS.

#### **2.4.3. ELISPOT assay**

For assessing IFN $\gamma$  secretion by individual cells, MultiScreen-IP plates (PVDF membranes, Millipore, Bedford, MA) were coated overnight at 4°C with 100  $\mu\text{g}/\text{well}$  of anti-mouse IFN $\gamma$  antibodies (BD Pharmingen, 551216) diluted at 1 mg/ml in carbonate/bicarbonate buffer (0.05 M, pH 9.6). After overnight incubation, plates were washed with PBS and saturated with MM for 2h at RT prior to use. Splenocytes ( $1 \times 10^5/\text{well}$  in 200  $\mu\text{l}$ ) were added to triplicate wells and stimulated by mitogen or by glioma GL261 cells ( $5 \times 10^4/\text{well}$ ). After incubation overnight at 37°C, supernatants were discarded and 200  $\mu\text{l}$  of cold pure water was added for 10 min to osmotically shock the cells. Plates were washed with PBS + 0.05% (v/v) Tween 20 (PBS-T) and 100  $\mu\text{l}$  of biotinylated rabbit anti-mouse IFN $\gamma$  detecting antibody (BD Pharmingen, 554410) diluted to 0.25  $\mu\text{g}/\text{ml}$  in PBS-T was added. After 2hrs incubation at 4°C, plates were washed with PBS-T, and streptavidin-alkaline phosphatase enzyme conjugate was added. After 30 min incubation at room temperature, plates were washed with PBS-T and the spots were visualised with BCIP/NBT Liquid Substrate system (Sigma, B1911) according to the manufacturer's instructions.

#### **3.4.4. Detection of caspase-3 activity.**

One of the commonly used ways to detect caspases activities is to incubate synthetic substrates such as peptides conjugated to a fluorochrome with lysates of cells and measure their cleavage. Substrate processing gives rise to increased fluorescence intensity, whose signal is proportional to the amount of cleaved substrate, which in turn is dependent on caspase activity and is related to the percentage of apoptotic cells in a cell population (Kohler et. al., 2002). Caspases activities was determined by adding 25 $\mu\text{l}$  of whole-cell extract

to caspase buffer (Hepes 50mM pH 7.4, NaCl 150mM, 0.1% CHAPS, 0.1% Triton X-100, EDTA 1mM, 10% Glycerol, DTT 10 mM) supplemented with caspase 3 (Ac-Asp-Glu-Val-Asp-AFC) substrate (1 mM, Calbiochem). The reaction was monitored for 1 hour on a Fluoroskan (Termolabsystem) measuring the emission at 510 nm wavelength with the excitation wavelength set at 390 nm.

### **3.5. Virus production**

MVMp was produced by “Virus production unit” INSERM U701. Briefly, MVMp virus was produced in A9 cells and purified with Iodixanol Density Gradient. The titer of virus was determined by Plaque Assay and Hybridisation Assay.

### **3.6. Statistical analysis**

Statistical differences were determined using the Student's t test. A  $P < 0.05$  was considered significant.

**List of Abbreviation**

(m)RNA (messenger) - Ribonucleic acid

AAV - adeno-associated virus

APC - antigen presenting cell

BM - bone marrow

BSA - bovine serum albumin

CNS - central nervous system

CTL - cytotoxic T lymphocyte

DC - dendritic cell

DMEM - Dulbecco's modified Eagle medium

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic acid

ds DNA - double stranded DNA

ECM - extra cellular matrix

EDTA - ethylenediaminetetraacetic acid

EGFP - enhanced green fluorescent protein

ELISA - enzyme-linked immunosorbent assay

EtBr - ethidiumbromide

FACS - fluorescence activated cell sorter

FCS - foetal calf serum

FITC - fluorescein isothiocyanate

GM-CSF - granulocyte-macrophage colony stimulating factor

HBSS - Hank's buffered salt solution

HSC - hemaetopoietic stem cells

HSV - herpes simplex virus

IL - interleukin

INFg - interferon gamma

Kb - kilobases  
kD - kilodalton  
LPS - lipopolysaccharide  
MHC - major histocompatibility complex  
MOI - multiplicity of infection  
MVMp - minute virus of mice prototype strain  
NDV - Newcastle disease virus  
NK - natural killer cells  
NS1 - parvoviral non-structural protein 1  
OD - optical density  
ODNs - oligodeoxynucleotides  
PBS - phosphate-buffered saline  
PFU - plaque forming unit  
PV - parvovirus  
RT - room temperature  
s.c. - subcutaneous  
TNF - tumor necrosis factor  
TGF - transforming growth factor  
WHO - World Health Organisation



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## 4. Results.

### 4.1. Oncolytic effect of PV MVMp in tumor cell lines.

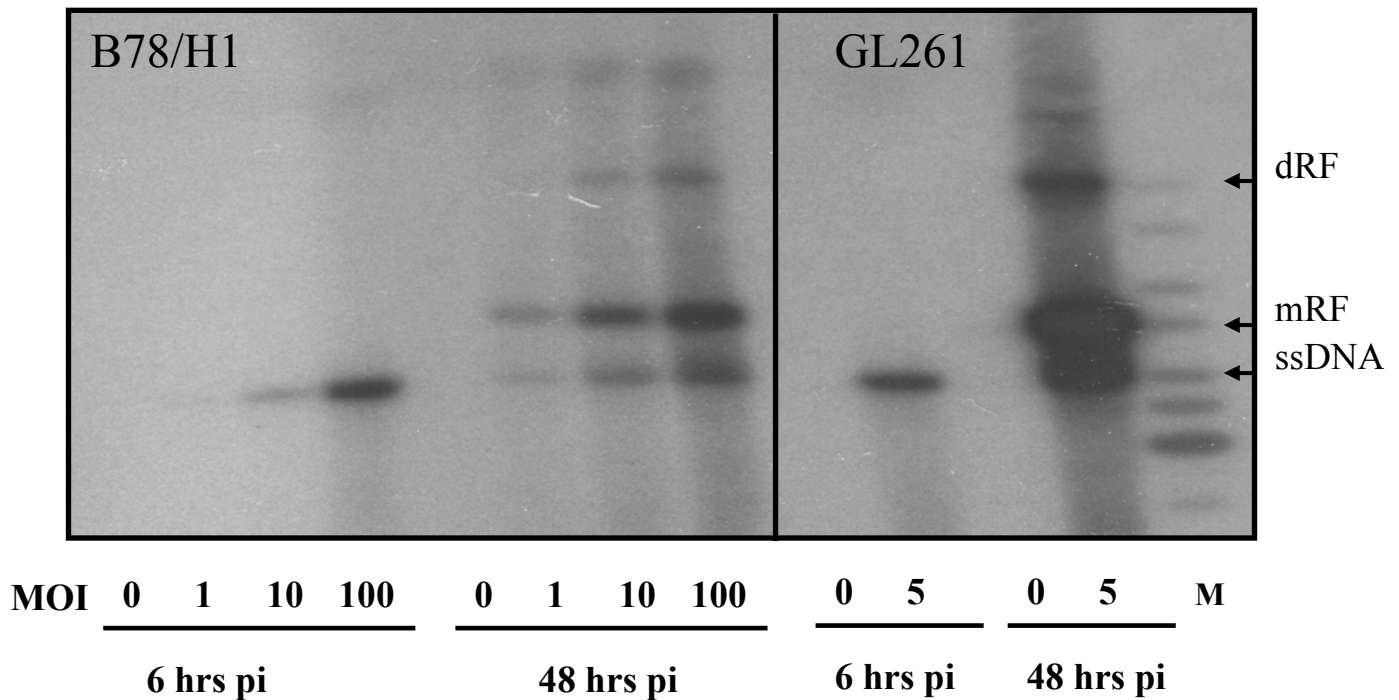
Previously, circumstantial evidence was provided that the oncolytic PV MVMp has the potential to elicit a tumor specific immune response (Moehler et.al., 2003; Guetta et. al., 1986). The main goal of the study presented here was to investigate the detailed mechanism by which PV MVMp is mediating a tumor-specific immune response in murine tumor models. A murine melanoma cell line, B78/H1, and glioma cell line, GL261, were used to analyze the induction of PV mediated oncolysis leading to a tumor-specific immune response *in vitro* and *in vivo*. MVMp vectors delivering immunostimulatory chemokines and cytokines have been evaluated to induce anti-tumor effects in the above-mentioned tumor models (Wetzel K. et. al. 2007; Lang S.I. et. al., 2006; Dinsart C. et. al. unpublished). However, the mechanisms involved, especially the contribution of the innate as well as adaptive immune response remains to be elucidated. A series of studies was conducted to verify the validity of both tumor models to assess their potential in the framework of this study inducing tumor-specific immune responses.

#### 4.1.1. Melanoma B78/H1 and glioma GL261 cell lines are both permissive for MVMp infection.

A sequence of experiments was performed to assess whether the murine melanoma B78/H1 and glioma GL261 cell lines are sensitive to PV MVMp infection. First of all the replication of MVMp in these two cell lines was investigated. It is known, that the single-stranded genome of PV is converted into a monomeric replication form in the nucleus. This monomeric intermediate form is amplified via multimeric forms and is also used as a template for the

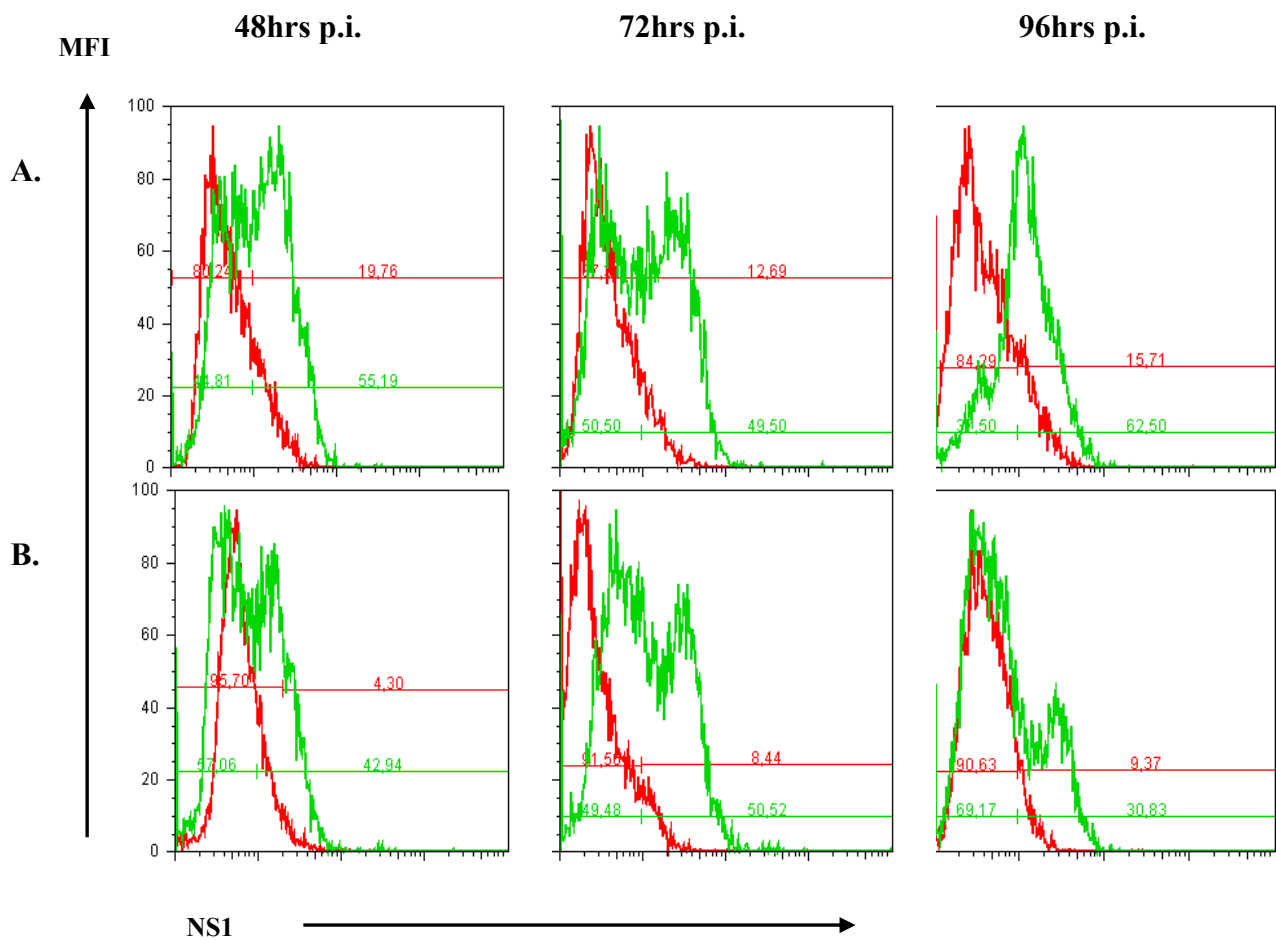
synthesis of mRNA. This process can be monitored by SouthernBlot according to the replicative forms described.

For this assay cells were left either uninfected or were infected with different MOI (1, 10, 100 for melanoma B78/H1 and 5 for glioma GL261). The amount of cell-associated viral single-stranded (ss) DNA was evaluated by Southern blotting analysis of low-molecular-weight DNA extracted 6hrs after virus infection. As shown in Fig.1, signals for the input of ssDNA could be detected in both melanoma B78/H1 and glioma GL261 cells (6hrs post infection), confirming the competence of these cell lines for MVMp uptake. Next, the formation and amplification of double-stranded (ds) DNA replicative forms (RF) of monomeric (mRF) and dimeric (dRF) size were analyzed at later time point after infection (48hrs post infection (p.i.)). Both replicative forms occurred in the two cell lines assessed (Fig.1). The glioma GL261 cell line showed a drastic increase in the ssDNA signal indicative for the production of viral progeny genomes, since the same amount of DNA was loaded. Together these data are the first indication that both cell lines are sensitive to PV MVMp infection and that MVMp can replicate in both cell lines albeit to a different extent with the GL261 cell line being the more susceptible one.



**Fig.1. Southern blotting analysis of viral DNA intermediates.** Glioma GL261 and melanoma B78/H1 cell lines were infected with different MOI (1, 5, 10, 100) of MVMp. Cells were harvested at the indicated times (6hrs and 48hrs post infection (p.i.)) and processed for the analysis of virus replication. 2 $\mu$ g of DNA were loaded. mRF, monomer replicative form; dRF, dimer replicative form; ssDNA, single-stranded genomic DNA; M – molecular weight marker.

Since the accumulation of viral genomes is coupled with their encapsidation (Cotmore, 1987), the expression of NS1 protein was investigated in both cell lines using flow cytometry. MOI 100 and MOI 10 were chosen as the optimal for melanoma B78/H1 and glioma GL261, respectively (data not shown). NS1 expression steadily increased in a time-dependent manner with the peak of expression at 96 hrs p.i. for the melanoma tumor cell line and at 72hrs p.i. for the glioma tumor cell lines (Fig. 2). Due to massive cell death upon MVMp infection, the glioma GL261 cell line shows less NS1 expression at 96 hrs p.i. Since NS1 plays a crucial role in the cytotoxic activity of PV, the cell survival might be affected due to MVMp infection.

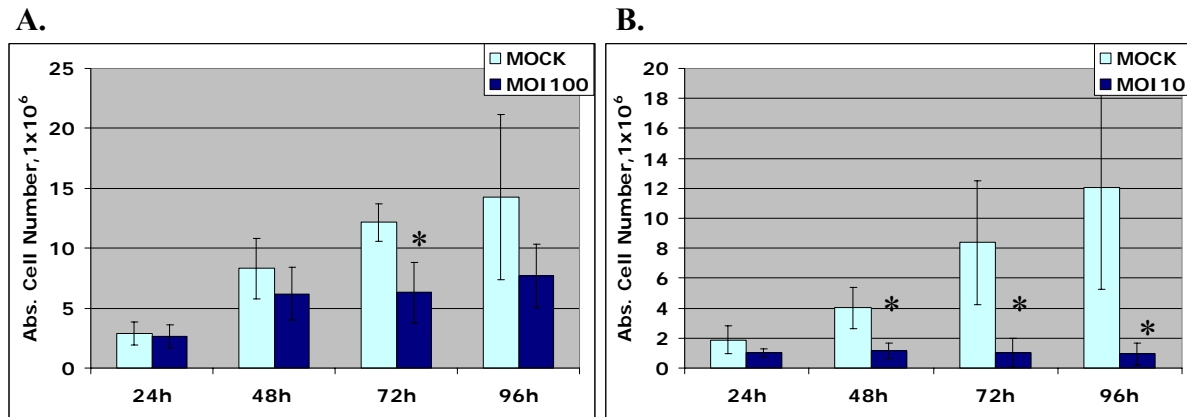


**Fig.2. Expression of NS1 protein in cell lines upon MVMp infection.** A. - Melanoma B78/H1, B. - glioma GL261 at 48hrs, 72hrs, 96hrs p.i. Red line histograms represents the MOCK-infected control samples, green line histograms depict MVMp-infected samples (melanoma B78/H1 cell line infected with MOI 100, glioma GL261 cell line infected with MOI 10). One representative experiment out of three is shown. Percentage of positively staining cells each indicated in the histograms.

#### 4.1.2. MVMp exerts a cytotoxic effect in tumor cells and inhibits cell proliferation.

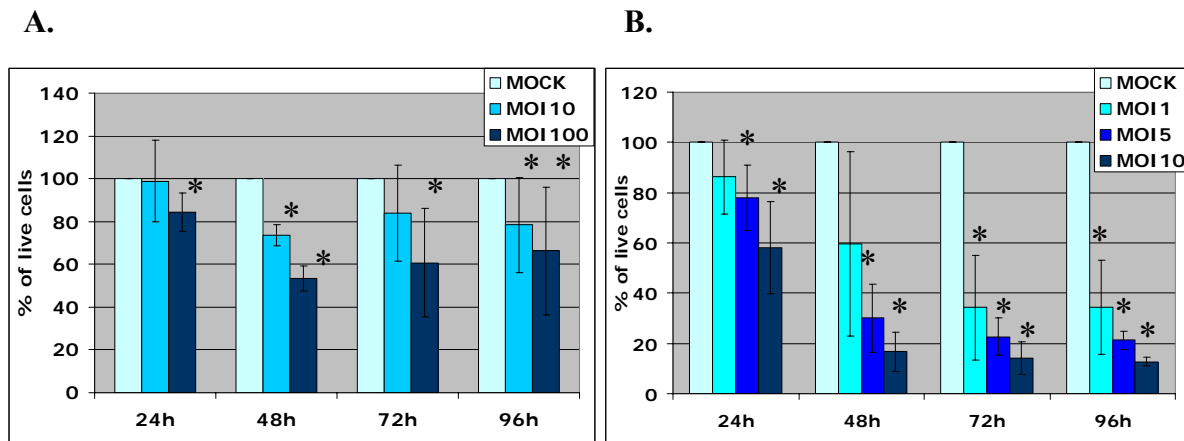
Autonomous PV are endowed with cytotoxic and oncolytic activities (Rommelaere J. 2001; Geletneky K. 2005; Wildner O. 2001). In order to establish whether PV MVMp infection was associated with the induction of cell

death in these tumor cell lines, the MTT assay was applied to verify cytotoxicity and the trypan blue dye exclusion method was used to assess the absolute number of live cells.



**Fig.3. Cell survival upon MVMP infection.** One million cells were infected with MVMP at A) MOI 100 for the melanoma B78/H1 cell line, or B) MOI 10 for the glioma GL261 cell line. The number of live cells was determined using the trypan blue exclusion method. Data represent the mean of three independent experiments. \* -  $p < 0.05$  (t-test) compared to MOCK infected samples.

Figure 3 shows the absolute cell numbers, which indicate the proliferation over time of the B78/H1 and GL261 tumor cell lines infected with different MOIs in comparison to non-infected control cells (MOCK). The two cell lines revealed a striking difference in their sensitivity to viral infection. Normal proliferation and increase in cell number in both uninfected cell lines was observed. Upon MVMP infection cell viability was partially reduced in the melanoma B78/H1 cells and dramatically in the glioma GL261 cells.



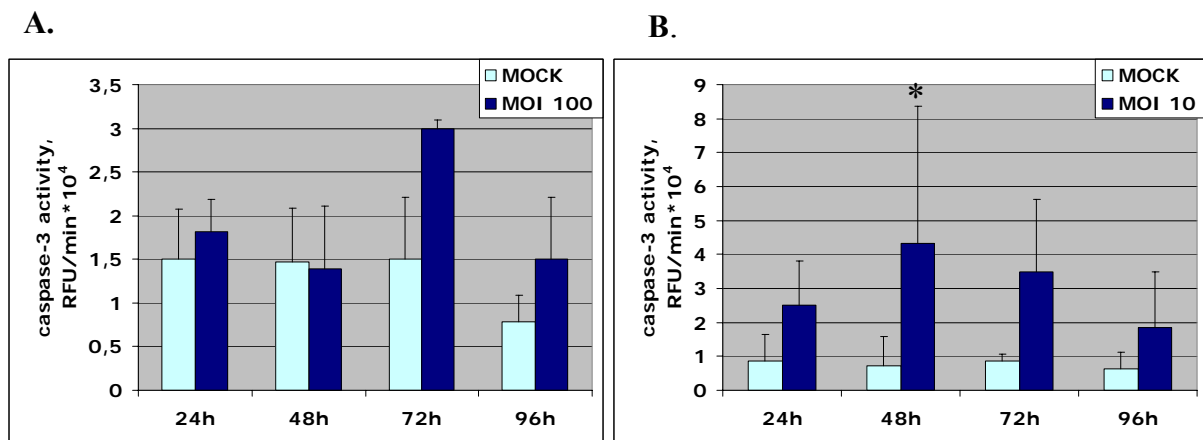
**Fig.4. Cytotoxic effect of MVMp on tumor cell lines.** A. Melanoma B78/H1 and B. glioma GL261 cells ( $2 \times 10^3$ ) were infected with different MOIs (B78/H1 – MOI 10, MOI 100: GL261 – MOI 1, MOI 5, MOI 10). The cytotoxic effect of MVMp was measured using the MTT assay after 24hrs, 48hrs, 72hrs and 96hrs p.i. Data represent the mean of three independent experiments. \* -  $p < 0.05$  (t-test) compared to MOCK infected samples.

Although the virus is able to replicate in the B78/H1 cell line and cell death is initially visible 2 days post infection, there are still about 30% living cells at day 4 using a MOI of 100. This might be due to the continuous proliferation of a population of possibly virus resistant cells (Fig.4). These cells were considered resistant since re-infection of cultures at the same MOI had no effect neither on survival nor proliferation (data not shown). The glioma GL261 cell line revealed high sensitivity to MVMp infection. The greatest cytotoxic effect of MVMp was observed at day 3 and day 4 post infection. Almost 90% of cells had died at day 4 upon MVMp infection with a MOI 10. Even though both the melanoma and glioma tumor cell lines are permissive for MVMp infection, glioma GL261 cells are more susceptible for the infection and more sensitive to the cytotoxic effect of the virus. Due to the high cytotoxic effect of MVMp on the glioma GL261 cell line, the mode of cell death was investigated.

#### 4.1.3. Induction of the apoptotic pathway following MVMp infection.

Rodent PV infection can induce either necrosis or apoptosis, depending on the tumor model considered (Moehler M. et. al. 2001; Rayet et.al. 1998; Ran et. al. 1999). The question arises whether PV MVMp would trigger the apoptotic death pathway or necrosis in either the melanoma B78/H1 or glioma GL261 cell line. There were no indications of necrosis in these cells upon MVMp infection in previous studies (Lang S. et. al., 2005). Therefore, the possible induction of the apoptotic death pathway was investigated in more detail.

Apoptosis is mediated by sequential activation of caspases, which are constitutively present in most cells as inactive proenzymes. Caspases operate in an intracellular cascade leading to the activation of downstream effectors caspases-3, -6 and -7 (Adams J.M. 2003; Green D.R. et al. 1998).



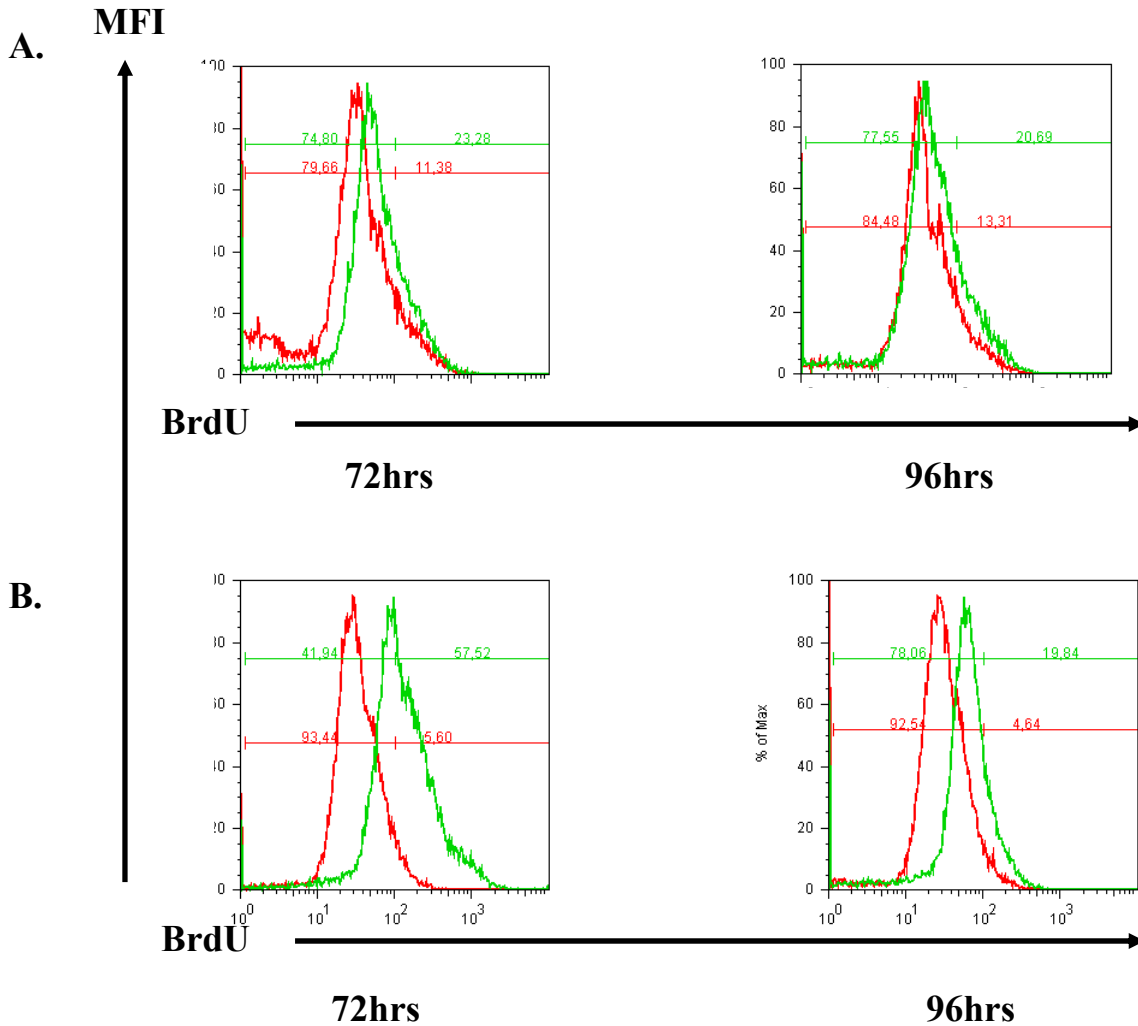
**Fig.5. Caspase-3 activation induced by MVMp infection** in **A.** melanoma B78/H1 and **B.** glioma GL261 cells. One million cells were plated and infected with different MOIs. At different incubation times (24hrs, 48hrs, 72hrs, 96hrs p.i.)  $1 \times 10^6$  cells were collected, lysed and caspase-3 activity was assessed in a fluorescence assay with synthetic substrates. Caspase-3 activity is expressed in relative fluorescence units (RFU) per minute. Data represent the mean of four independent experiments. \* -  $p < 0.05$  (t-test) compared to MOCK infected samples.

It is known that caspase-3 is a key mediator of nuclease activation, which results in DNA degradation during apoptosis. Therefore, we analyzed whether MVMP induced caspase-3 in infected cells. The presence of the active form caspase-3 was observed by flow cytometry in both cell lines investigated (data not shown). A fluorescence assay based on the enzymatic activity of caspase-3 was performed to verify at which time point caspase-3 activity was at its maximum upon MVMP infection. As depicted in Fig. 5, non-infected cells (MOCK) of both tumor cell lines showed constant caspase-3 activity within the first three days (24-72hrs). Upon MVMP infection caspase-3 activity in the melanoma B78/H1 was not significantly increased at either 72hrs p.i. (2-times fold increase compared to MOCK-infected cells) or 96hrs p.i. (1.5-times fold increase compared to MOCK-infected cells), which is in accordance with low replication potency of MVMP in these cells and viral cytotoxic effect. The outgrowth of MVMP resistant cells resulted in enhanced proliferation and reduced cell death of melanoma B78/H1. However, caspase-3 activity was significantly increased in infected glioma GL261 cells compared to MOCK. The highest activity was observed at 48hrs (5-times increase) and 72hrs p.i. (4-times increase) and this corresponds to massive cell death.

The final step of the apoptotic pathway is characterized by the breakdown of genomic DNA by endonucleases resulting in DNA fragmentation (Zhivotosky B. et. al. 2001). The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used to assay the endonuclease cleavage products by enzymatical end-labelling of the DNA strand breaks. To investigate whether DNA fragmentation as the final step in the apoptotic cell death pathway occurs upon MVMP infection, TUNEL labeling was assessed by flow cytometry. No considerable increase in DNA fragmentation was observed in the melanoma B78/H1 cell line after 72hrs (23%) or 96hrs (21%) p.i. (Fig. 6). In contrast, the glioma GL261 cells showed significantly increased DNA



fragmentation at 72hrs (58%) p.i. due to the higher sensitivity to PV MVMp infection (Fig. 6).



**Fig.6. DNA fragmentation upon MVMp infection in A. melanoma B78/H1 and B. glioma GL261 cells.** Cells were seeded at  $0.5 \times 10^6$  and infected with MVMp at MOI 10 for glioma GL261 and MOI 100 for melanoma B78/H1. Cells were labelled with FITC-BrdU and analysed by FACS at either 72hrs or 96hrs p.i.. Red line histograms represents the MOCK-infected control samples, green line histograms depict MVMp-infected samples. One representative experiment out of three is shown.

Taking all the data together we can conclude that both melanoma B78/H1 and glioma GL261 tumor cells are permissive to PV MVMp infection. Upon MVMp infection glioma GL261 showed higher sensitivity to the cytotoxic effect

of the virus and apoptotic features of cell death. These essential studies confirmed the usefulness of both tumor cell lines as models for the following investigations for the induction specific immune responses by MVMp albeit with the certain limitations. Due to the heterogeneity of the melanoma B78/H1 cell line and due to the resistance of a subpopulation of it to MVMp, this tumor cell line might only be useful for *in vitro* and *in vivo* assays within a very narrow window of time and at high MOIs. However, the melanoma cells B78/H1 provide a better model for *in vivo* studies due to the physiological relevance of the route of tumor transplantation subcutaneously. The glioma GL261 cells are more applicable for robust and reliable *in vitro* studies and can be used in *in vivo* models with the certain restrictions. The subcutaneous (s.c.) route of tumor transplantation does not mimic the physiological appearance of gliomas, however, a direct comparison between MOCK-infected and MVMp-infected cells, which are injected s.c., is still feasible and allows to test specific experimental parameters, such as innate and adoptive immune cells activation.

Due to the oncolytic activity of PV, MVMp-initiated tumor lysates are being generated in both tumor models. If this process takes place *in vivo*, lysed tumor particles might elicit the immune response. The first event of interaction between immune system and tumor cell lysates is antigen processing and presentation by APC, such as DCs or microglia in CNS.

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## 4.2. Activation of dendritic cells and microglia induced by MVMP.

We have clearly shown that both tumor cell lines (melanoma B78/H1 and glioma GL261) are permissive for MVMP infection. There are some strong differences between two tumor cell lines. The main difference is the sensitivity to the oncolytic activity of PV MVMP leading to apoptosis. We initially hypothesized that oncolysis of the tumor cells would initiate a tumor specific immune response and commenced on a detailed study in the generation of a specific immune response mediated by MVMP. It is well established that activated dendritic cells (DCs) provide the first line of defense in tumor cell recognition. It is also known, that there are two main subsets of DCs – the myeloid DCs and the lymphoid DCs. Both of these populations can be discriminated according to their phenotype (expressing specific cell surface receptors) and their functions. Myeloid DCs express the CD11c<sup>+</sup>CD11b<sup>+</sup>CD8<sup>-</sup> cell surface receptor combination and lymphoid DCs are CD11c<sup>+</sup>CD11b<sup>int</sup>CD8<sup>+</sup>. These two DC subsets have different antigen-presenting capacities. Whereas myeloid DCs initiate Th1 immune responses, lymphoid DCs regulate Th2 responses (Georgelas A., Schibler K. 2000). Whether MVMP infection and/or MVMP-mediated tumor cell lysates are responsible for the activation of DCs mediated specifically through either one of these populations, two basic approaches were established to generate either myeloid or lymphoid DCs.

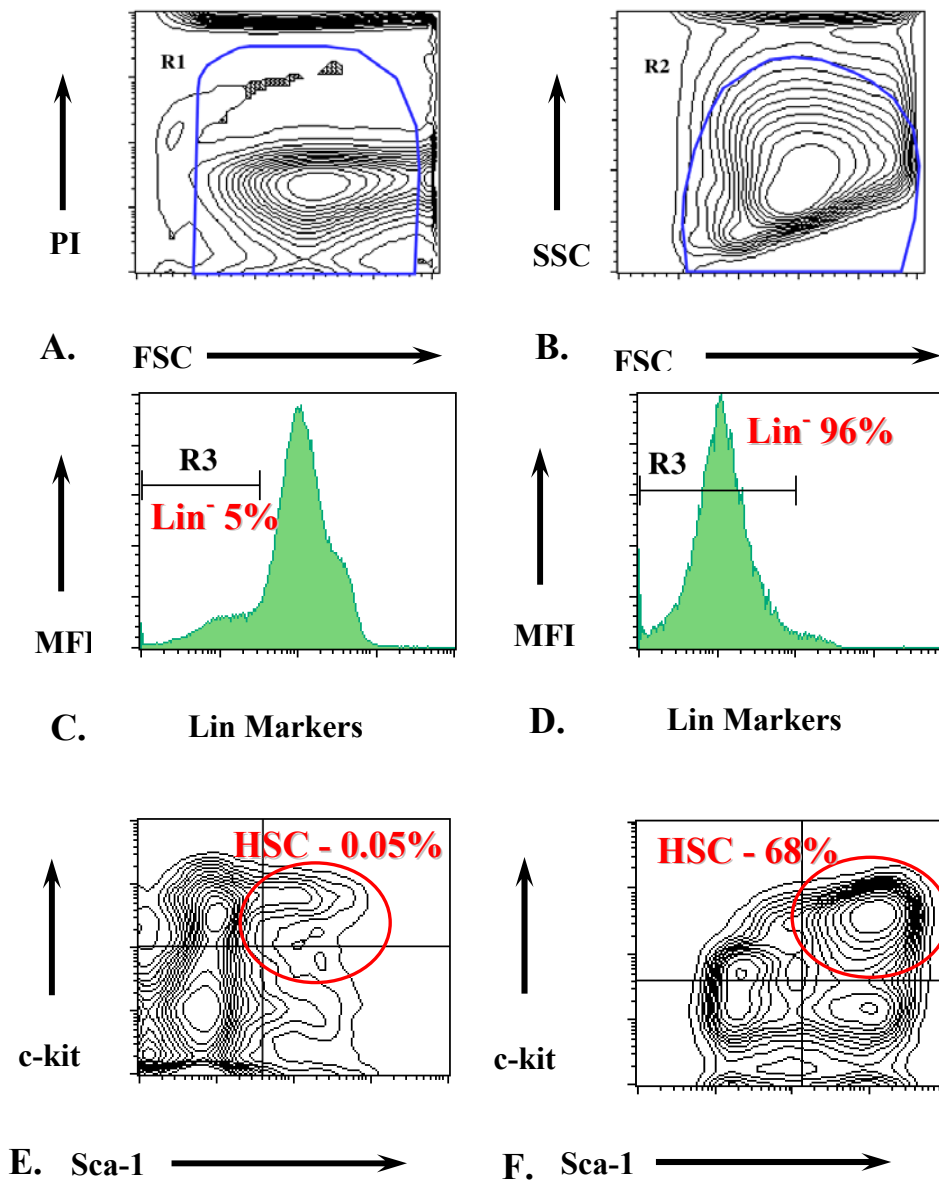
### 4.2.1. Generation of dendritic cells.

The first approach is referred to as *in vitro* hematopoietic stem cell (HSC)-derived DCs and the other as *in vivo* generated DCs of either the myeloid or lymphoid phenotype. *In vitro* HSC-derived DCs were obtained using highly purified HSC from bone marrow (BM) cells of C57/Bl6 mice that were treated for 5 days with 5-fluorouracil (5FU). A single injection of the pyrimidine analog

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5FU kills cycling hematopoietic cells, bringing the spared quiescent HSCs into cycle to repopulate the depleted bone marrow (Harrison and Lerner 1991; Randall and Weissman 1997). HSCs are cells isolated from BM which have self renewing potential and are omnipotent (H. Nakauchi, 1998). HSCs are defined through a combination of specific cell surface receptors namely c-kit (CD117) and Sca-1 and are negative for antigens of differentiated cells CD2, CD3, CD4, CD5, CD8, NK1.1, B220, TER-119, Gr-1 (Lin<sup>-</sup> cells) (W.H.B.M. Levering, et al., 2004). Negative selection for and sorting of Lin<sup>-</sup>/c-kit<sup>+</sup>/Sca-1<sup>+</sup> BM cells was performed (Fig.7.). HSCs were differentiated into either myeloid or lymphoid DCs using specific cytokine cocktails (SCF (100ng/mL), GM-CSF (20ng/mL) for myeloid DCs, and Flt3L (200ng/mL) or combination of GM-CSF and Flt3L for lymphoid DCs) for 9 days.

These cells were further assayed for their cell surface marker profile indicative of the specific DCs-subpopulation (data not shown). A 200-fold increase in absolute cell number was achieved for cells differentiated into the myeloid DC phenotype (CD11c<sup>+</sup>CD11b<sup>+</sup>CD8<sup>-</sup>) and a 30-50-fold increase was obtained for the lymphoid DCs (CD11c<sup>+</sup>CD11b<sup>int</sup>CD8<sup>+</sup>).



**Fig.7. Generation of DC progenitor populations prior culture with cytokine cocktail.** **A.** Selection of cells by size prior depletion (blue gate shows live cells (PI negative)). **B.** Selection of cells by granularity prior depletion (blue gate shows homogeneous size and granularity of cells). **C.** Lin<sup>-</sup> cells before depletion. **D.** Lin<sup>-</sup> cells after depletion. **E.** HSC before depletion (red gate) – 0.05%. **F.** HSC after depletion (red gate) – enriched to 68%.

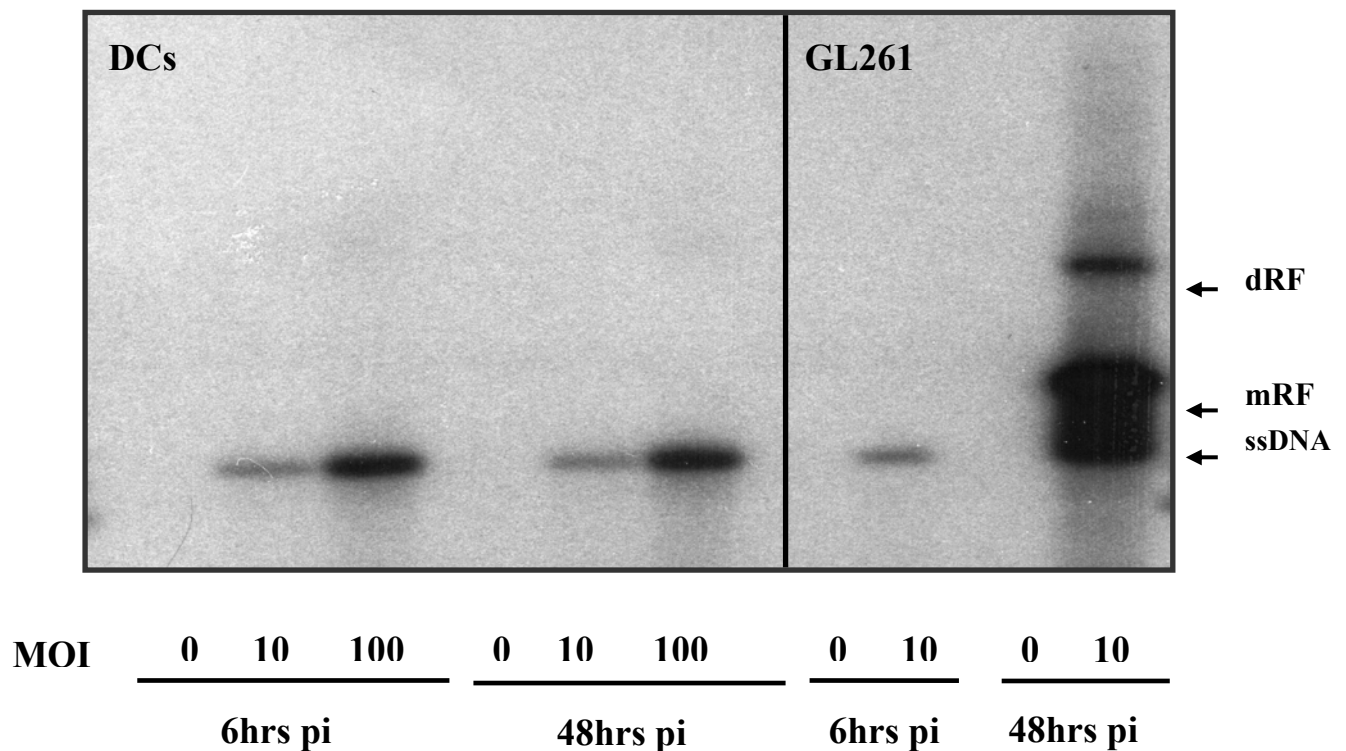
*Ex vivo* generated DCs were obtained using a tumor cell model described by G. Dranoff (Dranoff G. 1993). Myeloid DCs as well as lymphoid DCs were generated using stably transfected B16F10 melanoma cells expressing either GM-CSF or Flt3L which were subcutaneously injected into C57/Bl6 mice. The

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injected mice developed readily visible tumor burden and at the same time DCs accumulated in the spleen and lymph nodes of the mice. Myeloid DCs accumulated preferentially in the spleen of the B16F10/GM-CSF injected mice whereas lymphoid DCs massively infiltrated in the lymph nodes of the B16F10/Flt3L injected mice, respectively. DCs were isolated and enriched from mice injected with either one of the stably transfected melanoma cell lines and assayed for their surface marker profile (data not shown). Myeloid DCs revealed the  $CD11c^+CD11b^+CD8^-$  cell surface receptor phenotype and lymphoid DCs expressed the markers  $CD11c^+CD11b^{int}CD8^+$ . The purity of myeloid or lymphoid subpopulations was greater than 95%. These two different subsets of DCs were used in further assays to evaluate the MVMp-mediated activation of DCs cells.

#### **4.2.2. MVMp-mediated GL261 tumor lysates activate DCs.**

Since we had highly proliferative populations of cells upon DCs differentiation, an additional experimental parameter to establish was the level of viral replication in the various DCs populations. This parameter is essential for further clinical applications of PV as a potent oncolytic agent. It is known, that PV replicates preferentially in cells undergoing S phase and that replication of virus in DCs would be detrimental if the virus would also actually kill the DCs. Southern blot analysis was performed on highly enriched DC subpopulations infected with MVMp.



**Fig.8. Southern blotting analysis of viral DNA intermediates.** Myeloid DCs, generated ex vivo and glioma GL261 cells (positive control) were infected with two different MOIs (10, 100) of MVMp. Cells were harvested at the indicated time points (6hrs and 48hrs p.i.) and processed for the analysis of virus replication. mRF, monomer replicative form; dRF, dimer replicative form; ssDNA, single-stranded genomic DNA.

Viral input as well as viral replication was assayed at specific time points with different MOIs. Viral entry into the cells was observed, however, viral replication could not be detected (Fig. 8). The successful establishment of the experimental conditions to generate DCs as well as the deficiency of MVMp PV to replicate in DCs allows for a suitable experimental approach to further analyze the mechanisms of DC activation. The fact, that MVMp can infect DCs, although it does not replicate in these cells, could point that virus potentially initiates other signaling pathways to induce cytokine production and further activation of DCs.

Co-culture assay systems were established using PV-induced tumor cell lysates stimulating the different DC subpopulations to assess the potential of

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initiating an innate as well as an adaptive immune response. Induction of an innate immune response can be monitored through the activation status of DCs via the release of proinflammatory cytokines, expression of activation markers and TLRs. The generation of a specific immune response can be verified by the release of IFN $\gamma$  from T cells in response to tumor cells *in vivo* and *in vitro*.

In detail, DCs were employed in co-culture assays using B78/H1 or GL261 MVMp-induced tumor cell lysates. Forty-eight hour co-culture assays were performed, subsequently assaying the DC subpopulations for the expression of specific cell surface markers (CD80, CD86, CD40) and the release of proinflammatory cytokines (TNF, IL-6) indicating activation. Since the percent of CD80-, CD86-, CD40-positive DCs was quite high (80-98%) even without additional activation (Table 2), the mean fluorescence intensity (MFI) was measured for all of the activation markers. Such high percent of CD80-, CD86-, CD40-positive DCs was observed since DCs were generated in tumor-bearing mice (B16F10/GM-SCF or B16F10/FLT3L).

Thorough analysis of the expression levels of these activation markers revealed a significant increase in the MFI of CD80, CD86, CD40 on the myeloid and lymphoid DCs co-cultured with MVMp-induced GL261 tumor cell lysates, whereas myeloid and lymphoid DCs did not depict this increase in activation markers after co-culture with the B78/H1 tumor cell lysates. The induction was specific since the appropriate control samples did not reveal any noteworthy increase in the above-mentioned activation markers (Table 2, Table 3).

It was not clear why infected B78/H1 melanoma cells failed to stimulate DCs, although one possibility could have been the outgrowth of virus resistant melanoma cells within the short time window of four days. This would lead to an inefficient generation of tumor cell lysate able to activate DCs. On the other hand, B78/H1 cells infected with high MOI should induce significant cells lysis and viral replication at 72h post infection. Therefore, additional studies were



carried out to assess whether under these conditions considerable DC activation could be detected.

Co-culture assays of naïve and mature DCs with MVMp were performed to investigate whether high amount of virus alone can either stimulate DCs or induce a suppressive effect. No effect on naïve DCs was observed. Since MVMp did not induce the expression of activation markers (CD80, CD86, CD40) on the naïve myeloid and lymphoid DCs upon the infection (Table 2, Table 3). However, MVMp induces increased expression of CD80, CD86 and CD40 markers on the mature myeloid (Table 2) and lymphoid DCs (maturation with LPS treatment (Table 3)).

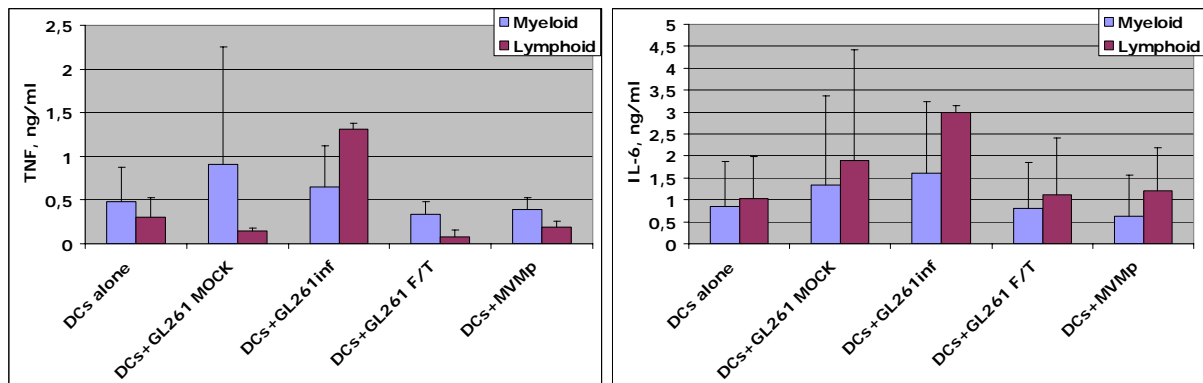
	CD80		CD86		CD40	
	%	MFI	%	MFI	%	MFI
<b>DCs alone</b>	93,2±3,6	824±241,3	75,1±12,0	860±416,8	58,5±16,1	182±63,0
<b>DCs+B78/H1</b>	85,7±4,1	954±106,1	<b>44,5±1,9*</b>	558±115,1	ND	ND
<b>MOCK</b>						
<b>DCs+B78/H1</b>	90,4±2,4	1163±215,7	<b>45,8±0,5*</b>	561±0,5	ND	ND
<b>infected</b>						
<b>DCs+B78/H1 F/T</b>	88,1±10,3	988±37,0	65,5±20,9	1426±1293,2	ND	ND
<b>lysates</b>						
<b>DCs+GL261</b>	91,2±10,5	992±40,1	67,3±10,3	986±195,3	35,0±35,9	86±23,0
<b>MOCK</b>						
<b>DCs+GL261</b>	94,7±5,4	<b>1513±142,4*</b>	<b>87,0±4,3*</b>	<b>1321±122,4*</b>	52,0±47,7	109±25,6
<b>infected</b>						
<b>DCs+GL261 F/T</b>	92,7±8,9	962±154,3	<b>86,7±2,6*</b>	1123±152,3	50,0±23,4	118±9,4
<b>lysates</b>						
<b>DCs+MVMp</b>	83,4±18,9	769±283,2	86,7±8,9	714±288,9	51,0±13,8	219±4,8
<b>DCs+LPS</b>	95,2±4,6	<b>2152±371,2*</b>	70,0±12,6	<b>1748±588,5*</b>	<b>73,7±20,6*</b>	276±134,5
<b>DCs+LPS+MVMp</b>	93,3±7,3	<b>2123±361,2*</b>	77,8±11,8	<b>2139±269,3*</b>	75,8±17,4	371±111,6

**Table.2. Expression of activation markers on myeloid DCs upon co-culture with MVMp.** %-percent of positive cells, MFI – mean fluorescent intensity of positive cells. \* -  $p < 0.05$  (t-test) compared to DCs alone. ND- not defined.

	CD80		CD86		CD40	
	%	MFI	%	MFI	%	MFI
<b>DCs alone</b>	56,5±29,1	516±143,3	60,7±23,0	924±608,3	20,4±14,9	78±68,0
<b>DCs+B78/H1</b>	71,0±26,5	<b>959±151,9*</b>	81,9±16,2	556±350,8	ND	ND
<b>MOCK</b>						
<b>DCs+B78/H1</b> <b>infected</b>	66,6±12,0	<b>876±22,2*</b>	68,7±3,8	452±208,1	ND	ND
<b>DCs+B78/H1 F/T</b> <b>lysates</b>	59,0±24,5	817±283,1	<b>90,8±4,1*</b>	416±193,6	ND	ND
<b>DCs+GL261</b> <b>MOCK</b>	78,2±3,4	<b>1034±101,6*</b>	70,8±5,9	1026±134,5	50,5±11,1	49±2,0
<b>DCs+GL261</b> <b>infected</b>	<b>88,7±3,2*</b>	<b>1547±266,8*</b>	<b>85,7±2,6*</b>	<b>1682±194,4*</b>	<b>78,0±13,8*</b>	80±23,6
<b>DCs+GL261 F/T</b> <b>lysates</b>	<b>87,3±3,3*</b>	<b>1092±135,0*</b>	62,9±3,7	1387±379,0	<b>75,8±6,4*</b>	54±3,5
<b>DCs+MVMp</b>	51,4±36,3	522±114,4	73,4±19,3	1167±987,2	26,5±14,9	124±6,7
<b>DCs+LPS</b>	<b>89,6±5,9*</b>	<b>2632±1113,1*</b>	<b>90,6±6,4*</b>	1874±847,1	<b>77,4±9,0*</b>	224±52,1
<b>DCs+LPS+MVMp</b>	<b>89,8±8,8*</b>	<b>3499±1185,7*</b>	<b>86,9±5,3*</b>	2446±1020,1	<b>78,8±10,7*</b>	248±73,8

**Table. 3. Expression of activation markers on lymphoid DCs upon co-culture with MVMp.** %-percent of positive cells, MFI – mean fluorescent intensity of positive cells. \* - p<0.05 (t-test) compared to DCs alone. ND- not defined.

Release of proinflammatory cytokines TNF, IL-6 is an additional indicator of DC activation. An increase of proinflammatory cytokine (TNF, IL-6) release was observed in culture supernatants from lymphoid DCs co-cultured with MVMp-mediated glioma GL261 lysates (Fig. 9). This was indicative for the activation of DCs and provided an additional signal for DC maturation. The supernatants from myeloid DCs co-cultured with MVMp-infected glioma GL261 cells did not show significant differences compared to unstimulated myeloid DCs.



**Fig.9. Release of proinflammatory cytokines from myeloid and lymphoid dendritic cells.** DCs were cultured alone, or in the presence of MOCK-infected glioma GL261, MVMp-infected glioma GL261 cells (MOI 10), freeze/thaw lysates from glioma GL261 cells, or were infected with MVMp (MOI 100). Forty-eight hour co-culture assays were performed, supernatants were collected and the absolute amount of cytokines produced was measured by ELISA. Data represent the mean of three independent experiments.

Concomitantly, MVMp does not stimulate the release of proinflammatory cytokines (TNF, IL-6) from naive myeloid and lymphoid DCs (Fig. 9). However, MVMp significantly increases the release of these proinflammatory cytokines from mature myeloid and lymphoid DCs (data not shown).

Since, as mentioned above, MVMp alone was also inefficient in stimulating DCs, we tentatively conclude that the release of apoptotic bodies (or

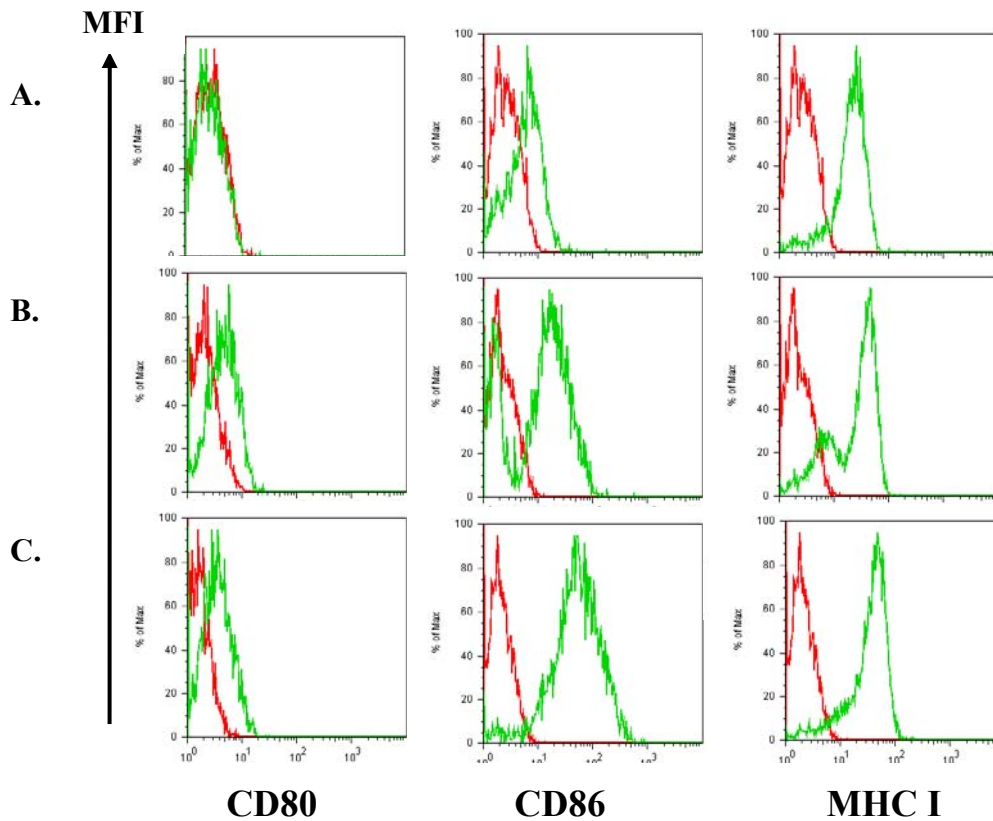
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other components) from glioma GL261 cells in comparison to B78/H1 melanoma upon MVMP infection may stimulate DCs.

#### **4.2.3. Microglia activation induced by MVMP-mediated glioma GL261 lysates.**

Under physiological conditions gliomas in the CNS interact within the microenvironment of a specialized form of antigen presenting cells namely the microglia. These cells resemble certain features of the DCs used in the above studies but might, however, at the same time provide a specialized signaling environment. Microglia have antigen-presenting and phagocytic capacities, they can secrete cytokines and chemokines (Aloisi F. 2001).

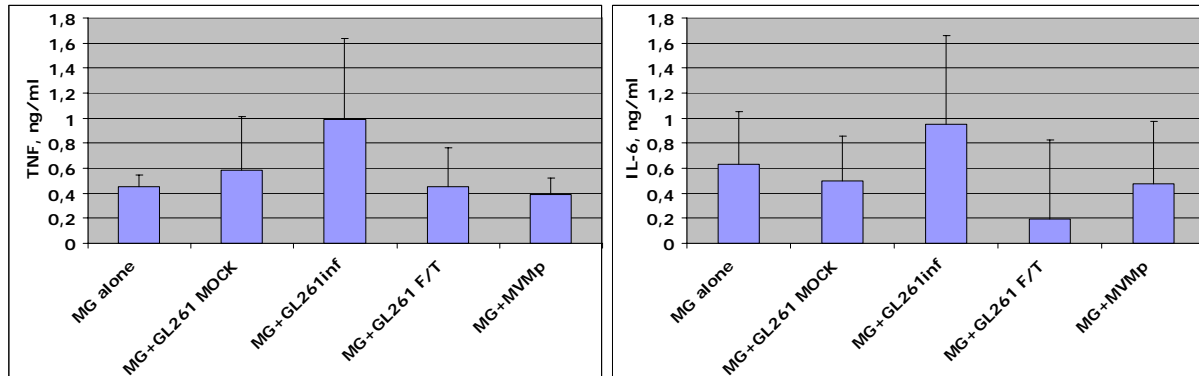
In order to examine the activation potential of microglia a similar co-culture assay as above was performed with MVMP-infected GL261 cells. It was previously shown that MVMP could infect but not replicate in microglia (Abschuetz A. et. al. 2006). It is well established that the expression levels of activation markers on microglia is lower than on other APCs, for instance DCs. In accordance with these findings, only a slight upregulation of CD86, CD80 and MHC class I surface markers compared to untreated microglia was observed upon the co-culture conditions with MVMP-induced glioma GL261 cell lysates (Fig. 10). The positive control (microglia stimulated with LPS (5 $\mu$ g/mL)/IFN $\gamma$  (30ng/mL) showed an up-regulation of the all markers (CD80, CD86, CD40, MHC class II, MHC class I), and the negative controls (microglia incubated with MOCK-treated glioma GL261 cells, and microglia incubated with freeze-thaw lysates from the glioma GL261 cells) showed no significant up-regulation of the same markers (data not shown for CD40, MHC class II).



**Fig.10. Microglia activation profile after 48h co-culture assay with MVMp-infected glioma GL261 cells.** A. Microglia alone. B. Microglia co-cultured for 48hrs with MVMp-infected (MOI 10) glioma GL261 cells. C. Microglia activated by LPS (5 $\mu$ g/mL)/IFN $\gamma$  (30ng/mL). Green histograms represent specific staining with antibodies against CD80, CD86, MHC class I, respectively. Red histograms represent the appropriate isotype controls. One representative experiment out of three is shown.

Since proinflammatory cytokines play a crucial role in microglia-tumor cell interaction, the levels of TNF and IL-6 produced were measured. Microglia co-cultured with MVMp-infected glioma GL261, showed a significant increase in the release of TNF and IL-6 (Fig.11). As a positive control, microglia were stimulated with LPS and IFN $\gamma$  and showed the highest level of proinflammatory cytokines released (more than 5ng/ml) (data not shown).

The data correspond with a similar finding, that microglia express increased amounts of IL-1, IFN $\beta$  and IL-6 upon co-culture with MVMp-infected GL261 cells (H.Hong, unpublished data).



**Fig.11. Release of TNF and IL-6 upon microglia activation after 48hrs.** Microglia were cultured alone, or in the presence of MOCK-infected glioma GL261, MVMp-infected glioma GL261 cells (MOI 10), freeze/thaw lysates from glioma GL261 cells infected with MVMp (MOI 100), respectively. Forty-eight hour co-culture assays were performed, supernatants were collected and the absolute amount of cytokines produced was measured by ELISA. Data represent the mean of three independent experiments.

Taken together, these data showed the potency of MVMp-mediated glioma GL261 lysates to stimulate the activation of microglia, which represent the physiological interacting cellular environment for gliomas. This stimulation might lead to increased phagocytosis of already lysed glioma GL261 and provide an additional stimulus for tumor antigen presentation by microglia. Since this stimulation leads to the production of proinflammatory cytokines, a crucial signaling pathway initiating the induction of transcription of the genes for these cytokines is the NF $\kappa$ B signaling pathway. We therefore investigated whether NF $\kappa$ B signaling is induced and specifically whether the signal induction is mediated through TLR receptor signaling.

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#### 4.2.4. GL261 tumor cell lysates induce NFκB signaling via TLR3 *in vitro* in DCs

DCs are known to express a broad pattern of TLRs which efficiently bind pathogen-associated–molecular-patterns (PAMP) and initiate an activating signaling cascade. One hallmark of this activation is the cytoplasmic to nuclear translocation of NFκB.

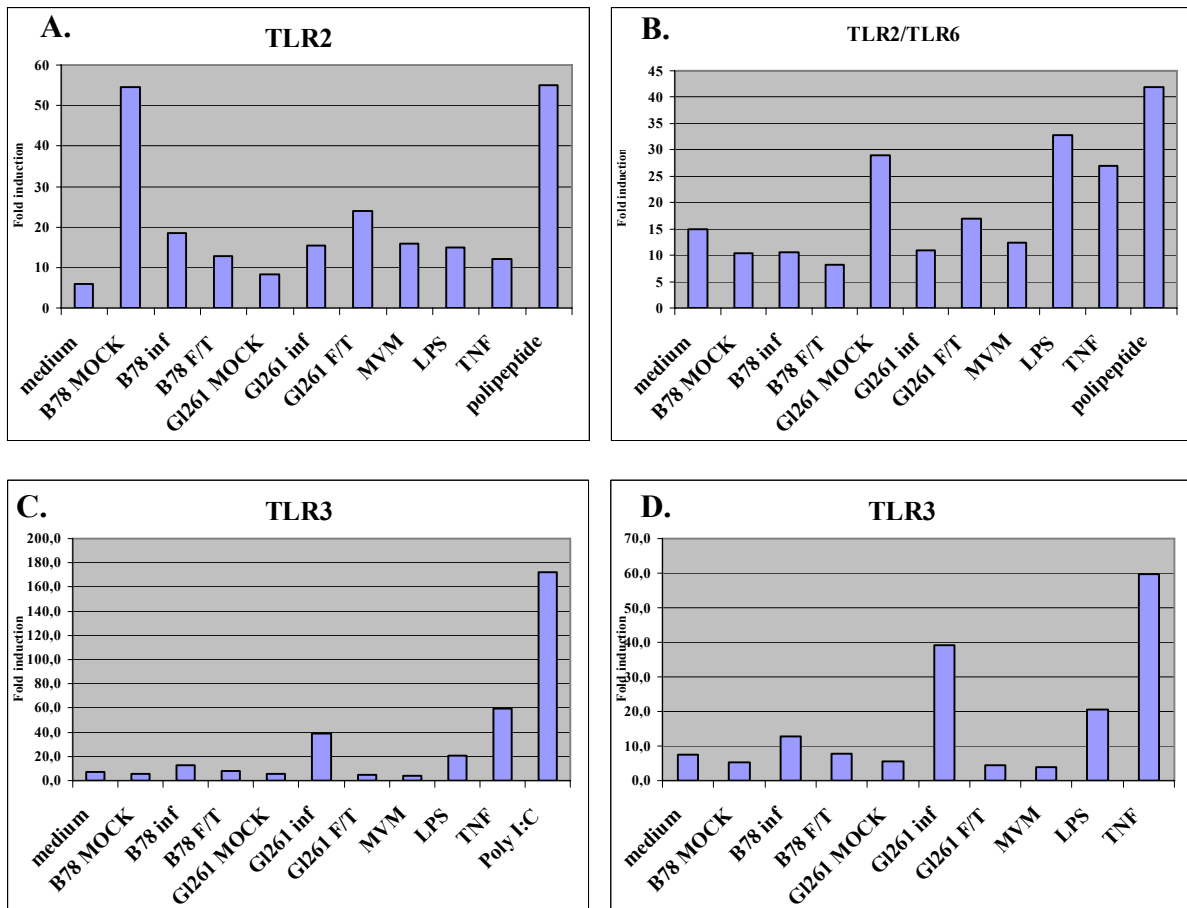
In view of the stimulatory capacity of MVMp-infected GL261 cell lysates towards DCs, it was hypothesized that DC activation is mediated through TLR signaling and would lead to specific upregulation of certain TLRs. Since the GL261 glioma cells express several TLRs the analysis of DCs derived from the coculture system was not feasible (H. Hong, unpublished data). The TLR expression profile was examined on the mRNA level in DCs infected with MVMp alone. Expression of TLR2, 3, 4 and 9 on myeloid DCs and TLR2, 6 and 9 on lymphoid DCs was increased upon virus infection (data not shown). The importance of this finding is still unclear since at the same time no increased expression of activation markers was detectable (Table 2, Table 3). Also, it is known, that the TLRs activation and upregulation of their expression might lead to upregulation of activation markers on different DCs (El Shikh, M.E., et. al. 2007; Hellman, P. et. al. 2007).

Unfortunately, the limited amount of DCs in these experiments did not allow for analysis of TLR signal transduction in cellular extracts from these cells. Therefore, heterologous expression of different TLRs was established in a tissue culture cell line HEK293 to study activation following contact with MVMp – infected glioma or melanoma tumor cells or with virus alone as a control. The main candidates selected were TLR2, TLR3, TLR4, TLR6, TLR9, since their mRNA expression was up-regulated on myeloid and lymphoid DCs upon MVMp infection.



Single candidate TLR expressing stable cell lines were generated to dissect the impact of specific receptors on downstream signaling pathways and the induction of proinflammatory cytokines. Specific TLR signaling upon stimulation with PV MVMp-induced tumor cell lysates was measured in cotransfection assays using a NF- $\kappa$ B luciferase reporter gene system. An *in vitro* culture assay system using stably transfected HEK cells allows for a much more precise analysis and will reflect the physiological situation within the DCs on a single receptor based analysis.

The data showed, that TLR2 and 6 (Fig. 12), as well as TLR4, TL4/MD2/CD14 and TLR9 (data not shown) are not involved in the activation of NF $\kappa$ B upon co-culture with MVMp-induced tumor cell lysates (melanoma B78/H1 and glioma GL261). However, untreated tumor cells showed significant influence on the activation of either TLR2 or TLR2/TLR6. Probably, some cellular proteins could directly activate these TLRs.



**Fig. 12. Activation of NF $\kappa$ B signalling through TLRs. A. TLR2 B. TLR2/TLR6 C. TLR3 D. TLR3 in the absence of positive Poly I:C control.** HEK293T expressing TLRs and transfected with NF $\kappa$ B-driven firefly luciferase reporter gene were seeded in 6 well plates at  $2 \times 10^5$  cells per well and stimulated for 6 hrs with MOCK infected melanoma B78/H1 cells (B78 MOCK); MVMp infected (MOI 100) melanoma B78/H1 cells (B78 inf); freeze/thaw lysates from non-infected melanoma B78/H1 cells (B78 F/T); MOCK infected glioma GL261 cells (GL261 MOCK); MVMp infected (MOI 10) glioma GL261 cells (GL261 inf); freeze/thaw lysates from non-infected glioma GL261 cells (GL261 F/T); MVMp MOI 100 (MVM). LPS (5 $\mu$ g/ml); TNF (10ng/ml), Poly I:C (10 $\mu$ g/ml), and Peptidoglycan from *Staphylococcus aureus* (10 $\mu$ g/ml) served as positive controls. Activation of NF $\kappa$ B was assessed by measuring firefly luciferase activity in cell lysates. Values shown represent the fold induction compared to control vector devoid of NF $\kappa$ B binding sites (control vector contains a minimal promoter). The results of one out of three similar independent experiments are shown.

TLR3 is involved in the DC activation by MVMp-mediated tumor cell lysates. Only MVMp-mediated tumor cell lysates showed a significant effect on

the activation of TLR3 compared to MOCK-infected cells and freeze/thaw lysates (Fig. 12). It is possible that dsRNA, which forms during the replicative cycle of virus, acts as a ligand for TLR3 in this case. However, MVMp alone does not affect the TLR3-mediated intracellular signaling.

Taken together, the *in vitro* results demonstrate significant activation of DCs and microglia upon co-culture with MVMp infected GL261 glioma cells. TLR3 was dissected as an important receptor initiating NF- $\kappa$ B signaling leading to the induction of proinflammatory cytokines. There is no specific activation observed in case of co-culture of these APC with MVMp-infected melanoma B78/H1.

These data gave a background for subsequent *in vivo* experiments. Significant activation of DCs and microglia might lead to a tumorspecific adaptive immune response, which is characterized by T-cell recruitment and functional activity at the site of the tumor.

### **4.3. Stimulation of anti-tumor specific immune response *in vivo* mediated by MVMP.**

We were able to show a significant increase of activation markers in DCs upon co-culture with MVMP-infected glioma GL261 cells and the induction of proinflammatory cytokines. Therefore, it is essential to assess the physiological relevance of the *in vitro* observations in an *in vivo* model.

We demonstrated that brain derived microglia, myeloid as well as lymphoid DCs displayed a comparable activation pattern of costimulatory surface markers. A study was initiated to monitor *in vivo* T-cell activation following s.c. administration of glioma GL261 cells into C57BL/6 mice, since this route is known to result in massive T-cell infiltration into the tumor tissue whereas too few T-cells would be recovered from the brain after intracranial application of glioma cell.

An *in vivo* model utilizing s.c. injection of GL261 glioma cells into C57BL/6 was established by Marta Enderlin, PhD (unpublished data) and modified to optimally assess innate as well as adaptive immune responses. The s.c. injection of MOCK-infected  $1 \times 10^6$  cells led to the moderate tumor development.

In our experiment, glioma GL261 cells were either MOCK infected, or infected with MVMP at various MOIs (MOI 0.3, 3, 30). The cells were subsequently injected into recipient mice 4 hours after infection (Tab. 4). This experimental approach allows to monitor not only a direct oncolytic effect, but also the activation of cells of the immune system. The same experimental approach was used for RAG2<sup>-/-</sup> mice (Tab. 4).

Treatment	C57BL/6	RAG2 <sup>-/-</sup>
MOCK-infected glioma GL261 cells	16	16
MVMp-infected glioma GL261 cells (MOI 0.3)	16	16
MVMp-infected glioma GL261 cells (MOI 3)	16	16
MVMp-infected glioma GL261 cells (MOI 30)	16	16

**Table 4. Experimental setup for *in vivo* experiment.** Number of mice injected per group is shown.

Mutations in the recombination-activating gene 2 (RAG2) result in an almost complete arrest of lymphocyte development due to the failure to properly rearrange the antigen receptor genes. Mice with genetically engineered mutations in the recombination-activating gene completely lack both mature T-cells and B-cells (Shinkai Y., et al. 1992). Therefore, it is possible to investigate also the impact of the innate immune response alone in these mice, but not acquired immune response.

#### 4.3.1. MVMp affects tumor growth in immunocompetent mice.

The GL261 glioma cells were infected *in vitro* with MVMp at MOI 0.3, 3, 30 or left uninfected. The cells, 4hrs p.i., were injected s.c. into immunocompetent C57/BL6J mice or into RAG2<sup>-/-</sup> mice. First signs of tumor development were observed at day 5 post injection (p.i.) in B6 and RAG2<sup>-/-</sup> mice. After that the tumor size was measured at day 9, 14, 18, and 21 p.i.. The immunocompetent B6 mice that were injected with glioma GL261 cells at a high infection dose (MOI 3 or MOI 30) did not develop any tumor at all (Tab. 5).

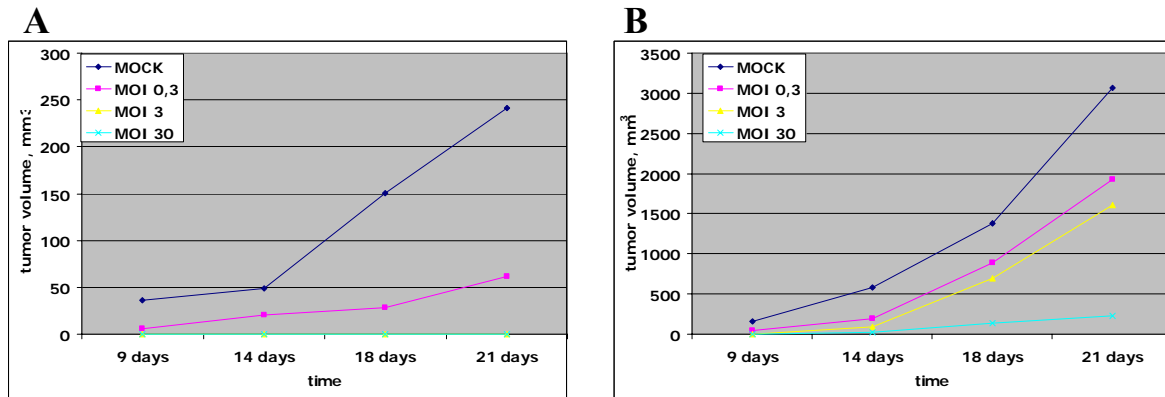
Almost all of the RAG2<sup>-/-</sup> immunodeficient mice had measurable tumors developed by day 21 p.i. All of the RAG2<sup>-/-</sup> animals injected with MVMp-infected GL261 cells eventually developed tumors by day 35 p.i. In addition, the RAG2<sup>-/-</sup> showed tumor onset earlier and tumor growth at a faster rate compared to the C57BL/6 mice (Tab. 5). Furthermore, the C57BL/6 mice injected with GL261 cells infected at MOI 3 or MOI 30, revealed long-term survival monitored over 6 months with no obvious tumor burden developing (data not shown).

Days p.i.	Group	s.c. injection of <b>MOCK</b> -infected glioma GL261 cells	s.c. injection of MVMp-infected glioma GL261 cells ( <b>MOI 0.3</b> )	s.c. injection of MVMp-infected glioma GL261 cells ( <b>MOI 3</b> )	s.c. injection of MVMp-infected glioma GL261 cells ( <b>MOI 30</b> )
9	C57/BL6	90%	37.5%	0%	0%
	RAG2 <sup>-/-</sup>	100%	57.1%	0%	0%
14	C57/BL6	90%	30.8%	0%	0%
	RAG <sup>-/-</sup>	100%	100%	100%	77,8%
18	C57/BL6	90%	38.5%	0%	0%
	RAG <sup>-/-</sup>	100%	100%	100%	60%
21	C57/BL6	83%	61.5%	0%	0%
	RAG <sup>-/-</sup>	100%	100%	100%	80%

**Table.5. Percent of mice developing tumors in each group.** The total number of animals per group was set to 100%, and mice, which developed tumors were calculated as a positive percentage.

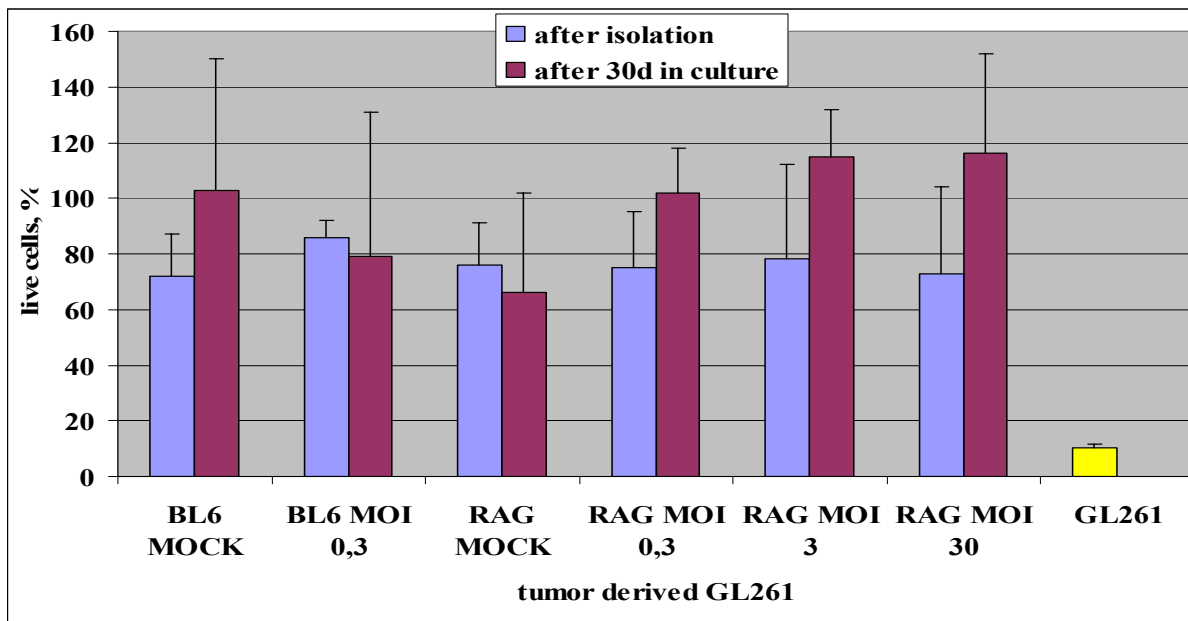
Tumors, which developed in the RAG2<sup>-/-</sup> recipient mice, showed at least a 10-fold increase in size in comparison to the immunocompetent C57BL/6 mice (Fig. 14). This was observed at all multiplicities of infection used. These results

point to the involvement of a specific immune response (most likely the T cell branch of the immune system) mediating the rejection of MVMP-infected tumors.



**Fig.14. Tumor growth following subcutaneous injection of glioma GL261 cells infected with MVMP.** One million glioma GL261 cells, infected 4hrs prior with MVMP (MOI 0.3, 3, 30) or left uninfected, were injected s.c. into the right flank of the recipients. Tumor volumes were measured at the indicated time points in **A**. C57BL/6 recipient mice **B**. RAG2<sup>-/-</sup> recipient mice.

Especially within the RAG2<sup>-/-</sup> recipients the MVMP-infected GL261 cells were not rejected and developed into sizeable tumors. We assessed the sensitivity of glioma GL261 cells, which escaped from rejection, to MVMP-reinfection *in vitro*. For this purpose tumors were isolated from the recipient mice and single tumor cell suspensions were reinfected *in vitro* with MVMP at MOI 10. Furthermore, tumor cell suspensions were cultured for 30 days under normal tissue culture conditions in the absence of viral infection. Subsequently, the sensitivity of these cells to MVMP infection was verified. As shown in Figure 15, neither any of the primary isolated GL261 tumor cells nor the cells cultured for prolonged periods of time showed a significant response to the cytotoxic effect of MVMP, which shows heterogeneity of glioma GL261 cells.



**Fig.15. Cytotoxic effect of MVMP on tumor isolated GL261 cells.** Tumor isolated GL261 cells ( $2 \times 10^3$ ) were infected with MVMP at MOI 10. Cytotoxic effect of MVMP was measured with the MTT assay after 72h. Data represent the mean of two independent experiments, 8 different tumors samples in each group. GL261 represents the sensitivity of glioma *in vitro*.

This result would support the initial hypothesis that virus induced tumor cell lysates elicit a tumor-virus specific immune response *in vivo* leading to the rejection of the tumor cells. The response is not solely mediated through natural killer cells, macrophages or DCs since these cell populations are present in RAG2<sup>-/-</sup> mice and the tumor cells were not rejected in these recipient mice. The *in vivo* experiments reveal that the adaptive immune response is initiated by MVMP-induced tumor cell lysates, since MOCK-infected tumor cells in immunocompetent C57BL/6 mice formed tumors. Most likely, some intracellular components are being recognized through the immune system that will call for the adaptive immune response and the rejection process.



#### **4.3.2. Hematopoietic cell distribution does not change within the spleen upon tumor induction of MVMp-infected glioma GL261 cells.**

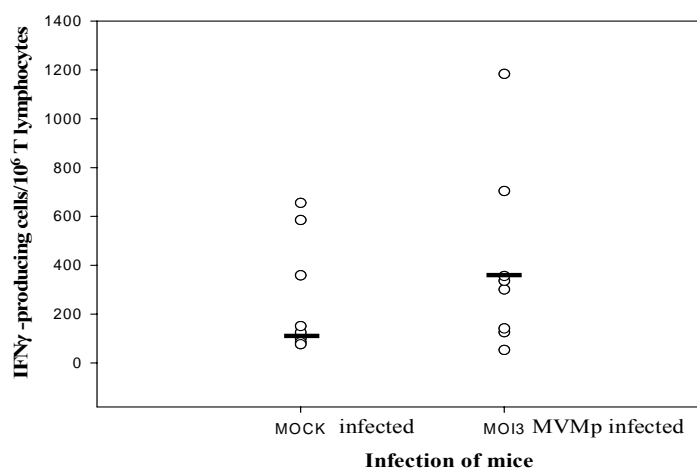
To assess possible changes in the immune cell compartment of the spleen after s.c. injection of MVMp-infected and non-infected glioma GL261 cells, populations of cells in spleen were analyzed by flow cytometry. The cells populations were assayed at day 7 and day 14 post injection. No significant differences were observed in the relative percentages of T, B, and NK cells as well as DCs, nor was the absolute cell number of any of these populations significantly affected. Additionally, the tumor infiltrating immune cell populations were analysed in immunocompetent mice. The massive leukocytes infiltration was also observed in MOCK-infected tumors. No significant differences were observed in the relative percentages or absolute numbers of immune cell populations in MVMp-infected tumors compared to MOCK-infected tumors (data not shown).

Day p.i.	Group	DCs, %	B cells (%)	T cells (%)	NK (%)	CD4 (%)	CD8 (%)	Absolute Cell Number (x10 <sup>6</sup> )
7days	MOCK (n=7)	0,74±0,38	48,9±3,57	32,59±6,02	3,65±0,59	21,55±6,65	15,19±2,85	67,5±32,32
	MOI 3 (n=8)	0,73±0,52	44,1±4,97	30,04±4,71	3,69±0,83	17,09±2,79	13,73±2,07	53,13±20,81
14days	MOCK (n=9)	1,08±0,29	44,69±6,53	22,10±8,63	2,49±0,65	15,11±5,43	11,59±2,84	49,28±17,65
	MOI 3 (n=9)	1,14±0,29	37,41±14,02	20,14±11,43	2,86±0,39	11,27±7,05	9,29±5,10	62,83±27,24

**Table. 4. *Ex vivo* analysis of splenocyte subpopulations after s.c. injection of MVMp-infected and non-infected glioma GL261 cells.** One million glioma GL261 cells were injected s.c. into the right flank of recipient mice. Four hours prior cells were infected with MVMp at MOI 3 or left uninfected. The immune cell populations were stained with specific mAbs and measured by flow cytometry at day 7 and day 14 p.i.

Since we did not observe any changes within the overall composition of the subpopulations analyzed in the spleen, it is possible that only a minute fraction of circulating CD8 and CD4 cells are in the spleen. However, upon restimulation those cells could mount a substantial IFN $\gamma$  response.

In order to verify the specific immune response towards tumor cells, the release of IFN $\gamma$  from splenocytes was measured. It is known, that specific cytotoxic T-cells release IFN $\gamma$  upon stimulation. We assessed the potential of a specific immune response towards the GL261 tumor cells by monitoring IFN $\gamma$  release of total splenocytes isolated from tumor bearing animals. The splenocytes were isolated 14 days post injection from mice bearing GL216 tumors. Two main groups of animals were assessed. One group, which was injected with MOCK-infected cells, and a second group, which was infected with MVMp at MOI 3, were used for the *ex vivo* analysis of IFN $\gamma$  production. Splenocytes were incubated in presence of non-infected glioma GL261 cells, and the release of IFN $\gamma$  was measured by ELISPOT.



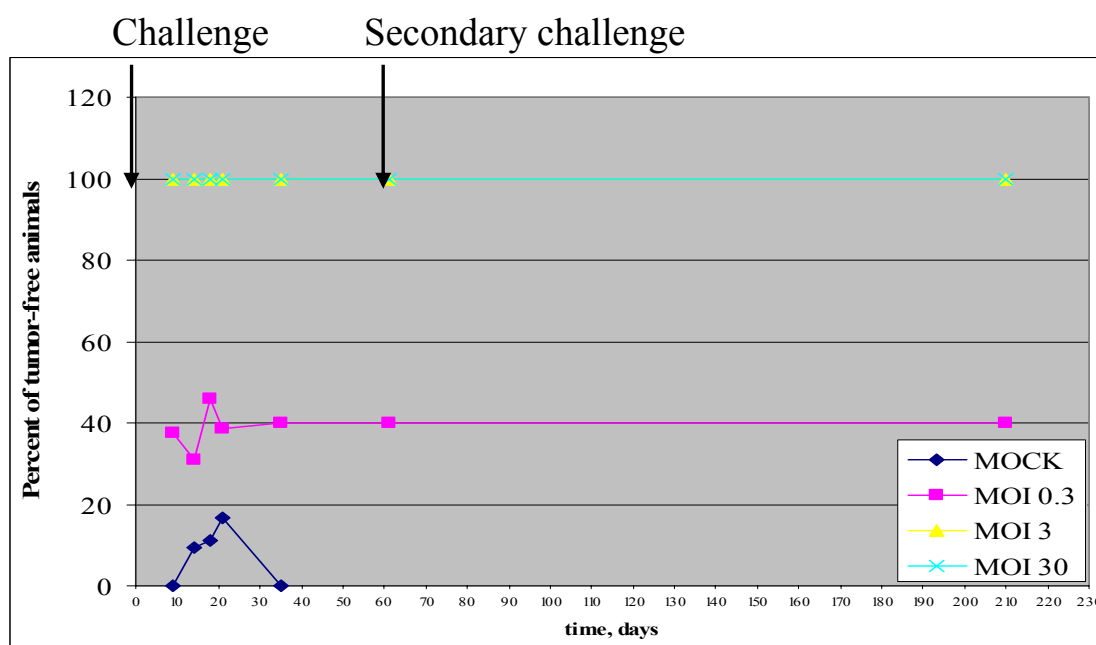
**Fig.16. Release of IFN $\gamma$  from splenocytes in response to uninfected glioma GL261 cells.** Splenocytes ( $1 \times 10^6$ ) were specifically restimulated *in vitro* with irradiated glioma GL261 cells ( $5 \times 10^5$ ). The release of IFN $\gamma$  was assessed as the number of cells-producing IFN $\gamma$  per  $1 \times 10^6$  of splenocytes.

Figure 16 depicts the median values of IFN $\gamma$  released. The cells isolated from spleens of mice, which were injected with MVM-infected glioma cells, is significantly higher compared to the cells derived from mice, which were injected

with MOCK-infected glioma cells. This result is indicative of a specific immune response in animals, which were injected with MVM-treated tumor cells.

#### 4.3.3. The T-cell memory response plays a crucial role against tumor development.

In order to verify, if surviving animals developed immunity against tumor cells, a challenge experiment was performed. There were 3 groups of mice, which previously received s.c. glioma GL261 cells infected with MVMp at MOI 0.3, 3 and MOI 30 (8 animals per group). None of the animals injected with glioma GL261 cells infected with MVMp at MOI 3 and MOI 30 developed tumors at day 35p.i. (0 out of 8 per group). At the same time point, some mice, which were injected with glioma GL261 cells infected with MVMp at MOI 0.3 developed tumors (5 out of 8). All tumor-free animals from these groups were challenged with  $1 \times 10^6$  GL261 tumor cells 60 days post primary challenge. After the challenge all mice survived and did not develop any visible tumors, with a survival range of 5 month after challenge (Fig. 17).



**Fig. 17. Survival rate of C57BL/6 mice after rechallenge.** C57BL/6 mice were injected s.c. with  $1 \times 10^6$  glioma GL261 cells, either MOCK-infected or MVMp-infected at MOI 0.3, 3 or 30. Mice were rechallenged with MOCK-infected glioma GL261 tumor cells ( $1 \times 10^6$  cells/mouse) at day 60. All mice survived after second challenge and non of them developed tumor at day 230 .

This experiment demonstrated that a specific memory immune response mediated by MVMp-induced tumor cell lysates plays a crucial role in rejection of tumor. Most likely the rejection reaction is mediated by memory T cells, since complete rejection of the tumor cells was observed in a secondary challenge experiment took place at 60-230 days after the first tumor challenge.

Taken together, the *in vivo* results demonstrate the activation of a T cell response upon s.c. injection of MVMp-infected glioma GL261 cells in immunocompetent C57BL/6 mice. This response is characterized by specific T-cell activation and T-cell memory formation towards tumor cells. Thus, we could conclude, that MVMp mediated a specific anti-tumor immune response against glioma GL261 in immunocompetent mice.

## 5. Discussion.

It was shown that due to the oncolytic activity of PV MVMp, formation of tumor lysates is possible in both of the described tumor models (glioma GL261 and melanoma B78/H1). Since the lysed tumor particles might elicit an immune response *in vivo*, the activation of APC, such as DCs or microglia in CNS, was investigated. The *in vitro* results demonstrated significant activation of DCs and microglia upon co-culture with MVMp infected GL261 glioma cells. There was no specific activation observed in case of co-culture of these APC with MVMp-infected melanoma B78/H1. These data gave a background for further *in vivo* experiments, which demonstrated the activation of T cell response upon s.c. injection of MVMp-infected glioma GL261 cells in immunocompetent C57BL/6 mice. This response is characterized by specific T-cell activation and T-cell memory formation towards tumor cells. Thus, we could conclude, that MVMp mediated a specific anti-tumor immune response against glioma GL261 in immunocompetent mice.

### 5.1. MVMp lyse glioma GL261 cells with high efficiency.

Autonomous rodent PV and recombinant derivatives provide promising and novel approaches for cancer treatment (Cornelis J. 2004.). However, the sensitivity of tumors towards PVs varies greatly and depends on tumor origin and the proliferative capacity of cells (Herrero et al. 2004). The analysis of genomic viral DNA in MVMp-infected glioma GL261 and melanoma B78/H1 cells clearly indicated that PV infected and replicated efficiently in both tumor cell lines. However, the two cell lines revealed a striking difference in their sensitivity to viral infection. Two to three days post infection it was observed that most of the glioma GL261 cells had rounded up, lost their adherent phenotype and started to die. Experimentally, cell cycle arrest and lysis of the glioma cells were assessed (data not shown) indicating that PV MVMp progressed through its life cycle within these cells exerting an

oncosuppressive effect. In addition, cell viability methods were performed to analyse the cell proliferation rate (cell growth) of glioma GL261 and melanoma B78/H1 cells upon MVMp infection.

These combined data strongly indicated that the glioma cell line was indeed very sensitive and permissive to PV infection. The melanoma B78/H1 showed less sensitivity to the oncolytic effect of MVMp. The growth of the melanoma cell line might be limited only partially by the cytotoxic effect of MVMp PV, and non-infected resistant cells continued to proliferate.

It seems that MVMp infection activates different death pathways in different cell lines. We had strong indications for the autophagy process leading to the induction of the death pathway in B78/H1 melanoma cells upon MVMp infection (data not shown). At the same time, caspase-3 activation in glioma GL261 upon PV MVMp infection pointed clearly to the apoptotic pathway of death in these cells.

Thus, evidence was provided that the both glioma GL261 and melanoma B78/H1 cells are useful models to study PV MVMp as a tool in future cancer treatment, but both models have certain limitations. The melanoma cells B78/H1 provide a better model for *in vivo* studies due to the physiological relevance of the route of tumor transplantation s.c. Due to the heterogeneity of the melanoma B78/H1 cell line and due to the resistance of a subpopulation to MVMp infection, this tumor cell line might only be useful for *in vitro* and *in vivo* assays within very narrow window of time and at high MOIs.

The glioma GL261 cells can be used in *in vivo* models with the certain limitations as well. The s.c. route of transplantation does not place the tumor cells at the physiological site for the glioma to develop, however, it proved very informative for the involvement of the innate and adaptive branches of the immune system and served successfully as a proof of principle model system.

## 5.2. MVMp induces activation of DCs and microglia.

Originally, we had hypothesized, that MVMp-infected tumor cells would induce an immune response. Most likely, apoptotic bodies or cellular components are involved in this process. It is known, that DCs provide the first line of defense in tumor cell recognition. DCs are a heterogeneous cell population of the haematopoietic lineage. Many DC subsets of different tissues have distinct cell surface receptor expression profiles. However, the ability to stimulate antigen specific naïve T cell activation and proliferation appears to be shared between these DC subsets. Although, the origin and lineage of many of these cells remain poorly understood, it was shown that lymphoid DCs are derived from common-lymphoid progenitor and myeloid DCs are derived from common myeloid progenitor cells. At the same time several studies suggest that plasmacytoid DCs develop from both lymphoid and myeloid sources (Shigematsu H. et al. 2004, Karsunky H. et.al. 2003; Karsunky H. et. al. 2005). However, all progenitor cells initially transform into immature DCs. These cells are characterized by high endocytic activity and low T-cell activation potential. Immature DCs uptake, process and present pathogens at their cell surface using MHC molecules. Simultaneously, they upregulate cell-surface receptors that act as co-receptors in T-cell activation such as CD80, CD86, and CD40 greatly enhancing their ability to activate T-cells (reviewed in Mellman and Steinman, 2001).

Depending on the co-stimulatory environment and signals, DCs can induce T activation, T cell anergy or tolerance (Steinman et al., 2000; Banchereau et al., 2003). Myeloid DCs are one of the promising tools in immunotherapy of tumors. In animal models and human clinical trials adoptively transferred myeloid DCs expressing tumor antigens mediate substantial antitumor immunity (Nestle F.O. et.al. 2001).

In terms to analyze whether MVMp infection and/or MVMp-mediated tumor cell lysates are responsible for the activation of DCs, two basic approaches were established to generate myeloid and lymphoid DCs. The first approach was referred to as *in vitro* hematopoietic stem cell (HSC) -derived DCs and the other as *in vivo* generated DCs of either the myeloid or lymphoid phenotype.



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The generation of DCs from HSC is more correlated with clinical application approaches, when human DCs are generated from patient monocytes upon stimulation with GM-CSF and IL-4. From another point of view, generation DCs *in vivo* (upon secretion of GM-CSF and FLT3L from stably transfected tumor cell lines) shows normal physiological activation of DCs towards foreign (tumor) antigens. Both approaches were used to compare the effect of tumor cell lysates on the DCs activation.

As a prerequisite, it was necessary to analyse whether MVMp infected and replicated in DCs or not. It was shown that MVMp infected DCs, but the virus did not replicate in these cells. Also DCs were infected with either low or high MOI, the virus, even at high concentration did not replicate and it was independent from DCs S-phase entry. Normally DCs have low proliferative potential, unless pushed through a challenge. This is crucial, since the oncolytic effect of PV is not exerted within the cells that are essential to mount a good activation of T cells.

Subsequently we investigated whether MVMp alone could stimulate DCs. Activation of DCs was assessed on the level of expression of specific activation markers (CD80, CD86, CD40) by flow cytometry and on the protein level of specific cytokines analyzed by ELISA. Infection at high MOI of naïve or mature, LPS-stimulated, DCs with MVMp was performed to investigate whether high MOI of the virus alone could either stimulate or suppress DC activation. No effect on naïve DCs was observed. MVMp did not stimulate the expression of the activation markers MHC class II, CD80, CD86 or CD40 on naïve myeloid and lymphoid DC and no production of TNF or IL-6 could be demonstrated. However, MVMp was able to increase further the enhanced expression levels of CD80 and CD40 markers on LPS-stimulated myeloid DCs, and CD80 and CD86 on LPS-stimulated mature lymphoid DCs and also to enhance the release of TNF and IL-6 from mature cells. This is an important finding, since PV MVMp could provide an additional activation signal to already mature DCs, which will ultimately lead to better antigen-presenting capacities and further more more efficient stimulation of the T-cell response.

Subsequently, we investigated the activation profile of DCs upon co-culture with MVMp-infected tumor cells. When MVMp-infected B78/H1 melanoma cells were co-cultured for 48hrs with myeloid or lymphoid DCs derived from syngenic C57/BL6 mice, there was no increase in the expression of activation markers on DCs, whereas a marked increase was observed in DCs stimulated with LPS as a positive control. Similarly, we also failed to detect any significant release of proinflammatory cytokines such as TNF and IL-6 in DCs co-cultured with infected B78/H1 cells. This finding strongly corresponds to the observation of partial resistance of melanoma B78/H1 cells to PV MVMp. Since we could not observe efficient lysis of these cells, we did not detect any significant stimulation of DCs with the MVMp-induced melanoma lysates.

The opposite effect was shown in co-culture experiments with MVMp-infected glioma GL261 cells. The results of these experiments clearly revealed a strong activation of DCs by infected GL261 cells compared with untreated DCs. Most likely, the DCs were activated by apoptotic bodies or specific virus-induced proteins in case of the infected glioma GL261 cells, since the melanoma B78/H1 cells did not show apoptotic features of death (no significant caspase-3 activation or DNA fragmentation) upon MVM infection.

Given that peripheral DCs do not represent the physiological environment for GL261 tumor cells, additional experiments using microglia derived from newborn brain tissues were performed. It is known, that microglia are considered as resident macrophages of the brain and display antigen-presenting, phagocytic capacities, and are able to secrete cytokines and chemokines.

It was previously shown, that the MVMp virus, even at high concentration did not replicate in microglia. (Abschuetz A., 2006). PV MVMp infection of microglia alone did not induce a robust immune response in microglia. Current data from the research group of Dr. A. Régnier-Vigouroux revealed that PV infection of microglial cells was not sufficient to induce TNF production, nor did PV alone induce the

expression of iNOS and the release of NO, a first line of reaction in terms of activation of these cells. (Abschuetz A., 2006). Neither were glioma GL261 cells alone able to activate microglia efficiently. We could also show, that MOCK-infected glioma GL261 cells did not stimulate microglia. The direct co-culture of microglia with untreated and uninfected GL261 cells was not conducted, as an overgrowth of the tumor cells in this system was to be expected. Therefore, glioma cells were arrested in cell cycle to prevent further proliferation. Additional data showed that microglia in co-culture with irradiated glioma GL261 cells did not reveal a different pattern of IL-1 $\beta$  expression at the RNA level compared to untreated microglia (H. Hong, diploma thesis, 2005). Glioma GL261 cells were also lysed by freeze/thaw (F/T) cycles, which led to necrotic lysis of cells. F/T lysed glioma GL261 cells did not induce significant secretion of TNF, nor did they have a measurable effect on IL-6 production. This observation confirmed data in the literature, which states that F/T lysed tumor cells had only a poor effect on the activation of DCs in a comparable co-culture set-up (Rad A.N. et. al. 2003). Taken together, the experimental data indicated that GL261 cells alone are unable to initiate a robust immune response in cultured microglia.

Upon co-culture of microglia with MVMp-infected GL261 cells we detected release of TNF and IL-6 as well as a significant and specific upregulation of the same set of activation markers analyzed on myeloid and lymphoid DCs. These data strongly correlate with observation of H.Hong showing that in co-culture assays microglia up-regulated IL-1 $\beta$  expression at the mRNA level, as well as IFN- $\beta$  expression. Furthermore, significant amounts of TNF could be detected by ELISA in the supernatants of co-cultured microglia, whereas GL261 cells did not secrete TNF in detectable amounts in any of the performed experiments. This provided evidence that murine primary microglia were specifically activated by PV MVMp infected tumor cells and that a tumor-specific immune response was induced. Therefore, PV MVMp-induced cell lysis of GL261 cells seems to be a crucial requirement to ensure efficient activation of microglia.

Thus, evidence was provided in support of the initial research hypothesis, that MVMp-infected tumor cells could specifically activate an immune response. The results were also consistent with the current view of microglia and their role as major immuno-competent cells in the brain (Aloisi F., 2001). They are able to discern a complex mass of various signals and they belong to the most diligent cytokine secreting cells in the brain. Microglia act as antigen-presenting cells and could therefore also initiate to a certain degree of T cell mediated immune responses in the brain.

An obvious question to address was to investigate the molecular pathways involved in the activation of DCs and microglia. A specific aim of this thesis work was to analyze the role of TLR signaling and the downstream NF $\kappa$ -B signaling pathway that might be involved in the activation of DCs and microglia.

DCs are the most efficient inducers of all immune responses. While plasmacytoid DCs and myeloid DCs share common functional features related to antigen uptake, processing and presentation, each type expresses unique sets of receptors that enables them to specifically recognize and respond to different pathogens (Colonna et al., 2004; Larsson et al., 2004; Iwasaki and Medzhitov, 2004). For example, all DCs and cells of the innate immune system express evolutionally preserved pattern recognition receptors, toll-like receptors. Viral pathogens can be sensed by TLR3 (double stranded RNA), TLR7 or TLR8 (single-stranded RNA), bacterial lipopolysaccharide and some viral proteins by TLR4, bacterial CpG sequences in DNA by TLR9 and flagellin by TLR5. TLR2 has a broad recognition profile that includes Gram-positive peptidoglycans and di- and triacetylated lipopeptides as well as selected viruses (Iwasaki and Medzhitov, 2004). Recently identified in human, TLR11 is activated by uropathogenic bacteria and *Toxoplasma gondii* in mice (Zhang et al., 2004; Yarovinsky et al., 2005), while no equivalent of TLR11 was found in other species. Plasmacytoid DCs express TLR7 and TLR9 that are pivotal in recognition of viruses and bacteria while myeloid DCs express TLR2,

TLR4, and TLR3 and monocytes express a broad profile of TLRs including TLR2, 3, 4, and 5 (Hornung et al., 2002). Microglia were shown to be activated by different PAMPs as well (Tran C.T. et al., 1998; Ebert S. et al., 2005). However, not only microbial and viral compounds but also tumour-derived and most probably host-derived components might play a role in the activation of microglia.

In view of the stimulatory capacity for DCs and microglia of MVMp-infected glioma GL261 cell lysates we were interested to know if this stimulation was associated with increased expression of one or more TLRs. Unfortunately, analysis of APC-specific TLRs expression was not possible in co-cultures since the glioma GL261 cells also express TLRs. Therefore, the TLR expression profile on the mRNA level in DCs infected with MVMp alone was studied. Expression of TLR 2, 3, 4 and 9 on myeloid DCs and TLR 2, 6 and 9 on lymphoid DCs was increased upon PV MVMp infection. Unfortunately, the limited amount of DCs in the experiments did not allow for analysis of TLR signal transduction pathways in cellular extracts from these cells. Therefore, heterologous expression of different TLRs in a tissue culture cell line was established to study activation following contact with MVMp-infected glioma or melanoma tumor cells or with virus alone as a control. The experiments were conducted with cell lines stably transfected with TLR2, TLR2/TLR6, TLR3, TLR4, TLR4/MD2/CD14 and TLR9. These TLRs were chosen as the most promising candidates. MD2 and CD14 are accessory molecules, which could further increase the efficiency of LPS recognition (Akira S. and K. Takeda, 2004). As it was shown, all investigated TLRs, except TLR3, were not activated either by MVMp-mediated tumor cell lysates, or by virus alone. Only TLR3 showed significant activation in response to MVMp-mediated tumor cell lysates. The level of activation was significantly higher towards glioma GL261 cell lysates compared to melanoma B78/H1 cell lysates, which confirm the DC activation data overall.

It was extensively discussed that live glioma GL261 cells did not induce any efficient activation of DCs and microglia, nor did F/T lysed GL261 cells induced a

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strong immune response. This provided evidence that PV infection and PV MVMp-induced lysis of glioma GL261 cells was required to mediate a robust activation of APC. Thus, the mode of cell lysis might play an important role. During the lysis of the glioma cells by the virus, cellular compounds are released, which would not be set free by F/T cycles. These yet unknown cellular molecules might be recognized by receptors displayed on the surface of APC and stimulate these target cells.

Further research on the molecular mechanisms of PV-mediated cell killing is required. Cell lysis upon PV infection of tumor cells can either be accomplished by necrosis, by apoptosis or autophagy formation and seems to be dependent from the cellular system, in which the experiments are conducted. For instance, Möhler et al. showed that the rat PV H1 is cytotoxic to human hepatoma cells. In their experiments, two of the three hepatoma cells lines presented over 50% apoptotic cells at days 3-6 post infection as detected by flow cytometry analysis (Moehler M., et al. 2001). Another study by Ran et al. suggested that NAD might play a role in the regulation between apoptosis and necrosis. Inhibition of NAD consuming enzymes interfered with PV induced NAD depletion, resulting in an increased number of infected cells lysed with apoptotic features (Ran Z., et. al., 1999).

Möhler and colleagues published additional evidence describing and characterizing PV induced cell killing in a human melanoma cell line (give the name of the melanoma cell line), which was highly susceptible to rat PV H1 infection. Assuming that HSPs can be detected in cells in stress situations, they analysed the constitutive and inducible form of HSP70 after H1 infection in this melanoma cell line. Interestingly, they found that intracellular levels of inducible HSP72 decreased continuously from day 2 post infection, whereas the HSP72 levels were elevated in the cell culture medium. A release of HSPs post infection was therefore observed (Moehler M., et al. 2003).

Taking together all these *in vitro* results demonstrate significant activation of DCs and microglia upon co-culture with MVMp-infected GL261 glioma cells. This

activation might lead to an initial innate and subsequently tumor specific adaptive immune response.

### **5.3. MVMp-mediated tumor cell lysates stimulate a specific antitumor immune response.**

So far, all the experiments conducted in this study were *in vitro* approaches. The final aim of this work was to show the generation of a specific immune response *in vivo*. So far all the promising results concerning the activation of APC were obtained in the glioma model, the all further experiments were conducted in the same model. As a matter of fact, the complexity of the physiological environment in the CNS is surely far beyond any *in vitro* approach. Seligman and Shear induced the “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 both intracranial (Ausman J.I. et al. 1970; Ehtesham M. et al. 2002; Saito R. et al. 2004) and s.c. (Miyatake S. et al. 1997; Schueneman A.J. et al. 2003) induction of 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 our study it was shown that glioma GL261 cells injected s.c. induce tumor growth. As it was clearly shown, the oncolytic effect of MVMp was not complete in immune deficient RAG2<sup>-/-</sup> mice. The growth of tumor was much more aggressive and the size of tumor was significantly larger in the RAG2<sup>-/-</sup> animals compared to normal immunocompetent C57BL/6 mice. Eighty percent of RAG2<sup>-/-</sup> mice developed significant tumor burden 21 days post injection even in the case of infection with high titer of virus (MOI 30). This clearly showed undoubtedly the role of an adaptive immune response in complete rejection of s.c. glioma GL261 tumor cell transplantation.

Additionally, ELISpot assays showed a specific recognition of tumor cells by host cells obtained from the spleen. The recognition and the respective specific immune response were significantly higher in the group of animals, which received MVMp-infected glioma GL261 cells s.c.. Which specific cell subset contributed to this effect remains however undetermined. From the literature it is not clear which cells, CD4<sup>+</sup> or CD8<sup>+</sup>, play the major role in the antitumoral immune response (Segal B. M. et al. 2002, Lumnitzky K. et al. 2002, Horton H.M. et al. 1999, Yang S.Y. et al. 2004). In a therapeutic vaccination approach against intracranial GL261-derived tumors depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes equally prevented the antitumor effect of the vaccine (Lumnitzky 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<sup>+</sup> T cell-dependent. Functional analyses demonstrated that CD8<sup>+</sup> 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 embryonic neural stem cells engineered to release IL-12 were implanted intratumorally (Yang S.Y. et al. 2004). It is known, that IL-12 is a critical cytokine involved in the transition from the innate to the adaptive immune response. One of the hallmarks of the biological functions of IL-12 is its ability to regulate the balance between Th1 and Th2 responses of T cells. Th1 cells secrete IFN- $\gamma$  and IL-2, whereas Th2 response is manifested by IL-4, IL-5, IL-6, IL-10 and IL-13 secretion. One promotes cell-mediated immunity, whereas the other facilitates humoral immunity (Paul W.E. et al., 1994). IL-12 favors Th1 responses promoting the differentiation of naïve T cells into Th1-cells, which are capable to produce large amounts of IFN- $\gamma$ . Furthermore, IL-12 acts as a co-stimulus, which is necessary for maximum production of IFN- $\gamma$  by differentiated Th1 cells responding to specific antigen. IL-12 also stimulates the development of IFN- $\gamma$  secreting Th1 cells from resting memory T cell populations by interacting with an antigen to which the T cells have been previously exposed (DeKruyff R.H. et al., 1995). Similarly to



many other cytokines, IL-12 can impart functions contradicting each other. For example, IL-12 was also shown to support Th2 responses. Thus, the role of IL-12 appears to depend on the cytokine micro-environment and on the state of maturation of T-cells. Besides its role in T cell activation, IL-12 also induces secretion of other cytokines, including anti-inflammatory cytokines such as IL-10, which might be involved in negatively regulating its own effects (Tripp C. S. et al., 1993). For these reasons, it would be interesting to analyze whether APC co-cultured with PV MVMp infected GL261 cells could induce the release of IL-12.

Another possibility to analyze the activation of a specific immune response furthermore would be the investigation of the TLR expression patterns on APC prior to and after stimulation with MVMp-induced tumor cell lysates. Doyle et al. examined the up-regulation of TLR3 and TLR4 after stimulating macrophages with TLR specific ligands (Doyle S.E., et al., 2003). TLR3 as well as TLR4 induced the expression of IFN- $\beta$ . IFN- $\beta$  in turn is believed to activate secondary antiviral gene induction by autocrine and paracrine pathways.

As a summary, PV MVMp infection and lysis of tumor cells could encompass two beneficial effects. On the one hand, upon completion of the PV life cycle in glioma cells there will be a drastic increase in the release of newly generated viral particles. Newly produced viral particles could lead to re-infection and thus enhance the cytotoxic effect in gliomas. On the other hand, lysis of glioma cells could elevate the immunogenicity of the tumor as lysed tumor cell debris might be a target for APCs like microglia. Upon culture of single TLR expressing stable cell lines with MVMp-mediated tumor cell lysates, the cell line stably expressing TLR3 had an eight fold higher activation of NF- $\kappa$ B reporter gene expression compared to control. This finding called for a role of TLR3 mediated signaling in the activation NF- $\kappa$ B and downstream target genes such as pro- and inflammatory cytokines. Activated microglia could then in turn activate invading T cells and promote either a Th1 or a Th2 response.

So far, no effective therapy is available to treat glioma and all conventional attempts have failed to cure this disease. In this study, PV was discussed as a promising candidate as an alternative strategy to treat cancer.

Vaccination strategies against cancer aim at priming immune cells against tumor antigens. Many problems have to be faced and overcome, such as the usually low immunogenicity of tumors, the immuno-suppressive features in the tumor environment, and possible side-effects associated with vaccination therapies, for instance possible autoimmune reactions. PVs could enhance the immunogenicity of tumors by releasing tumor antigens in the process of cell lysis. Therefore, the use of PV in regard to tumor vaccination strategies seems promising and future research in this area might soon include clinical trials.

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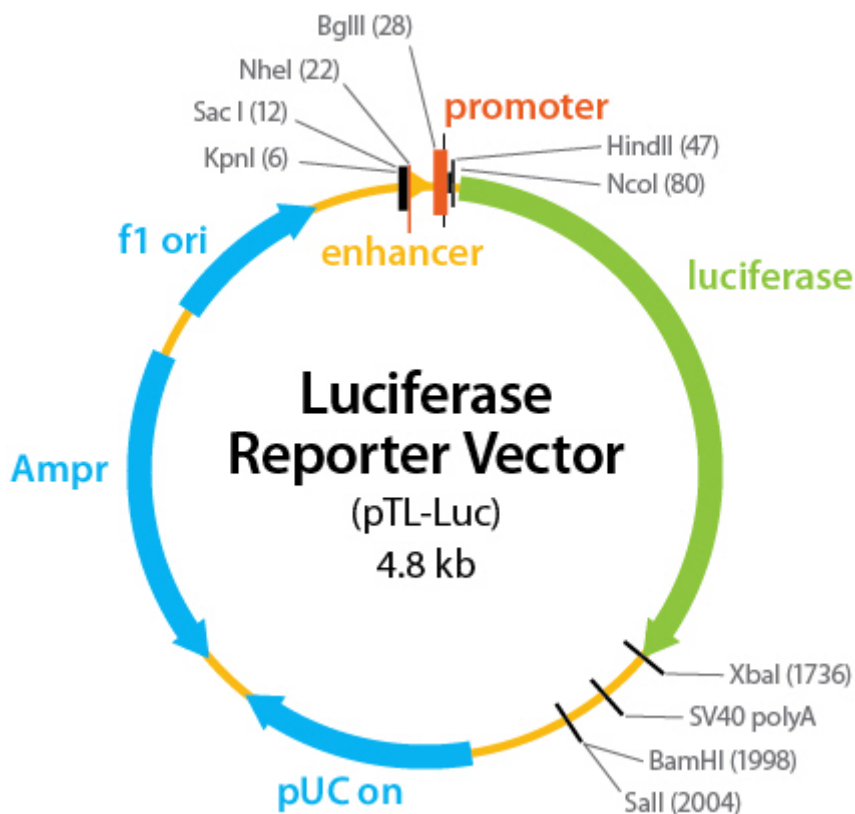
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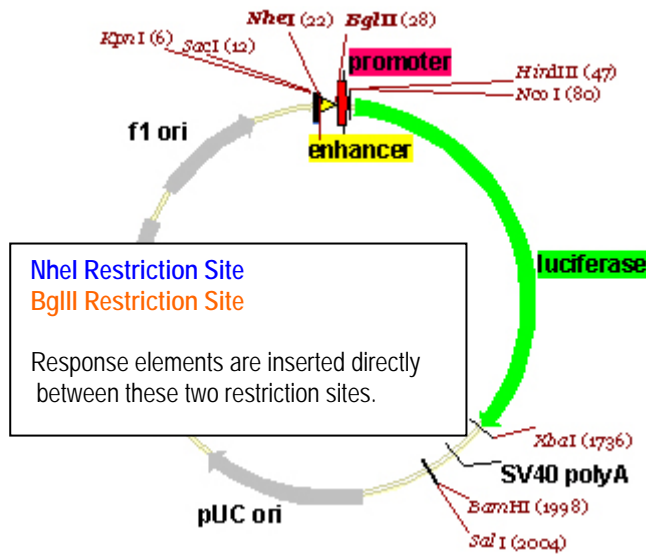
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## TransLucent Reporter Control Vector Sequence, Catalog #: LR0000



The cis-acting enhancer element sequence resides between the *NheI* and *BglII* restriction sites, upstream of the TATA box promoter, which drives expression of the firefly luciferase reporter gene upon transcription factor binding. Unique restriction sites are listed on the map. The length of the enhancer element differs for each Reporter Vector, therefore the positions indicated on the vector map should be adjusted accordingly. All of the Vectors contain the Panomics signature sequence. These vectors are intended for research use only and should not be used commercially.

Vector contains minimal TA promoter immediately upstream of luciferase gene (*Photinus pyralis*).



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