CHARACTERIZATION OF CANDIDATE ONCOLYTIC VACCINIA VIRUSES WITH DELETIONS IN VIRAL GENES BLOCKING THE ACTIVATION OF INTERFERON REGULATORY FACTOR 3 IMMUNE SIGNALING

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Inaugural-Dissertation zur Erlangung der Doktorwürde
der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität
München

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München 2021

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Lehrstuhl für Virologie

Arbeit angefertigt unter der Leitung von: Univ.-Prof. Dr. Gerd Sutter

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Gedruckt mit Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.
Berichterstatter:	Univ.-Prof. Dr. Gerd Sutter
Korreferent:	Univ.-Prof. Dr. Dr. h. c. Hans-Joachim Gabius

Tag der Promotion: 17. Juli 2021

Meine Liebe.

Für Dani.

Für meine Familie.

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LIST OF ABBREVIATIONS

%	Percentage
µl	Microliter
µm	Micrometer
µM	Micromolar
ALL	Acute lymphoblastic leukemia
AP-1	Activator protein 1
APC	Antigen-presenting cell
ATC	Adoptive T cell therapy
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AU	Arbitrary units
bp	Base pair
CAR	Chimeric-antigen receptor
CD8	Cluster of differentiation 8
CD19	Cluster of differentiation 19
CD28	Cluster of differentiation 28
CD40	Cluster of differentiation 40
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
cDNA	Complementary DNA
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase

X
LIST OF ABBREVIATIONS

CMC	Carboxymethylcellulose
CO2	Carbon dioxide
CR	Complete response
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated Protein-4
CVA	Chorioallantois VACV Ankara strain
CXCL-11	C-X-C motif chemokine 11
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DDX3	DEAD-box RNA helicase 3
DNA	Deoxyribonucleic acid
DNA-PK	DNA protein kinase
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double stranded RNA
EDTA	Disodium ethylenediaminetetraacetic acid
EFC	Entry-fusion complex
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic translation initiation factor 2A
ELISpot	Enzyme Linked Immuno Spot
EV	Extracellular enveloped virion
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
g	gram

LIST OF ABBREVIATIONS

GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GFP	Green fluorescent protein
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HMGB1	High mobility group box 1 protein
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)
HRP	Horseradish peroxidase
HSP	Heat Shock Protein
HSV-1	Herpes simplex virus type 1
ICD	Immunogenic cell death
ICI	Immune checkpoint inhibitor
IFN	Interferon
IFNAR	IFN receptor
IgG	Immunglobulin G
IKKε	IκB kinase-ε
IL-2	Interleukin 2
IL-10	Interleukin 10
IL-12	Interleukin 12
IMV	Intracellular mature virion
IRAK2	IL1-receptor-associated kinase 2
IRF-3	Interferon regulatory factor 3
IRF-7	Interferon regulatory factor 7

LIST OF ABBREVIATIONS

ISG	IFN-stimulated gene
ISG	Interferon-stimulated gene
ISGF	IFN-stimulating gene factor
ISRE	IFN-stimulated response element
ITR	Inverted terminal repeats
JAK	Janus kinase
kb	Kilobase
kDa	Kilodalton
LAG-3	Lymphocyte Activation Gene-3
mAB	Monoclonal antibody
MAL	Myd88 adaptor-like
MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation antigen 5
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
ml	Milliliter
MLKL	Mixed lineage kinase domain like pseudokinase
mM	Millimolar
mm^3	Cubic millimeter
MOI	Multiplicity of infection
mRNA	Messenger-RNA
MV	Mature virion
MVA	Modified Vaccinia virus Ankara
Myd88	Myeloid differentiation primary response gene 88

LIST OF ABBREVIATIONS

NAP-1	Nucleosome assembly protein 1
NDV	Newcastle disease virus
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NK-cell	Natural Killer Cell
nm	Nanometer
OAS	2`-5`-oligoadenylate synthase
ORR	Objective response rate
OV	Oncolytic virus
P/S	Penicillin-Streptomycin
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1, -2	Programmed cell death ligand 1, -2
PEG2	Prostaglandin E2
Pexa-Vec	Pexastimogene devacirepvec
PFU	Plaque-forming unit
P-IRF3	Phospho-IRF3
PKR	Protein kinase R
PMA	Phorbol-12-myristat-13-acetat
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
RIG-I	Retinoic acid inducible gene I

XIV
LIST OF ABBREVIATIONS

RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFC	Spot forming cell
SINTBAD	Similar to NAP1 TBK1 adaptor
ssRNA	single-stranded RNA
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TAA	Tumor-associated antigen
TAE	Tris-Acetat-EDTA
TAM	Tumor associated macrophage
TANK	TRAF family member-associated NF-kappa-B activator
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
TIL	Tumor-infiltrating lymphocyte
TIR	Toll/interleukin-1 receptor
TK	Thymidine kinase

LIST OF ABBREVIATIONS

TLR	Toll like receptor
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-α
TRAF-6	TNF-receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
T_{regs}	Regulatory T cells
TRIF	TIR-domain-containing adaptor-inducing interferon-β
T-Vec	Talimogene laherparepvec
VACV	Vaccinia virus
VEGF	Vascular endothelial growth factor
VGF	Vaccinia growth factor
VSV	Vesicular stomatitis virus
WHO	World Health Organization
WR	Western Reserve

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I INTRODUCTION

Despite the significant progress made in the treatment of cancer, it remains one of the leading causes of death worldwide with about 10 million estimated cancer deaths in 2020 (Sung et al. 2021). To improve the safety and off-target effects, as well as the effectiveness of cancer therapies, research in novel cancer therapeutics is fundamental.

Cancer immunotherapy includes a variety of strategies to activate and boost a patient's own immune system to detect and destroy cancer cells. In this context, oncolytic virotherapy is an emerging branch of cancer treatment, which takes advantage of the capacity of natural or genetically modified viruses to selectively replicate in cancer cells, leading to tumor cell lysis. Many different viruses have been tested as oncolytic viruses, including vaccinia virus (VACV), which provides several advantages such as a fast and lytic replication, an extensive safety record in humans and a high capacity to harbor transgenes. Importantly, replication of VACV within the tumor can result in cellular immune responses targeting relevant tumor antigens and in a transient overcoming of the localized immune suppression in the tumor microenvironment. However, this mechanism demonstrated in clinical trials to be not efficient enough for activating effective antitumor immune responses in a wide number of tumor patients.

The goal of this study was to investigate novel modifications introduced to oncolytic VACV to enhance their capacity to induce potent anti-tumor immune responses. As robust antitumor cytotoxic T-cell responses demonstrated to be key for the successful treatment of cancer, and activation of the TLR3-IRF3 pathway directly correlates with activation of such immunities, we constructed a panel of oncolytic VACV combining deletions in genes involved in the inhibition of IRF3 pathway activation. We evaluated the replication capacity in cancer cells, their ability to induce anti-tumor T cell response, and their antitumor efficacy in mouse tumor models.

II LITERATURE REVIEW

1. Cancer and the immune system

Cancer is characterized by the accumulation of cells with genetic defects in regulatory circuits controlling cell proliferation and homeostasis, leading to uncontrollable proliferation. However, this is not the only feature that distinguish normal cells and cancer cells. Hanahan and Weinberg described in 2000 six “hallmarks of cancer”, including resisting cell death, evading growth suppressors, sustaining proliferative signaling, enabling replicative immortality, induction of angiogenesis, and activation of invasion and metastasis (Hanahan and Weinberg 2000). With new developments in the understanding of the biology of cancer, they added two emerging hallmarks a decade later: reprogramming of cellular energy metabolism to support excessive cellular proliferation and active evasion by cancer cells from the immune system to avoid destruction (Hanahan and Weinberg 2011).

Since Paul Ehrlich first proposed the idea that the development of cancerous cells in our body can be suppressed by the immune system (Ehrlich 1909), the role of the immune system regarding cancer was controversially discussed for decades until the role of Interferon-γ (IFN-γ) in promoting immunologically induced rejection of transplanted tumors was described (Dighe et al. 1994). When it was also described that mice lacking an adaptive immunity were more susceptible towards carcinogen-induced or spontaneous tumor formation, the immune surveillance hypothesis postulated by Sir Frank Mac Farlane Burnet was reinforced. Such hypothesis, postulated that tumor cell-specific neo-antigens could provoke an effective immunologic reaction that would lead to regression of the tumor (Kaplan et al. 1998; Shankaran et al. 2001; Burnet 1957; Burnet 1970). In addition, the discovery that tumors formed in mice without an effective

immune system were more immunogenic than those formed in immunocompetent mice leaded to the cancer immunoediting hypothesis (Shankaran et al. 2001; Dunn et al. 2002; Schreiber et al. 2011). Such hypothesis discusses the different roles of immunity in tumor development: within the first phase ("elimination"), transformed cells are being detected and destroyed by cells of the innate and adaptive immunity, successfully suppressing tumor development. However, if some cancer cells are not destroyed, they enter the so-called "equilibrium phase", in which tumor dormancy is induced by immunologic mechanisms preventing its outgrowth. This is also the phase where tumor editing occurs due to the constant immune selection pressure. This pressure leads to the rise of cancer cells that enter the escape phase where their outgrowth can no longer be blocked by the immune system. Those tumor cells can evade immune recognition or prevent immune destruction by the immune system and cause a clinically apparent tumor (Schreiber et al. 2011).

1.1. Immune-mediated destruction of tumors

Important steps to an efficient antitumor immune response are summarized in the cancer-immunity cycle (Chen and Mellman 2013). It starts with the release of cancer cell antigens from tumor cells that can be captured and processed by dendritic cells (DCs). In the next step, DCs present the captured antigens on MHC-I and -II molecules in the lymph nodes to T lymphocytes, leading to the priming and activation of those naive T cells against antigens derived from the tumor. The activated cytotoxic T lymphocytes (CTLs) traffic via blood vessels to the tumor and infiltrate it. In the tumor bed CTLs recognize cancer cells carrying their antigen and leads to the killing of the cancer cell and the release of more tumor-associated antigens (Chen and Mellman 2013) (**Figure 1**). Promoting an effective T cell response while overcoming the immune suppressive mechanism of the tumor is the key to successful destruction of cancer cells.

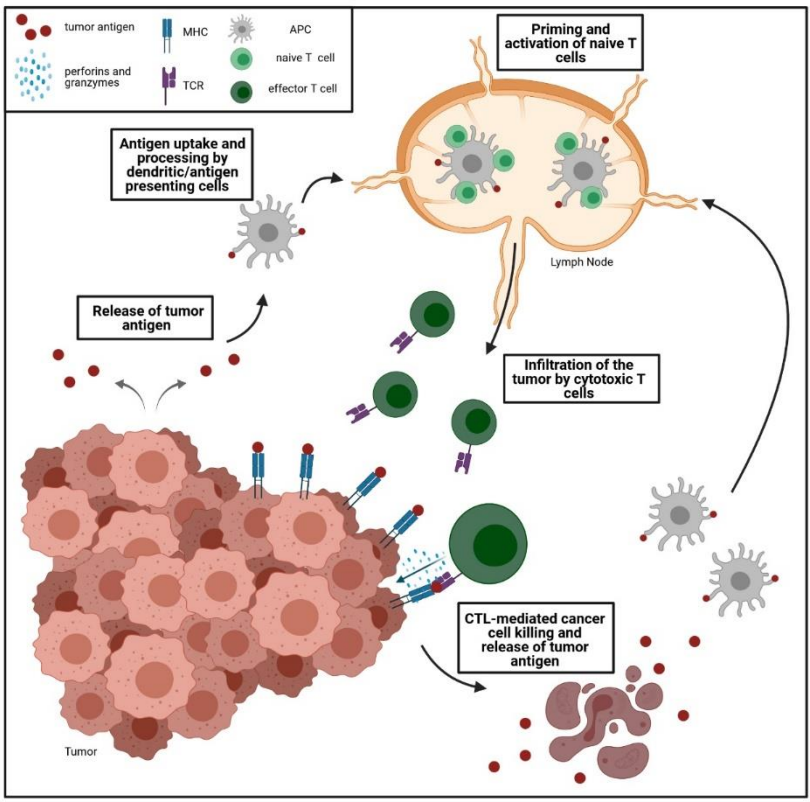


Figure 1. **Generation of anti-tumor immunity** (created with BioRender.com)

1.2. Tumor-immune evasion strategies

As previously indicated, tumors can evade destruction by the immune system through changes acquired at cell level or to the tumor microenvironment (TME). During their progression, tumors are able to acquire a large variety of these immune-evasion mechanisms in order to escape immune recognition. One example involves impaired antigen

processing and presentation, including the loss or downregulation of the antigen presenting machinery (Khong and Restifo 2002; Leone et al. 2013; Seliger et al. 2000). Another form of alteration on tumor cell level is an increased resistance to immune-mediated apoptosis, for instance by the upregulation of anti-apoptotic molecules (Fernald and Kurokawa 2013). Furthermore, tumors are able to establish an immunosuppressive microenvironment by producing immunosuppressive cytokines such as the vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β) or interleukin-10 (IL-10) (Gabrilovich et al. 1996; Geissmann et al. 1999; Wrzesinski et al. 2007; Steinbrink et al. 1999). Tumor cells can also express inhibitory molecules like PD-L1, which suppresses T cell function (Hamanishi et al. 2007). Another way tumor cells lead to impaired T cell function involves their metabolic activity: as rapidly dividing cells, tumor cells require high glucose uptake for fast energy production, even in the presence of oxygen (Hanahan and Weinberg 2011). As tumor cells can express higher level of nutrient transporters, T cells are exposed to a restricted level of glucose resulting in reduced T cell infiltration and antitumor activity (Singer et al. 2011; Chang et al. 2015). Overexpression of the glucose transporter GLUT1 in tumor cells is linked to a decreased level of CD8 T cells and a poorer survival rate in patients with ovarian cancer (Cho et al. 2013).

Tumors are also able to recruit immunosuppressive immune cells into the tumor bed, including regulatory T cells (T_{regs}) or myeloid-derived suppressor cells (MDSCs). T_{regs} are able to suppress T cell function in several ways, for example by secreting TGF-β or IL-10 (Facciabene et al. 2012; Strauss et al. 2007), or by expressing inhibitory molecules on their surface such as CTLA-4 or PD-1 (Takahashi et al. 2000; Zhang et al. 2016). T_{regs} can also inhibit T cells by starving them of IL-2 an important cytokine for T cell function (Pandiyan et al. 2007). Another class of efficient inhibitors of effector T cells are MDSCs, a heterogeneous group of myeloid progenitors with multiple functions. MDSCs are not only able to attract T_{regs} (Huang et al. 2006), but

they also produce immunosuppressive cytokines (Gabrilovich and Nagaraj 2009) and induce angiogenesis (Yang et al. 2004). Like T_{regs}, MDSCs can sequester amino acids needed for T cell function (Srivastava et al. 2010).

II LITERATURE REVIEW

2. Cancer immunotherapies

During the last two decades, a novel kind of antitumor therapy has been added to the anticancer arsenal: cancer immunotherapies. The goal of these therapies is to overcome the acquired immune modulatory mechanisms of the tumor by stimulating and boosting the patient's own immune system. There are different approaches to reinstall the immune system´s capability to induce an efficient, targeted antitumor immune response, and they include cancer vaccines, adoptive T cell transfer, immune checkpoint inhibitors and oncolytic virotherapy, which are detailed in the following sections.

2.1. Cancer vaccines

Antigen presentation is the first step in generating an antitumor immune response, so one attempt to induce tumor antigen specific T cells is the exogenous delivery of cancer antigens. Nevertheless, the effectiveness of cancer vaccines is limited, mostly due to suboptimal vaccine design and to the immunosuppressive tumor microenvironment. Improvements that need to be done involve the choice of antigen to immunize with, the delivery mode and possible combinational treatment to overcome tumors' immunosuppressive mechanisms (Palucka and Banchereau 2013; Melief et al. 2015).

2.2. Adoptive T cell therapy (ACT) and T cell engineering

For adoptive T cell therapy (ACT), T cells are isolated from patient's blood or tumor, modified or selected ex vivo, expanded and injected back into the patient, mostly after lymphodepletion to eliminate immunosuppressive cells like T_{regs} or MDSCs (Hinrichs and Rosenberg 2014).

TILs (Tumor-infiltrating lymphocytes) therapies consist on isolating T cells from a tumor biopsy, select, and expand those able to recognize tumor cells. Studies using TILs in melanoma patients demonstrated an objective

response rate between 50% and 70% with some complete responses (22%) (Rosenberg et al. 2011). Despite this encouraging result, this approach demonstrates some limitations, such as difficulty, time, and cost of selecting and expanding TILs. Furthermore, melanomas are the only type of cancer in which TIL therapy displayed clinical activity, probably due to high immunogenicity of melanomas compared to other tumor types (Hinrichs and Rosenberg 2014).

Strategies to improve ACT and increase the application to other tumor types include the genetic engineering of the T cell receptor. One approach is the expression of transgenic T cell receptors (TCR) with higher antigen-specificity and affinity on lymphocytes derived from a patient's blood. However, limitations arise, as this technology is MHC-restricted and only of use in patients, whose tumor present the targeted antigen; tumors can downregulate MHC expression, limiting the clinical use of TCR-technology (Park et al. 2011).

The second approach are the so-called chimeric-antigen receptor (CAR) T cells, which combine the antigen-recognition ability of antibodies with T cell activating functions. Firstly described by Eshhar et al., CARs consist of an antigen-binding single-chain fragment, which is variable, a transmembrane domain, and a signal transduction domain (Eshhar et al. 1993). That way, CAR-T cells do not depend on a cancer cells' functioning antigen-expressing machinery, but target any potential cell surface antigen. One of the most investigated targets for CAR-T cells is CD19, which shows encouraging results in hematologic malignancies such as B cell leukemia and lymphoma (Turtle et al. 2016; Davila and Brentjens 2016; Strati and Neelapu 2019; Grupp et al. 2013). However, in solid tumors, the results of CAR-T cell therapies remain modest due to different obstacles. For example, the lack of suitable antigen targets, the complex tumor microenvironment, which makes it difficult for the T cells to enter the tumor bed, and the highly

immunosuppressive milieu existing within the tumor (Dai et al. 2016; Klebanoff et al. 2016; Yang 2015). Another major difficulty that needs to be addressed in CAR-T cells are the serious side effects, which include neurotoxicity and cytokine release syndrome (CRS) as a consequence of the uncontrolled release of proinflammatory cytokines (Morgan et al. 2010; Hartmann et al. 2017; Santomasso et al. 2019).

2.3. Immune checkpoint inhibitors

Non-specific immunotherapies boost cells from the innate and adaptive immune system without specifically targeting tumor antigens. These therapies include, for example, administration of interleukins, interferons, enzyme inhibitors, or immune checkpoint inhibitors (ICIs) (Berraondo et al. 2019; Shirota et al. 2012; Baek et al. 2005; Vonderheide 2020).

Immune checkpoints are inhibitory receptors expressed on the surface of immune cells that trigger immunosuppressive signaling pathways. The immune system has several of these immune checkpoints (e.g. Cytotoxic T Lymphocyte Antigen-4 (CTLA-4), Programmed Death-1 (PD-1), Lymphocyte Activation Gene-3 (LAG-3) and others) to regulate the amplitude and duration of induced responses in order to minimize collateral tissue damage during infection, and to generate and maintain self-tolerance. However, some tumors, take advantage of those mechanisms to suppress T cell activation and prevent destruction by the immune system resulting in hyporesponsiveness or T cell exhaustion (Pardoll 2012; Sharpe et al. 2007; Nirschl and Drake 2013). An approach to reinstall T cell function and enabling immune cells to destroy cancer cells is the use of antibodies that block the function of these “immune checkpoints” (Darvin et al. 2018). Ipilimumab, a monoclonal anti-CTLA-4 antibody, was the first immune checkpoint inhibitor approved by the FDA in 2011 for its use against metastatic melanoma, demonstrating prolonged overall survival (Hodi et al. 2010; Yang 2015). CTLA-4 is a negative regulatory receptor on the surface

of T cells whose expression gets rapidly upregulated upon the activation of T cells (Krummel and Allison 1995). As a homologous to CD28, a key co-stimulatory receptor in T cells, CTLA-4 competes with it for both, CD80 and CD86 ligands, but has higher affinity to them (Greene et al. 1996). Blocking of CTLA-4 enhances T cell activation as well as the depletion of T_{regs} in the TME (Peggs et al. 2009; Simpson et al. 2013) and therefore demonstrated encouraging clinical results. However, it also displayed immune-related toxicities in a subset of patients. The importance of CTLA-4 as an immunomodulator could be observed in CTLA-4 knockout mice showing a fatal autoimmune phenotype (Hodi et al. 2010; Waterhouse et al. 1995).

Following the success of the CTLA-4 blockade, other immune checkpoints were investigated as potential targets for increased antitumor immune response. PD-1, which is expressed on activated T cells, B cells, antigen-presenting cells (APCs) and NK-cells, inhibits T cell activity upon interaction with PD-L1 and PD-L2 (Ishida et al. 1992; Agata et al. 1996; Keir et al. 2008; Freeman et al. 2000; Latchman et al. 2001). The expression of PD-L1 has been detected in a variety of tumor cells to avoid destruction by the immune system (Jadus et al. 2012). These tumor cells revealed increased resistance to T cell-mediated lysis and Fas-induced apoptosis (Hirano et al. 2005; Azuma et al. 2008). Several immune checkpoint inhibitors targeting PD-1 (e.g. nivolumab, pembrolizumab) and PD-L1 (e.g. atezolizumab) have reached the clinic and earned FDA approval (Topalian et al. 2014; Ansell et al. 2015; Brahmer et al. 2012; Mahoney et al. 2015; Powles et al. 2014). In addition, PD-1 and PD-L1 antagonists result in less severe side effects compared to CTLA-4 blockade, but severe pneumonitis has been observed in a small fraction of patients (Brahmer et al. 2010; Topalian et al. 2012).

With multiple potential targets identified for immune checkpoint blockade, other immune checkpoint inhibitors are being investigated and hundreds of clinical trials are ongoing. Yet, the biggest hurdle of immune checkpoint

therapy is that only a subset of patients is responsive. Different studies investigate potential biomarkers to predict which patients are most likely to respond to these therapies (Zappasodi et al. 2018; Rizvi et al. 2018; Snyder et al. 2014).

2.4. Oncolytic virotherapy

A rather new branch of cancer immunotherapy is the use of oncolytic viruses (OVs) as an intratumoral danger signal for the immune system. These oncolytic viruses were initially used as anticancer agents based on their restricted replication in malignant cells (Kirn et al. 2001; Guo et al. 2008). Cancer cell selectivity can be achieved by using viruses that are non-virulent in humans, but present replication in certain cancer cells due to their defects in interferon-responsiveness. These viruses include Newcastle disease virus (NDV) (Zamarin and Palese 2012; Pecora et al. 2002), vesicular stomatitis virus (VSV) (Lichty et al. 2004) and parvovirus (Angelova et al. 2015). Another wild-type virus that is used as an oncolytic agent is reovirus, which merely causes mild symptoms in humans and present a natural restricted replication to cells with an activated Ras signaling pathway (Norman and Lee 2000; Hashiro et al. 1977; Duncan et al. 1978), such are most tumor cells (Norman et al. 2004; Maitra et al. 2012). In addition to these viruses presenting natural tropism for tumor cells, cancer-selective replication can also be achieved by genetic engineering. In the 1990s, genetic viral engineering started a new chapter in the oncolytic field, when a genetically engineered herpes simplex virus type I (HSV-1) with a mutated thymidine kinase gene showed successful application in human brain tumors (Martuza et al. 1991). Genetic modifications for this increased selectivity involve deletions of virulence genes redundant for viral replication in tumors (Guo et al. 2005; McCart et al. 2001), as many tumor cells display mutations in antiviral signaling pathways and therefore naturally favor viral replication (Hanahan and Weinberg 2011, 2000). Other alterations include use of tissue specific promoters for essential viral genes (Rojas et al. 2010).

II LITERATURE REVIEW

Ever since a connection between viral infection and tumor regression was observed, people tried to treat cancer with different viruses (Hoster et al. 1949; Taqi et al. 1981; Kelly and Russell 2007). Up to date, a wide range of different viruses are investigated for their use as OVs, with more than 90 clinical trials reported during the last 20 years, mainly adenoviruses, HSV-1, reoviruses, and poxviruses (Macedo et al. 2020).

Generally, oncolytic viruses promote antitumor responses through several distinct mechanisms. Because of viral replication within the tumor, tumor cells are lysed, which also leads to an amplification of the initial viral dose administrated. Importantly, such replication within the tumor environment can also result in indirect induction of antitumor immune responses (Lichty et al. 2014). Viral replication within tumor cells leads to tumor cell lysis and thereby to the release of tumor-associated antigens (TAAs), as well as different damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), which can activate the innate and adaptive immune system (Rubartelli and Lotze 2007; Bartlett et al. 2013; Chiocca and Rabkin 2014) (**Figure 2**). This local intratumoral inflammation milieu can overcome the immunosuppression existing within the TME and promote antitumor immunity. The effect of oncolytic viruses can be enhanced by the expression of therapeutic transgenes, including different cytokines and anti-angiogenic proteins.

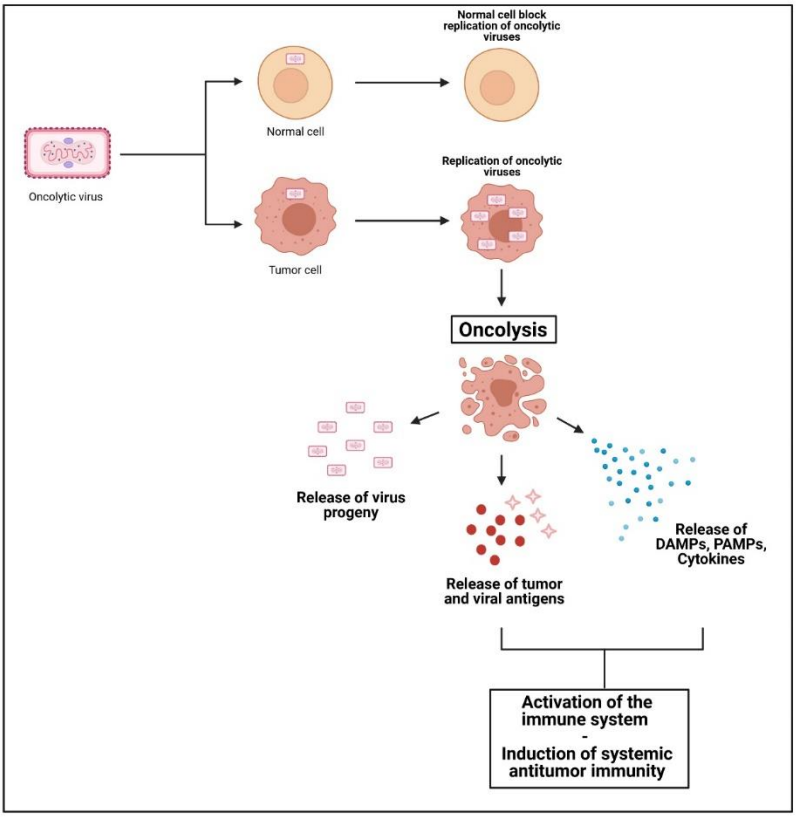


Figure 2. **Antitumor activity of oncolytic viruses** (created with BioRender.com)

One of the oncolytic viruses most advanced in clinical development is a modified oncolytic herpes simplex virus type I called Talimogene laherparepvec (T-VEC). T-VEC displays a deletion in both the γ34.5 and the α47 genes, and an insertion of the human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hu et al. 2006; Liu et al. 2003). While γ34.5 was deleted to achieve cancer cell-selective replication on one hand and to attenuate the virus neuropathogenicity on the other hand, α47 was deleted to enhance antigen presentation and therefore improve antitumor immune

responses (Chou and Roizman 1992; He et al. 1997; Goldsmith et al. 1998). To further increase the induction of antitumor immunity, the immunostimulatory cytokine GM-CSF was inserted into the viral genome (Hercus et al. 2009). After demonstrating its success in numerous clinical trials, T-VEC became the first oncolytic virus to be approved by the FDA in October 2015, followed by approval in Europe and Australia (Andtbacka et al. 2015; Ledford 2015; Senzer et al. 2009; Coffin 2016).

Despite promising results achieved by oncolytic virotherapy, there are limitations faced by oncolytic viruses. The main issue is that oncolytic viruses can be recognized by the immune system as pathogens and therefore be cleared before they could induce a sufficient antitumor response. Especially when administrated intravenously, the antitumor effect is limited due to neutralizing antibodies, the complement system, and the sequestration in the liver or spleen (Gong et al. 2016; Russell et al. 2014). Additional obstacles for successful systemic administration are the dilution of the injected virus in the bloodstream and the limited permeability of tumor blood vessels, as well as other physical barriers faced within large tumors (Russell et al. 2012; Miller and Russell 2016). When administrated intratumorally, on the other hand, the virus may not be able to reach tumors in locations difficult or impossible to inject, leaving systemic administration as more effective, especially in case of metastatic cancer.

To enhance the efficacy of oncolytic viruses, their combination with classical form of cancer treatments and immunotherapies like immune checkpoint inhibitors is under investigation (Rajani et al. 2016; Rojas et al. 2015; Puzanov et al. 2016).

II LITERATURE REVIEW

3. Poxviruses as oncolytic agents

3.1. Vaccinia virus – taxonomy and viral life cycle

Vaccinia virus (VACV) is a member of the family Poxviridae, which can be divided into two subfamilies: Entomopoxvirinae specific for insects, and Chordopoxvirinae, which infect vertebrates. The Chordopoxvirinae consists of nine genera with Orthopoxvirus being the best-known genus because of its two famous members: variola virus and vaccinia virus. Variola virus is the causative agent of the human smallpox, and vaccinia virus is the vaccine used between 1958 and 1977 in the smallpox eradication campaign by the WHO (Fenner et al. 1988).

Like all poxviruses, VACV is a large, oval to barrel-shaped, enveloped double-stranded DNA virus with 250 nm in diameter and 360 nm in length. An outer lipid membrane surrounds the biconcave core, containing an S-shaped genome with linear dsDNA (130-300 kbp) and two lateral bodiesFehler! Verweisquelle konnte nicht gefunden werden.. Inverted terminal repeats (ITR) at the end of the genome form two single stranded hairpin loops (Baroudy et al. 1982). Genes located in the terminal region are often variable and mostly dedicated to host range or immune evasion functions, whereas genes in the central regions of the genome are highly conserved amongst poxviruses and essential for viral replication (Moss 1996; Werden et al. 2008). Poxviruses exist in two infectious forms: intracellular mature virions (IMVs) and extracellular enveloped virions (EVs). IMVs are the majority of infectious progenies, which are released by cell lysis and responsible for host-to-host transmission. EVs possess an additional outer lipid membrane with associated proteins absent in IMVs and induce cell-to-cell as well as long-range spread (Payne 1978; Blasco and Moss 1992).

Poxvirus replication takes place in the cytoplasm (**Figure 3**) and, therefore,

poxviruses encode their own transcription machinery (Moss 1996). The multi-step process is regulated at a transcriptional level, with three classes of genes (early, intermediate, late), by transcription factors produced in each stage to promote gene expression onto the next level (Moss 1996; Baldick and Moss 1993; Broyles 2003; McFadden 2005; Moss 2013). Initially, the virion enters the host cell: in the case of IMVs, via plasma membrane fusion or actin-dependent micropinocytosis, and in the case of EVs, via disruption of the outer membrane and followed by fusion of the inner membrane with the cell's plasma membrane (Law et al. 2006; Moss 2016). Cell entry of IMV is initiated by phosphatidylserine in the viral membrane (Mercer and Helenius 2008) and, for the entry of EVs, the F13 protein promotes the rapid entry into the cell (Bryk et al. 2018). The fusion of both infectious forms depends on the entry-fusion complex (EFC), which consists of twelve viral proteins of the IMV membrane (Moss 2012), leading to the release of the viral core, early transcription factors and DNA-dependent RNA-polymerase into the cytoplasm of the host cell (Moss and Earl 2001). Early gene expression starts immediately with those products leading to the uncoating and release of the viral genome and DNA replication (McFadden 2005). DNA replication takes place in the so-called viral factories, specific cytoplasmic sites surrounded by rough endoplasmic reticulum (Yuen and Moss 1987; Katsafanas and Moss 2007). Intermediate and late gene expression only occurs from replicated genome leading to the production of proteins necessary for DNA packaging, as well as virion morphogenesis and assembly; this includes structural proteins as well as early transcription factors to be packaged into newly assembled virions (Broyles 2003; Moss and Earl 2001). Most of the newly assembled IMVs are released from the cell approximately 72 hours after infection by cell lysis (Moss 2013). However, some of these IMVs are being wrapped with two additional membranes derived from the trans Golgi or endosomal membranes (Hiller and Weber 1985; Tooze et al. 1993; Schmelz et al. 1994). Subsequently,

microtubules transport them via microtubules to the cell surface and leave the cell by exocytosis (Ward and Moss 2001; Smith et al. 2002; Ward 2005; Blasco and Moss 1992). Some EVs are released from the cell surface and are responsible for wide-range transmission within the host (Payne 1980; Vanderplasschen et al. 1998) while the majority promotes efficient cell-to-cell spread via an actin tail (Cudmore et al. 1995; Leite and Way 2015).

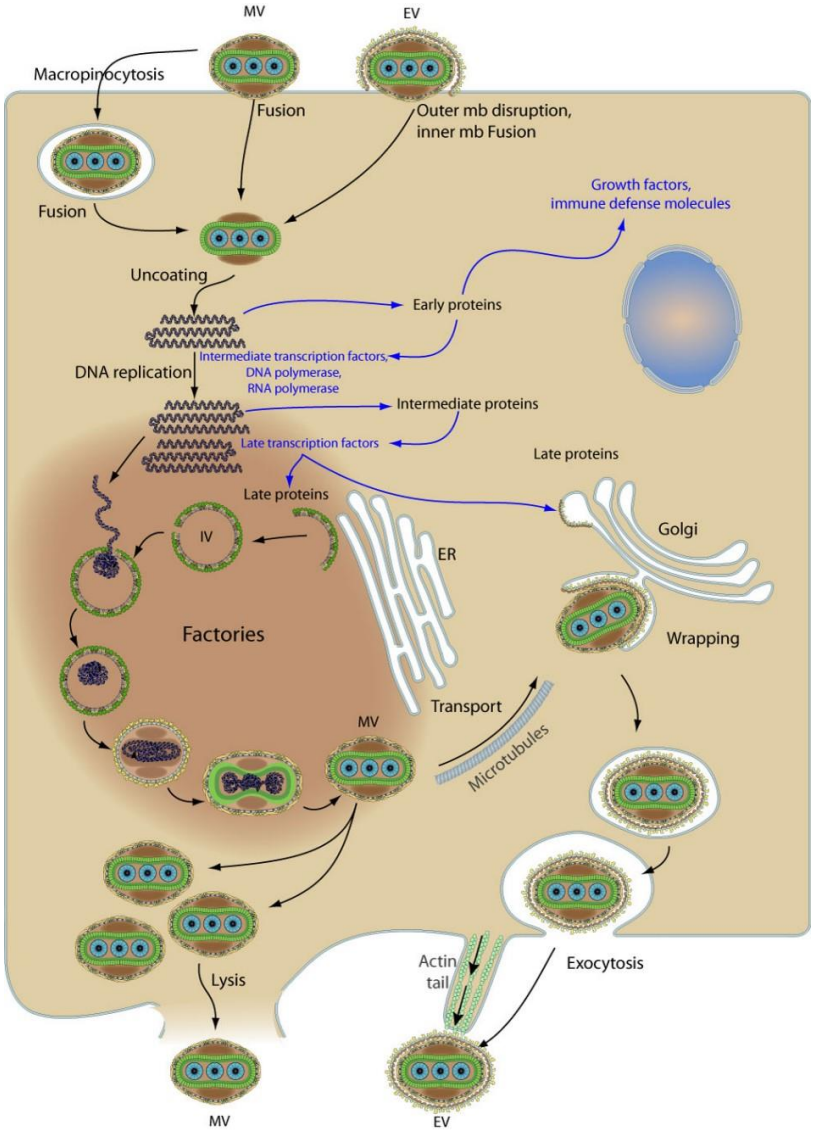


Figure 3. **Poxvirus replication cycle** (obtained from *ViralZone; https://viralzone.expasy.org/4399, SIB Swiss Institute of Bioinformatics*)

3.2. Immunomodulation by poxviruses

The mammalian immune system is composed of two parts: the innate and the adaptive immunity. Upon viral infection, phagocytes, as part of the innate immune system, are activated by recognizing pathogen-associated molecular patterns (PAMPs) via their pattern recognition receptors (PRRs). This recognition leads to the expression of different cytokines and interferons (IFNs) to restrict viral replication, and to the induction of adaptive immunity. However, viruses have developed different immunomodulatory strategies to avoid detection and destruction by the immune system. Poxviruses, for example, dedicate almost half of their genomes to such immunomodulatory proteins, with most of them being expressed early during infection to counteract the innate immunity (Smith et al. 2013).

3.2.1. Interferon response upon viral infection

Upon viral infection, many different cytokines are produced, and type I Interferon (IFN-I) are in the first line of defense against viral infection. IFNs are secreted glycoproteins with potent antiviral effect (Honda et al. 2006). There are three classes of IFN (Pestka et al. 2004); type I IFN, first discovered over 60 years ago (Isaacs and Lindenmann 1957), includes IFN-α, -β, -κ, -δ and -ω, which all bind to the ubiquitously expressed type I IFN receptor. IFN-α/-β are those type I IFNs induced directly upon viral infection and can be produced by all nucleated cells (Stark et al. 1998)). IFN-γ, as the single member of type II IFN, is only secreted by activated immune cells. Type III IFNs (IFN-λ) act, like IFN-α/-β, as a direct response to viral infection (Kotenko et al. 2003). The main function of IFNs, next to inducing apoptosis of infected cells, is to activate the expression of a set of proteins with antiviral activity, the so-called IFN-stimulated genes (ISGs). These include, protein kinase R (PKR), 2`-5`-oligoadenylate synthase (OAS), and the Mx protein (de Veer et al. 2001; Samuel 2001; Williams 1999; Silverman 1994; Haller and Kochs 2002), within others. Apart from inducing an antiviral state, IFNs

also have an impact on systemic immunity, especially regarding DC maturation and NK cell activation (Le Bon and Tough 2002; Stetson and Medzhitov 2006).

Antiviral immune responses are initiated upon sensing of nucleic acids by PRRs in the cytosol or endosomes (Stetson and Medzhitov 2006; Pichlmair and Reis e Sousa 2007; Kawai and Akira 2010). Binding of PAMP to its PRR starts a signaling cascade, that includes the recruitment of specific adaptor molecules and the activation of different kinases, that ultimately leads to the activation of different transcription factors (IFN regulatory factor 3 (IRF3), IRF7, NF-κB and activator protein 1 (AP-1)). Those transcription factors translocate into the nucleus where they activate the promoter of genes encoding type I IFNs. Therefore, IFN-α/-β are secreted from the cell, binding to type I IFN receptors (IFNAR) in an auto- or paracrine manner, which triggers the Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway. The JAK/STAT signaling pathway culminates with the formation of a complex called IFN-stimulating gene factor 3 (ISGF-3) that binds to IFN-stimulated response elements (ISREs) in the promoter region of IFN-stimulated genes (ISGs) leading to the expression of hundreds of antiviral proteins (Haller et al. 2006; Stark et al. 1998; Villarino et al. 2017).

The importance of type I IFN as a defense mechanism against viral infections is demonstrated in different *in vivo* mouse models lacking IFN receptors or IFNs (Müller et al. 1994; Deonarain et al. 2000; van den Broek et al. 1995). Moreover, also humans with defects in the IFN signaling system are more susceptible to viral infections (Dupuis et al. 2003; Sancho-Shimizu et al. 2011).

3.2.2. Blocking of IFN induction and IFN signaling pathways by VACV

Poxviruses, including Vaccinia virus (VACV), inhibit IFN induction and block

IFN signaling pathways by multiple mechanisms at different levels (Smith et al. 2013). The first mechanism involves a specific arrangement of its genome for reducing production of PAMPs, such as dsRNA (Smith et al. 1998). In addition, VACV encodes for a variety of immunomodulatory genes to interfere with the IFN response. Such gene products can prevent that PAMPs or IFN reach their receptor, or block signaling pathways required for IFN induction. Because of the objective of this work, the focus here will be on VACV proteins that are known inhibitors of the IRF-3 signaling, as IRF-3 is a critical participant in the regulation of type I IFN gene induction (Sato et al. 2000; Hiscott 2007).

Under normal conditions, IRF-3 is constitutively expressed and resides in the cytoplasm in its inactive form. Upon sensing of viral infection by PRRs such as retinoic acid inducible gene I (RIG-I), melanoma differentiation antigen 5 (MDA5), DNA protein kinase (DNA-PK), or Toll like receptor 3 or 4 (TLR3, TLR4), IRF-3 is phosphorylated by the kinases TANK-binding kinase 1 (TBK1) and IKKε, undergoes dimerization, and translocates into the nucleus. Here it binds to its binding sites within promoters of IRF-3-dependent genes (mainly IFN-β) (Sharma et al. 2003; Fitzgerald et al. 2003; Lin et al. 1998; Yoneyama et al. 1998). To block this signaling, VACV codifies for different proteins with capacities to interfere the pathway at different levels (see also **Figure 4**):

- Accelerated mRNA turnover is mediated by the highly conserved proteins D9 and D10, promoting the removal of the 5'-end m7GpppN cap (Parrish and Moss 2006, 2007; Parrish et al. 2007). This contributes to an increased host mRNA degradation and the prevention of viral mRNA accumulation and, consequently, the activation of dsRNA-responsive host innate immune sensing pathways, including PKR and OAS (Liu et al. 2014; Liu et al. 2015a; Burgess and Mohr 2015).

- E3 is a multifunctional protein, consisting of a C-terminal RNA-binding domain and a N-terminal Z-DNA-binding domain. The sequestration of dsRNA via the C-terminal domain prevents activation of protein kinase R (PKR) and 2'-5'-oligoadenylate synthetase (OAS), the dsRNA-depending PRRs (Chang et al. 1992; Brandt and Jacobs 2001; Kim et al. 2003). By preventing PKR activation, E3 interferes with three different signaling pathways: mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and IRF3 (Langland and Jacobs 2002; Myskiw et al. 2009). Furthermore, E3 blocks sensing of RNA derived from AT-rich dsDNA after transcription by RNA polymerase III (Marq et al. 2009; Valentine and Smith 2010) and prevents virus-induced necroptosis in IFN-treated cells (Koehler et al. 2017). The importance of the immunomodulatory function of E3 is apparent as its deletion blocks viral replication in most mammalian cell lines (Beattie et al. 1996).
- Another viral protein preventing binding of PAMPs by PRRs is C10 (also named C16 in the Western Reserve strain of VACV). By binding to the Ku subunits of DNA-PK, the C-terminal part of C10 disrupts recognition of dsDNA by DNA-PK and thereby inhibits IRF3 activation (Ferguson et al. 2012; Peters et al. 2013).
- The protein A46 is able to bind to different adaptor molecules downstream of Toll-like receptor signaling, including TIR-domain-containing adaptor-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), myeloid differentiation primary response gene 88 (Myd88) and Myd88 adaptor-like (MAL), which are associated with the cytoplasmic parts of TLRs (Bowie et al. 2000; Stack et al. 2005). The blocking of TRIF and TRAM, both adaptor molecules required for the signaling of TLR3 and TLR4, leads to inhibition of the IRF3 signaling pathway (Lysakova-Devine

et al. 2010; Stack and Bowie 2012). In addition, the binding of these adaptor molecules blocks activation of MAPK and NF-κB as well.

- The VACV protein C6 prevents activation of IRF3 by its interaction with the scaffold adaptor proteins of TBK1 and IKKε, the two kinases phosphorylating IRF3 (Unterholzner et al. 2011). In addition, C6 can inhibit the JAK/STAT signaling pathway in the nucleus by binding to STAT2 (Stuart et al. 2016).
- Protein K7 blocks IRF-3 signaling by binding to DEAD-box RNA helicase 3 (DDX3), an adaptor of TBK1 and IKKε (Schröder et al. 2008). In addition, K7 also binds to IL1-receptor-associated kinase 2 (IRAK2) and TNF-receptor-associated factor 6 (TRAF6), both important ligands in the NF-κB pathway (Schröder et al. 2008; Oda et al. 2009).
- Another protein interfering with IRF-3 signaling is N2, which inhibits the signaling pathway downstream of IRF-3 phosphorylation and nuclear translocation by yet unknown mechanism (Ferguson et al. 2013).
- Recently, a new inhibitor of the IRF-3 signaling pathway, encoded by the B2R gene, was identified by Eaglesham et al. and named poxvirus immune nuclease (poxin) (Eaglesham et al. 2019). Poxin neutralizes the effect of cGAMP, which is generated upon the sensing of dsDNA by the cyclic GMP-AMP synthase (cGAS) and acts as a second messenger and activator of the stimulator of interferon genes (STING). STING, once activated, is a scaffolder to TBK-1, inducing IRF-3 activation (Sun et al. 2013; Ablasser et al. 2013; Liu et al. 2015b; Ishikawa et al. 2009).

As can be observed, many immune evasion proteins encoded by VACV have multiple immunomodulatory functions on the IRF3 pathway (Smith et al. 2013). Furthermore, virus mutants lacking one or more of such genes are attenuated *in vivo*, demonstrating that, while some of those proteins may not

be essential, their functions are non-redundant (Benfield et al. 2013; Stack et al. 2005; Ferguson et al. 2013; Fahy et al. 2008).

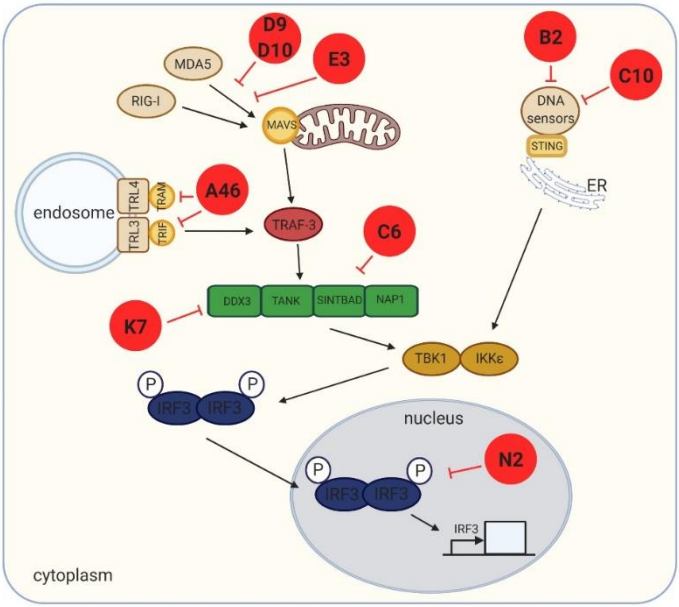


Figure 4. **IRF3 signaling inhibition by VACV** (obtained from *Smith et al. 2013*, modified with BioRender.com)

3.3. Poxviruses as viral vectors

As mentioned before, due to the global vaccination program with VACV, smallpox was successfully eradicated in 1980, which makes it the first infectious disease that has been eradicated (Fenner et al. 1988). After that, the study of VACV for its use as expression and viral-vector based vaccine was continued (Mackett et al. 1982; Panicali and Paoletti 1982; Moss 1996). In terms of vaccine development, the use of VACV demonstrates multiple advantages, such as a potent inflammatory immune response that makes the additional use of adjuvants redundant (Akira et al. 2006; Ura et al. 2014).

In addition, poxviruses, due to their own large genome, are able to incorporate large inserts of foreign DNA, even multiple genes at once (Smith and Moss 1983; Perkus et al. 1985). Furthermore, poxviruses have a good safety profile as the viral life cycle occurs completely in the cytoplasm of cells without any integration into the host genome (Roberts and Smith 2008). Because of side effects, especially in immunocompromised people, highly attenuated viruses, such as Modified Vaccinia virus Ankara (MVA), are of particular interest for vaccine development (Lane et al. 1969). MVA was generated after more than 500 passages of the chorioallantois VACV Ankara strain (CVA) in chicken embryo fibroblasts. Consequently, the virus lost large portions of its genome resulting in drastically impaired replication capacity, but in highly increased immunogenicity (Mayr and Munz 1964; Meyer et al. 1991; Antoine et al. 1998; Sutter and Moss 1992; Moss et al. 1996; Drexler et al. 1998). Due to mutations and deletions in many immunomodulatory genes, MVA is able to efficiently induce type I IFN secretion after infection (Dai et al. 2014; Waibler et al. 2007). Nowadays, MVA is extensively studied as an expression vector and many recombinant MVAs have been developed as vaccine candidates against different infectious diseases or even therapeutic cancer vaccines (Volz and Sutter 2017; Altenburg et al. 2014; Veit et al. 2018; Acres and Bonnefoy 2008).

3.4. Oncolytic Vaccinia viruses

In addition to its use as a viral vector for vaccine development, VACV is a promising candidate as an oncolytic agent. For the generation of oncolytic VACVs, different strains have been investigated, including Lister, Copenhagen, Wyeth, and specially, Western Reserve, which was derived from the Wyeth strain after several passages in mice (Zhang et al. 2007; Foloppe et al. 2008; Kim et al. 2006; Thorne et al. 2007; Kirn and Thorne 2009).

The most clinically-advanced oncolytic VACV is JX-594 (Pexa-Vec), which

is based on the Wyeth strain and it is modified by inactivating the thymidine kinase gene for achieving cancer cell selectivity, and it is armed with the transgene GM-CSF to enhance activation of antitumor immunity (Kim et al. 2006). To date, Pexa-Vec has been tested in a variety of different tumors by intratumoral as well as intravenous administration, with highly promising results (Cripe et al. 2015; Park et al. 2015). It was able to reach distant metastasis when systemically administrated (Park et al. 2008) and, when administrated intralesional in melanoma-patients, regression of non-injected regional dermal metastases could be observed in 4 of 7 patients (Mastrangelo et al. 1999). More importantly, it was demonstrated that there is a correlation between administrated viral dose and prolonged survival (Heo et al. 2013).

In order to achieve tumor cell selectivity in poxviruses, the deletion of viral genes such as the thymidine kinase gene (TK) is one of the most common strategies. By deleting TK, the virus is no longer able to produce high pools of nucleotides needed for viral replication (Buller et al. 1985). Yet, in most tumors, cellular TK expression is constitutively upregulated and allows VACV replication, whereas in normal cells TK is only expressed during the S phase in proliferating cells (Hengstschläger et al. 1994). The additional deletion of vaccinia growth factor (VGF), which induces proliferation in infected and surrounding non-infected cells by binding to the epidermal growth factor receptor (EGFR), demonstrated an increased tumor selectivity compared to the single deletion of TK (Buller et al. 1988; McCart et al. 2001; Thorne et al. 2007). Yet, replication-capacity is impaired in large panel of cancer cells when the two deletions are combined.

As already mentioned, oncolytic viruses have different mechanisms of action to destroy cancer cells. In addition to the direct destruction of infected cancer cells due to viral replication, cell lysis leads to the release of different DAMPs and PAMPs as well as tumor and viral antigens (Rubartelli and Lotze 2007;

Bartlett et al. 2013; Chiocca and Rabkin 2014). This release of immune stimulatory-molecules can overcome the excessive immunosuppression that exists within the tumor and induce an in-situ vaccination effect for the released tumor antigens (Thorne 2011; Thorne et al. 2010). In order to improve this immune activation, the expression of transgenes to further enhance this antitumor effect has been explored. Different therapeutic transgenes have been tested, including cytokines, costimulatory molecules, or anti-vascular agents (Hermiston and Kuhn 2002; Kim et al. 2006; Kirn et al. 2007; Rojas et al. 2016). However, the main hinderance of excessive immune activation is the fine balance between activation of the immune system to destroy the tumor and premature clearance of the OV, which can result in reduction of the oncolytic efficacy.

In addition, VACV displays another mechanism of action, thanks to their capacity to replicate in tumor-associated endothelial cells (Kirn et al. 2007). The destruction of these cells leads to a vascular collapse within the tumor, a disruption of the tumor blood flow, and, finally, to tumor necrosis (Breitbach et al. 2007). This viral-mediated destruction of vessels is restricted to the tumor due to a high pool of Vascular Endothelial cell Growth Factor (VEGF) within tumors, which activates endothelial cells and allows VACV replication (Arulanandam et al. 2015).

All the poxvirus characteristics, combined with its different mechanisms of action against tumor cells, make VACV an attractive oncolytic vector. Advancements in the understanding of poxvirus biology, gene functions, and immunogenicity enables the improved logical design of genetically engineered oncolytic poxviruses and their use for cancer treatments.

III OBJECTIVES

Recently, oncolytic vaccinia viruses (VACV) have demonstrated their potential to provide for clinically effective cancer treatments. The reason for this clinical success is not only the direct destruction of infected cancer cells, but also the activation of immune responses directed against tumor antigens. For eliciting a robust antitumor immunity, a dominant Th1 cell differentiation of the response is preferred, and such polarization can be achieved by activating the Toll-like receptor 3 (TLR3)-interferon regulatory 3 (IRF3) signaling pathway and, thus, activation of such pathway is suboptimal. However, current VACV used to date as oncolytic viruses still encode several immune evasion proteins involved in the inhibition of this signaling pathway. By inactivating genes of selected regulatory virus proteins, we aimed for a candidate virus with increased potency to activate cellular antitumor immunities but at the same time presents a fully maintained replicative capacity in cancer cells.

For achieving this general objective, we stablished the following objectives:

i. Generation of oncolytic VACV with deletions in immunomodulatory genes inhibiting IRF3 activation

ii. Characterization of *in vitro* features and functional analysis of deleted VACV viruses

iii. Evaluation of *in vivo* replication and anti-tumor immune response, and anti-tumor activity of deleted VACV viruses in syngeneic mouse tumor models

IV MATERIAL and METHODS

1. Cells

1.1. Cultivation of permanent cell lines

All cell lines used in this work (MA104, HeLa, Renca, B16 and THP-1 cells) were obtained from the American Type Culture Collection (ATCC) and maintained in recommended culture media containing 5-10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) at 37°C, 5% CO_2. **Table 1** and **Table 2** detail the media used for culturing each cell line. Cell cultures were split 2 times per week when about 90% confluent, for which they were detached with Trypsin-EDTA.

Table 1. **Media/additives/cell culture**

Media/additives/cell culture	**Supplier**
Fetal bovine serum (FBS)	SIGMA-ALDRICH, St. Louis, USA
Penicillin-Streptomycin (P/S)	SIGMA-ALDRICH, St. Louis, USA
Dulbecco's Modified Eagle's Medium (DMEM)	SIGMA-ALDRICH, St. Louis, USA
RPMI-1640	SIGMA-ALDRICH, St. Louis, USA
RPMI 1640	Anprotec, Bruckberg, Deutschland
L-Glutamine	Lonza, Verviers, Belgium
Sodium Pyruvat	SIGMA-ALDRICH, St. Louis, USA
MEM Non-Essential-Amino Acid Solution	SIGMA-ALDRICH, St. Louis, USA

Trypsin-EDTA	SIGMA-ALDRICH, St. Louis, USA
Antibiotic Antimycotic Solution	SIGMA-ALDRICH, St. Louis, USA

Table 2. Cell lines

Cell lines	**Culture medium**
HeLa (human cervical carcinoma) MA104 (African green monkey kidney)	Dulbecco's Modified Eagle's Medium (DMEM) + 5% FBS + 1% P/S
Renca (mouse renal adenocarcinoma)	RPMI 1640 + 10% FBS + 1% P/S
B16 (mouse melanoma)	Dulbecco's Modified Eagle's Medium (DMEM) + 10% FBS + 1% P/S + 1% L-Glutamine + 1% Sodium Pyruvat + 1% MEM non-essential Amino Acid Solution
THP-1 (human leukemic monocyte)	RPMI 1640 (anprotec) + 10% FBS + 1% P/S

1.2. Cell count

Cells were trypsinized, diluted (1:2 or 1:4) and stained with Trypan blue (SIGMA-ALDRICH) solution for live/death staining before counting using a Neubauer Chamber.

IV MATERIAL and METHODS

2. Viruses

2.1. Construction of recombinant viruses

All recombinant replication-efficient viruses constructed or used in this research or constructed are based on the Vaccinia virus (VACV) strain Western Reserve (WR). To enhance selective replication in cancer cells, VACV WR/TK- was constructed by inactivation of the viral thymidine kinase gene (TK) though insertion of an expression cassette for the mCherry reporter gene under transcriptional control of the VACV late promoter P11. VACV WR/TK- was constructed prior to this study by Dr. Juan J Rojas and served as backbone for deleting VACV genes in this study.

The C6L, C10L and N2L genes were inactivated by homologous recombination replacing the original gene sequence with a synthetic construct containing two 350 base pair DNA sequences upstream and downstream of the genomic site targeted for insertion. In addition, the start codon in the synthetic gene sequence was mutated. For generation of recombinant oncolytic VACVs, $1x10^6$ MA104 cells were seeded in 6-well plates and infected with the parental backbone virus at a MOI of 1 and, 3 hours later, cells were transfected using Lipofectamine 2000 (Fisher Scientific) with recombinant pUC18-GFP plasmid containing the respective synthetic gene construct. Such plasmid contains a green fluorescent protein (GFP) for facilitating isolation of recombinant viruses. At 48 hours after infection, cell cultures were harvested, and the GFP marker was used for isolation of clones (**Figure 5**: transitory recombinant viruses express now red (mCherry) and green (GFP) fluorescence). Once recombinant clones of this transitory form were isolated, viruses incorporating a second homologous recombination between the synthetic gene and the original gene are selected. In such recombination, we have a 50% of chances of successfully replace the original gene with our deleted version (see **Figure 5**: now recombinant viruses express only mCherry). PCR analysis using

oligonucleotide primers flanking deletion sites are necessary to confirm the correct modification in the isolated clones. PCR analyses were performed with oligonucleotide primers flanking the deletion sites and sequencing were further necessary to confirm the correct genetic modification of plaques with red fluorescence. **Figure 5** depicts schematic diagram of the construction of the deletion viruses, presented on the example of the deletion of the C6L gene from WR/TK-.

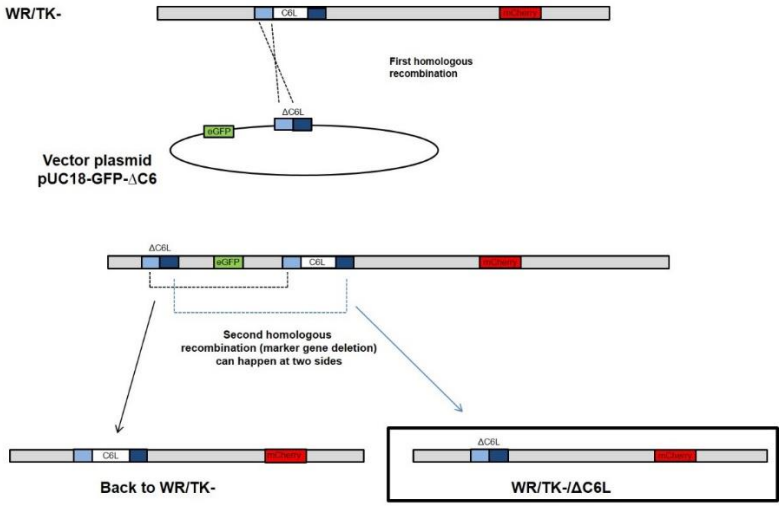


Figure 5. **Scheme of the construction of the deletion viruses**

The non-replicative strain MVA was generated prior to this study by Prof. Dr. Gerd Sutter and Astrid Freudenstein and served as positive control for the Western Blot analysis and mRNA-expression analysis. This recombinant MVA contains a mCherry-expression cassette under transcriptional control of the P11 promoter inserted into deletion III. It was based on the MVA clonal isolate F6 (Sutter 1990 LMU thesis).

2.2. Virus amplification, purification and titration

All deletion viruses were propagated in HeLa cells for purification. 60 flasks of 150 cm^2 containing HeLa cells monolayer were infected at a MOI of 1 and incubated for 2-3 days at 37° C until extensive cytopathic effects could be observed. Then, cells were mechanically de-attached, transferred into 50 ml falcons and centrifuged at 1250 rpm for 5 minutes at 4°C. The supernatant was discarded and cells were resuspended with 45 ml 10mM Tris-HCl pH 9, before being frozen to -80°C.

After three freeze-thaw cycles, cell-suspensions were homogenized using a douncer homogenizer and submitted to three cycles of sonication. Samples were centrifuged at 1250 rpm for 5 minutes at 4°C and the virus-containing supernatant was carefully collected. In 6 ultracentrifuge tubes, 18 ml of a 36% sucrose solution (SIGMA-ALDRICH, in 10 mM Tris-HCl pH 9) were carefully overlaid with 7,5 ml of virus-sample, before they were ultracentrifuged at 15 000 rpm for 1 hour and 20 minutes at 4°C. Afterwards, the supernatant was discarded and the virus pellets resuspended in 20 ml of a 10 mM Tris-HCl pH 9. For digestion of contaminating DNA, 3µl of Benzonase (VWR) were added to the samples and incubated 2 hours at room temperature. Consecutively, the virus suspension was filtered (Spectrum Labs™ MICROKROS HOLLOW FIBER FILTER MODULE 1XFL PS 0.05, Fisher Scientific) to discard endotoxins, concentrating the volume to 2ml. After readjusting the volume to 15 ml with fresh 10 mM Tris-HCl pH 9, the sample was submitted to another round of ultracentrifugation as described above, dividing the volume within 2 ultracentrifuge tubes. Again, the supernatant was discarded and the remaining virus pellets were finally resuspended in 1.2 ml 10 mM Tris-HCl pH 9, aliquoted and stored at -80°C. Every time before usage, virus stocks were thawed on ice and submitted to three cycles of sonication.

To determine an accurate viral titer, viruses were tittered three times in

parallel in confluent 6-well plates of MA104 cells. In duplicates, the wells were infected with prepared virus dilutions (10^{-6}, 10^{-7}, 10^{-8}) and overlaid with a 1:1 mixture of 3% CMC (Carboxymethylcellulose sodium salt, low viscosity, SIGMA-ALDRICH) and cell culture media, cultured for three days at 37°C, and dyed with crystal violet. Viral titer was calculated in plaque forming units per milliliter (PFU/ml) by counting the plaques formed in each dilution.

3. Virus growth assay and plaque size

$2x10^5$ cells were seeded in 24-well plates and infected with a multiplicity of infection (MOI) of 5 or 0.05. One hour after infection, cells were washed with PBS and new pre-warmed media was added. At different time points (0, 4, 12, 24, 48 and 72 hours after infection), samples were harvested and frozen at -80°C. Viral titer was determined by plaque assay after three freeze-thaw cycles.

To assess the size of the plaques formed by different viruses, MA104 cells were infected at a MOI of 0.05 and, 72 hours post infection, the diameter of plaques was measured after crystal violet dying (SIGMA-ALDRICH).

4. *In vitro* cytotoxicity assay

Cytotoxicity assays were performed by seeding $5x10^4$ cells in 96-well plates. Cells were infected with 1/5-serial dilution starting at a MOI of 150 (ranging from 150 to 0.0001) and incubated at 37°C for 72 hours. After three days, cells were checked for remaining metabolic activity using a non-radioactive cell proliferation assay (CellTiter96® Aqueous Non-radioactive cell proliferation assay, Promega) following the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm (Sunrise™, Tecan Trading AG) and metabolic activity was quantified.

IV MATERIAL and METHODS

5. Polymerase chain reaction (PCR)

To obtain viral DNA samples for PCR, one well of a confluent 6-well plate of HeLa cells was infected at an MOI of 1 and incubated for 3 days. 200µl of infected culture were used for DNA extraction using a QIAmp DNA Mini Kit (Qiagen). DNA concentration was determined using the NanoDrop® (PEQLAB Biotechnology GmbH).

Polymerase chain reaction (PCR) was performed with 50-150 ng of DNA (10µl) per sample and 40µl of a PCR Master Mix, consisting of 1 µl of 10 µM forward Primer, 1 µl of 10 µM reverse Primer, 25 µl of OneTaq® Mix (New England Biolabs) and 13 µl of distilled water, using a peqSTAR 2x thermocycler (PEQLAB Biotechnology GmbH). Primers used for the different PCRs performed in this work are summarized in **Table 3**, and conditions of the different PCRs are summarized in **Table 4**.

Table 3. **Specific oligonucleotide primers**

Primer	Sequence	Size
C10-F	5' – AGT AAA ATC TAG TTA CCT TG – 3'	1311 bp (ΔC10 = 670 bp)
C10-R	5' – TAT AAT TCT ATT ACA CCG GC – 3'	
C6-F	5' – ACT GTA AAT TTC TCA ACG CG – 3'	1083 bp (ΔC6 = 682 bp)
C6-R	5' – ATC TTA AAC ATG GTA TTA CG – 3'	
N2-F	5' – ATG TAC ATA CAT CGC CGT CA – 3'	1126 bp (ΔN2 = 693 bp)
N2-R	5' – GTA GAC TTT GTA GTT AAC GG – 3'	

IFN-β-F (human)	5’ – TGC TCT CCT GTT GTG CTT CTC C – 3’	459 bp
IFN-β-R (human)	5’ – CAG TGA CTG TAC TCC TTG GCC TTC – 3’	
GAPDH-F (human)	5’ – AGC CAC ATC GCT CAG AAC AC – 3’	606 bp
GAPDH-R (human)	5’ – GAG GCA TTG CTG ATG ATC TTG – 3’	
E3L-F (human)	5’ – GAT CTA TAT TGA CGA GCG TTC TG– 3’	201 bp
E3L-R (human)	5’ – GTT GTC ATA AAC CAA CGA GGA G– 3’	
IFN-β-F (mouse)	5’ – ATG GAA AGA TCA ACC TCA CCT AC – 3’	502 bp
IFN-β-R (mouse)	5’ – TAG ATT CAC TAC CAG TCC CAG AG – 3’	
GAPDH-F (mouse)	5’ – GAC AAC TCA CTC AAG ATT GTC AG – 3’	540 bp
GAPDH-R (mouse)	5’ – GTA GCC GTA TTC ATT GTC ATA CC – 3’	

Table 4. **Thermocycling conditions**

C10-/C6-/N2-PCR		
Step	**Temperature**	**Time**
Initial denaturation	94°C	30 seconds
30 cycles	94°C 55°C 68°C	30 seconds 30 seconds 1 minute 20 seconds
Final extension	68°C	5 minutes
Store	4°C	forever
IFN-β-mRNA-PCR		
Step	**Temperature**	**Time**
Initial denaturation	95°C	3 minutes
28 cycles	95°C 59°C 72°C	30 seconds 30 seconds 45 seconds
Final extension	72°C	5 minutes
Store	4°C	forever
GAPDH-/E3L-mRNA-PCR		
Step	**Temperature**	**Time**
Initial denaturation	95°C	3 minutes
24 cycles	95°C 59°C 72°C	30 seconds 45 seconds 45 seconds
Final extension	72°C	5 minutes
Store	4°C	forever

The size of the PCR products was analyzed by gel electrophoresis using 1% agarose gels with added GelRed (VWR) and 1X TAE buffer (Fisher Scientific) as running buffer. A 1kb DNA ladder (New England Biolabs) was used as a molecular weight marker. The nucleic acid bands were visualized with a ChemiDoc™MP Imaging system (Bio-Rad).

6. Protein analysis

Indicated cells were seeded in 24-well plates and infected with a MOI of 10. 5 hours after infection, cells were harvested using Trypsin-EDTA, resuspended with suitable medium and centrifuged at 1250 rpm for 10 minutes at room temperature. Cells were washed with PBS and lysed using RIPA buffer (Abcam) supplemented with 1% Protease-Phosphatase-Inhibitor Cocktail (Half™ Protease & Phosphatase Inhibitor Cocktail, Fisher Scientific). After an incubation time of 30 minutes at 4°C, supernatant and cell debris was separated by centrifugation for 10-15 minutes at 4°C and 13 000 rpm. The supernatant was collected and frozen at -20°C.

For protein quantification, a BCA assay kit (Pierce™ BCA Protein Assay Kit, Fisher Scientific) was used according to the manufacturer's instruction before absorbance was measured at 560 nm and protein amounts calculated.

To load equal amount of proteins into the gel, samples were diluted with lysis buffer and 4X loading buffer (9 parts Laemli buffer (Bio-Rad), 1 part β-Mercaptoethanol (Carl-Roth GmbH). The samples were then boiled at 95°C for 5 minutes before being loaded onto a 10% SDS-gel (Mini-PROTEAN® TGX™ Gels, Bio-Rad). The Precision Plus Protein™ Dual Color Standard (Bio-Rad) was used as a molecular weight marker. The protein electrophoresis was performed in 1X Tris/Glycin/SDS running buffer (Bio-Rad) at 80-130 V for about 90 minutes (Mupid®-One, Mupid). Proteins were

then transferred to a nitrocellulose blotting membrane (Amersham™ Protran™ Premium 0.2µm NC, GE Healthcare Life Science) with 1x transfer buffer using the Trans Blot Turbo system (Bio-Rad). The membrane was blocked with 1X Tris buffered saline with 0.1% Tween®20 (Promega) (TBS/Tween) and 5% BSA (Bovine serum albumin, SIGMA-ALDRICH) for 1 hour at 4°C. Afterwards, the blot was incubated overnight at 4°C with a rabbit anti-P-IRF3 primary antibody diluted 1:1000 in TBS/Tween 1% BSA. The following day, the blot was washed 4 times 10 minutes with TBS/Tween before incubation with a secondary anti-rabbit IgG HRP-linked antibody, 1:5000 diluted in TBS/Tween-1% BSA, for 1 hour at room temperature. Again, the blot was washed 4 times for 10 minutes with TBS/Tween before ECL Plus kit (Pierce™ ECL Plus Western Blotting Substrate, Fisher Scientific) was used following manufacturer's instructions and bands were analyzed using ChemiDoc™ MP Imaging System (Bio Rad). See **Table 5** for used antibodies.

Table 5. **Antibodies used for Western Blot analysis**

Antibody	**Dilution**	**Company**
P-IRF-3 (S396) rabbit mAB	1:1000	Cell Signaling, Danvers, USA
rabbit mAB GAPDH-antibody	1:1000	Cell Signaling, Danvers, USA
anti-rabbit IgG HRP-linked antibody	1:5000	Cell Signaling, Danvers, USA

For the Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) loading control, the blot was stripped using Restore™ Western Blot Stripping Buffer (Fisher Scientific) for 45 minutes at 37°C and washed 3 times, 10 minutes with TBS/Tween. Again, the blot was blocked with TBS/Tween-5% BSA for 1 hour at 4°C before overnight incubation with rabbit anti-GAPDH antibody,

1:100 diluted in TBS/T-5% BSA. After further washing, incubation with the secondary anti-rabbit IgG HRP-linked Antibody (1:5000) in TBS/Tween-1% BSA was performed and ECL Plus kit was used as described above.

7. mRNA expression analysis

$1x10^6$ cells were seeded in 24-well plates and infected at a MOI of 5. At 6 hours post-infection, cells were harvested using Trypsin-EDTA and centrifuged at 1300 rpm for 5 minutes at 4°C. Total RNA was purified, using an RNeasy-Plus Kit (Qiagen) following manufacturer's instructions. cDNA was synthesized from the template RNA using a Omniscript RT Kit (Qiagen), following the manufacturer's instructions and PCR was performed using specific primers for the mRNA of interest (see **Table 3**).

8. *In vivo* experiments

8.1. Mouse models

All animal experiments were handled in compliance with the German regulations for animal experimentation (Animal Welfare Act, approved by the Government of Upper Bavaria, Munich, Germany). 6-8 weeks old female BALB/c (Renca tumor model) or C57Bl/6 (B16 tumor model) mice were purchased from Charles River Laboratories and housed in an isolated (ISO) cage unit with free access to food and water.

8.2. Tumor implantation and virus administration

Tumor cells for implantation were maintained *in vitro* at standard conditions. At the day of implantation, cells were trypsinized, centrifuged at room temperature for 7 minutes at 1200 rpm, washed with PBS, counted, and resuspended in an appropriate volume of PBS for implanting $5x10^5$ cells in

100µl. The tumor cells were subcutaneously injected into the flank of mice. Prior to injection, mice were anesthetized using 3% isoflurane (cp-pharma) and the fur on the flank was clipped for easier observation of the tumor growth.

When the tumors reached 50-100 mm^3 in size, mice were randomized and viruses were administrated intratumorally at a dose of $1x10^7$ PFU in a volume of 10 µl.

Tumor volume was defined by the following equation:

$$V(mm^3) = \frac{\pi}{6} \, x \, L \, x \, W^2$$

W stands for width and L for the length of the tumor. These parameters were determined by caliper measurements.

After virus injection, weight of mice was checked daily and the mice were scored daily following the scoresheet described in **Table 6.**

Table 6. Scoresheet for *in vivo* mouse experiments

Scoresheet		
Parameter		**Score**
Subcutaneous tumor volume	1500-2000mm³	5
	≥ 2000mm³	8
Apparence of the tumor	No findings	0
	Incipient necrosis	4
	Necrosis, >1/2 of the tumor surface	8
	Reddened, uneven surface	2
	Reddened, heavily sunken surface	4
	Ulcerated/bloody	8
Invasive tumor growth	Tumor immovable	8
Body weight	Consistent	0
	5% reduction	2
	10% reduction	4
	20% reduction	8
Apparence of the mice	Normal	0
	Ruffled coat	2
	Reduced coat care	2
	Hunchback posture	2
	Severly reduced coat care (sticky eyes/nose, soiled with feces)	4
	Emaciated, pale, sunken flank	6
	Unphysiological, abnormal body posture	8
Activity	Normal	0
	Inactiv (reduced reaction)	2
	Notable defense reaction/aggressiveness if palpating tumor	8
	Prolonged self-isolation	8
	Apathy (complete inactivity, no reaction to external stimuli)	8
	Self mutilation	8
Movement	Normal	0
	Careful, no climbing, reluctance to move	2
	Unsteady gait (ataxia)	4
	Signs of paralysis	8
Breathing	Increased frequency	2
	Noticeable respiratory problems	6

Rating:	
Burden level A	score 0
Burden level B	score ≥2
Burden level C (low-moderate)	score ≥4
Burden level D (moderate)	score ≥6
Measures:	
Non	score 0
Specific observation	score 2
Immediate veterinary examination	score 4
Euthanasia if persistent burden (>6h)	score 6
Termination: immediate euthanasia	score 8

8.3. Study of viral replication *in vivo*

Tumors were established as described above. After randomization of mice (n = 4-6), they received at day 0 a single intratumoral dose of $1x10^7$ PFU. Mice were sacrificed 4 days after virus administration and tumors were harvested, washed with PBS, and fluorescence signal emitted from virally expressed mCherry acquired using a Geldoc imaging system (Bio-Rad) and quantified using ImageJ.

For determining the viral titer within the tumors, mice were treated as described above and sacrificed at day 4 after viral administration. Tumors were harvested, weighted, and 300µl of cold PBS supplemented with 20% Antibiotic Antimycotic Solution (SIGMA-ALDRICH) was added. Tumors were homogenized using metal beads and a tissue homogenizer (TissueLyser II, Quiagen) for 1 minute, before centrifugation at 2000 rpm for 2 minutes at 4°C. After centrifugation, the supernatant was collected and virus titer was determined by plaque assay as previously described, adding 1% Antibiotic Antimycotic Solution for avoiding contaminations.

8.4. *In vivo* antitumor activity

Tumors were established as described above. Mice were treated twice (day 0 and day 4) with an intratumoral dose of $1x10^7$ PFU of indicated virus. Mice were monitored daily, tumors were measured 3 times per week using a caliper, and tumor volume was calculated as described before. Mice were euthanized by cervical dislocation when tumors reached termination criteria.

8.5. IFN-γ ELISpot

Tumors were established as described above and mice were treated twice (day 0 and day 4) with an intratumoral dose of $1x10^7$ PFU of indicated virus. 5 days after the second virus injection, mice were sacrificed and the spleens harvested. After passing through a 70 µm strainer (Falcon®, A Corning

Brand) and incubating with Red Blood Cell Lysis Buffer (SIGMA-ALDRICH), splenocytes were washed with RPMI-1640 supplemented with 10% FBS. $2x10^5$ cells were cultured for 48 hours at 37°C and 5% CO_2 in anti-IFN-γ (MABTECH) pre-coated 96-well plates together with 2µg/ml of peptides. The synthetic peptides used for restimulation were $B8R_{20-27}$, gp100 and B16-M30mut (see **Table 7**, peptides were dissolved in PBS to a concentration of 2mg/ml). Cells treated with phorbol myristate acetat (PMA) (SIGMA-ALDRICH) and ionomycin (SIGMA-ALDRICH) were used as a positive control. The ELISpot kit was used following the manufacturer's instructions. An automated ELISPOT reader software (A.EL.VIS Eli.Scan, A.EL.VIS ELISPOT Analysis Software) was used for counting and analyzing.

Table 7. **Synthetic peptides used for IFN-γ-ELISpot analysis**

Synthetic peptide	**Amino acid sequence**
$B8R_{20-27}$	TSYKFESV
gp100	EGSRNQDWL
B16-M30mut	PSKPSFQEFVDWENVSPELNSTD

9. Statistical analysis

Standard student's t test (two-tailed) was used for analyzing results in Figure 9 and 10. A one-way ANOVA and Tukey's Multiple Comparison test was used for analyzing Figure 13 and 15. In Figure 14, a Two-way ANOVA and Bonferroni posttest was chosen for analyzing tumor growth curves and a log rank test for survival curves. In all cases, significance was achieved if $p<0.05$.

V RESULTS

1. Generation of oncolytic VACV with deletions in key genes blocking activation of IRF3 pathway

To improve cellular immune responses, we modified the candidate oncolytic VACV WR/TK- (Rojas et al. 2016) (Western Reserve strain of VACV with a deleted thymidine kinase gene) by inactivating a set of viral genes involved in interfering with the IRF3 signaling pathway. Three genes were selected and sequentially deleted (**Figure 6**): C6L, N2L and C10L. C6 interacts with the scaffold proteins NAP1, TANK, and SINTBAD (Unterholzner et al. 2011; Smith 2018); N2 inhibits nuclear IRF3 (Ferguson et al. 2013); and C10 (named C16 in the WR strain) inhibits DNA-PK-mediated DNA sensing (Peters et al. 2013; Scutts et al. 2018). **Figure 6** schematically depicts deletions present in the genomes of the viruses tested in this study (WR/TK-/Δ, WR/TK-/2Δ, and WR/TK-/3Δ).

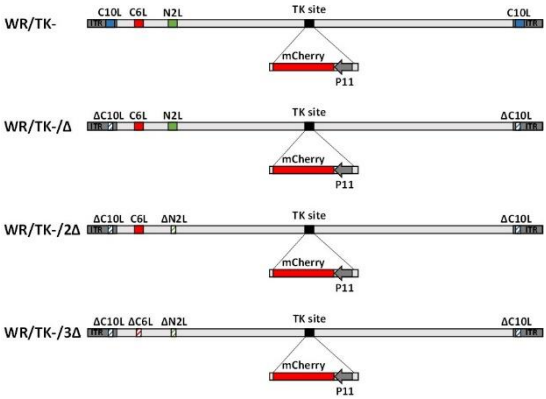


Figure 6. **Generation of oncolytic VACV with accumulated deletions in key genes blocking activation of IRF3 signaling pathway.**

Schematic diagram of VACV genomes indicating the positions of the viral genes targeted by sequential deletion. For the prospect of monitoring viral replication, an expression cassette encoding the red fluorescent marker protein mCherry was inserted into the Thymidine Kinase (J2R) site of the virus genomes.

Correct genetic modifications of the viral genomes were confirmed by PCR analysis with oligonucleotide primers flanking the deletion sites (**Figure 7**) and by sequencing.

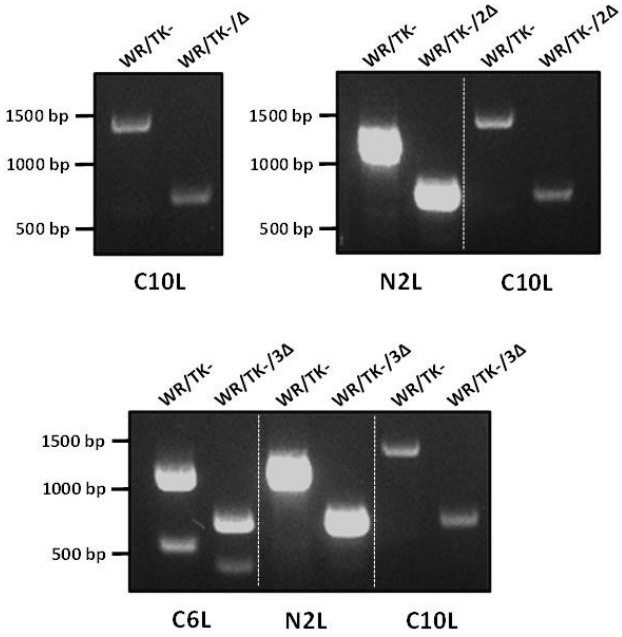


Figure 7. **PCR-analysis to confirm deletions in target genes**.

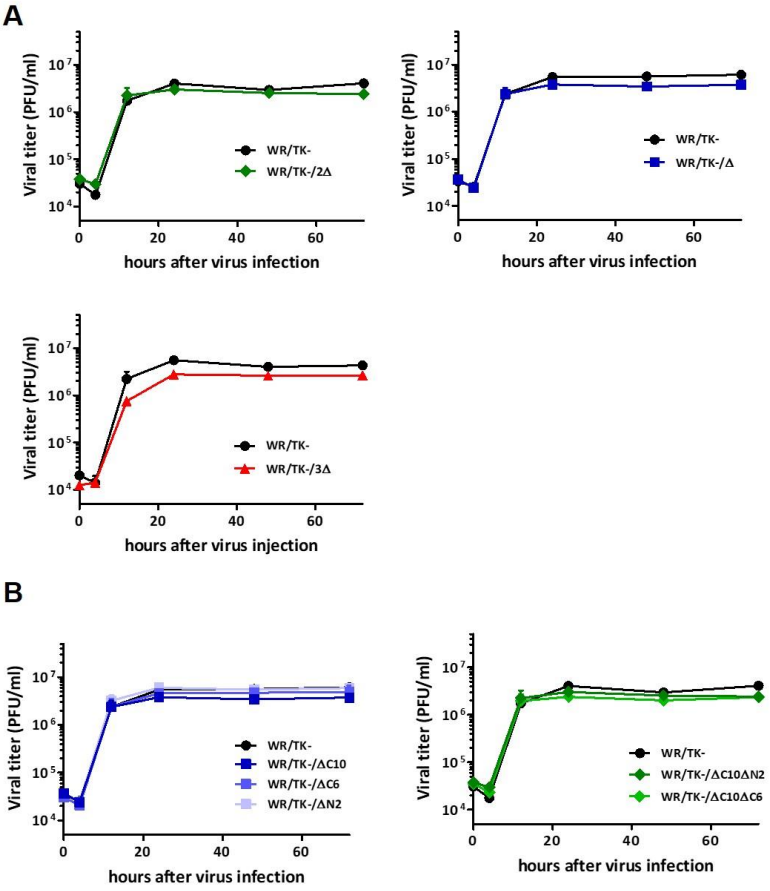
Expected size of the PCR products are: C10L = 1311 bp, ΔC10L = 670 bp; N2L = 1126 bp, ΔN2L = 693bp; C6L = 1083 bp, ΔC6L = 682 bp

V RESULTS

2. Deletion of genes blocking the IRF3 pathway mainly do not interfere with oncolytic VACV *in vitro* features

2.1. Growth analysis of oncolytic deletion VACV

Maintaining an efficient replication of the vector virus in cancer cells is important for achieving an effective oncolytic activity. Therefore, we evaluated whether the deletions or the combination of deletions in the viral genomes have an influence on VACV replication in cancer cells. For one-step-growth or multiple-step-growth analysis, we infected HeLa cells with candidate viruses at multiplicities of infection (MOI) of 5 or 0.05 and, at indicated time points, cultures were harvested to determine viral titers by plaque assay. Both under one-step-growth (**Figure 8. Mainly unimpaired replication of mutant VACV witch single and accumulating deletions in genes inhibiting the IRF3 signaling pathway.A+B**) or multiple-step-growth conditions (**Figure 8C**), all candidate viruses replicated to titers similar to those obtained with the parental WR/TK- virus. All VACV with single, and double, deletion in candidate genes were tested under one-step-growth conditions in order to discard effect of single mutations in candidate viruses with multiple genes deleted. All candidate viruses replicated to titers similar to those obtained with the parental WR/TK- virus. The size of virus plaques formed in cell monolayers after infection can serve as an indicator of the viral capacity to destroy target cells upon propagation. In MA104 cells, the plaques lesions formed after infection with the candidate viruses were not significantly different in size compared to those formed after infection with WR/TK-, although we observed a tendency for plaque size reduction with accumulation of genomic deletions (**Figure 8D**).


A
Viral titer (PFU/ml)
hours after virus infection
WR/TK-
WR/TK-/2Δ
WR/TK-/Δ
WR/TK-/3Δ
hours after virus injection
B
WR/TK-/ΔC10
WR/TK-/ΔC6
WR/TK-/ΔN2
WR/TK-/ΔC10ΔN2
WR/TK-/ΔC10ΔC6

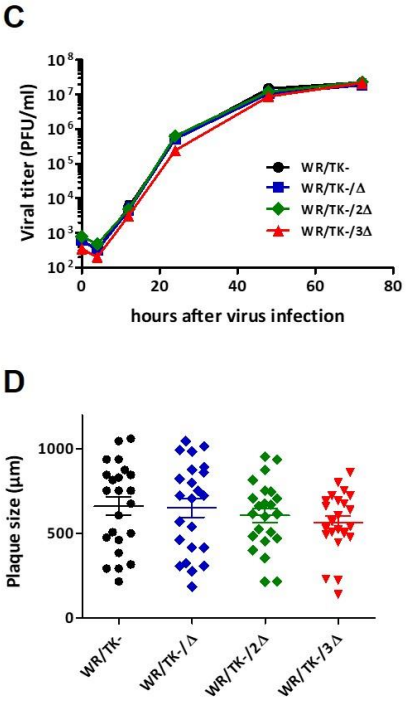


Figure 8. Mainly unimpaired replication of mutant VACV witch single and accumulating deletions in genes inhibiting the IRF3 signaling pathway.

Productive multiplication of deletion mutant viruses. HeLa cells were infected with a multiplicity of infection (MOI) of 5 (A+B) or 0.05 (C) and, at indicated time points, samples were collected and viral titers were determined by plaque-assay. Virus yield was evaluated in quadruplicate. (D) Plaque size analysis in MA104 cells. MA104 cells monolayers were infected at a MOI of 0.05 and, 72hours post infection, stained with crystal violet solution before the diameter of plaques was measured. The diameter size (µm) of 25 representative plaques per virus and mean ±SD are depicted.

2.2. Evaluation of the cytotoxic capacity of oncolytic deletion VACV

Then, we assessed whether the candidate oncolytic VACV conserve an unimpaired capacity to kill cancer cells. We infected both human (HeLa) and mouse cancer cell lines (Renca and B16) at different MOI (ranging from 0.0001 to 200) and, 72 hours after infection, the remaining metabolic activity of cells was determined (**Figure 9A**). The capacity to kill cancer cells was not affected by the accumulation of gene deletions and resulted in very similar patterns of cell death for infections with WR/TK-/Δ, WR/TK-/2Δ and WR/TK-/3Δ compared to the parental virus WR/TK-. Again, all the possible combinations of single and double deleted viruses were tested in order to discard effects of single deletions **Figure 9B**).

V RESULTS

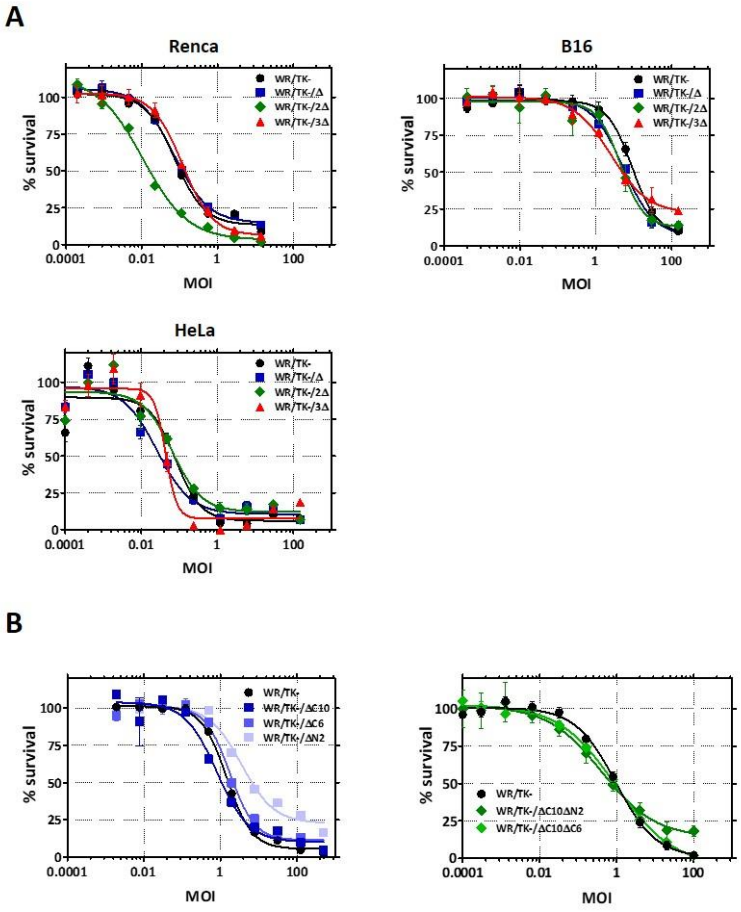


Figure 9. **Viruses with deletions in genes involved in interfering with the IRF3 signaling pathway maintains capacity to kill cancer cells**.

Comparative cytotoxicity in human and mouse tumor cell lines. Cells were infected with indicated viruses at doses ranging from 200 to 0.0001 PFU/cell. After 72 hours, the percentage (%) of killed cells was determined.

3. Deletion of viral genes interfering with the IRF3 signaling pathway leads to phosphorylation of IRF3 and expression of IFN-β-mRNA

To evaluate whether infection with candidate oncolytic VACV (WR/TK-/Δ, WR/TK-/2Δ, and WR/TK-/3Δ) leads to activation of the IRF3 pathway, we tested the phosphorylation status of IRF3 by Western Blot. As a positive control for the activation of the IRF3 pathway we used infections with the replication-deficient Modified Vaccinia Virus Ankara (MVA), which is a natural VACV mutant with many inactivated viral genes and it is known to efficiently activate IRF3 (Lehmann et al. 2016). In HeLa cells, levels of phosphorylated IRF3 were not increased by the presence of deletions compared to the parental virus WR/TK- (**Figure 10A**). However, both in THP-1 cells (**Figure 10. Activation of the IRF3 pathway by candidate oncolytic VACV due to accumulation of genomic deletions.B**) and in the mouse tumor cell line B16 (**Figure 10C**), we detected increasing amounts of phosphorylated IRF3 upon infection with viruses accumulating inactivations in genes interfering with the IRF3 pathway. Effects of single gene deletions were discarded by Western Blot analysis of extracts from THP1 cells infected with mutant viruses including all possible combinations of C10L, N2L, and C6L gene deletions (**Figure 10D**).

V RESULTS

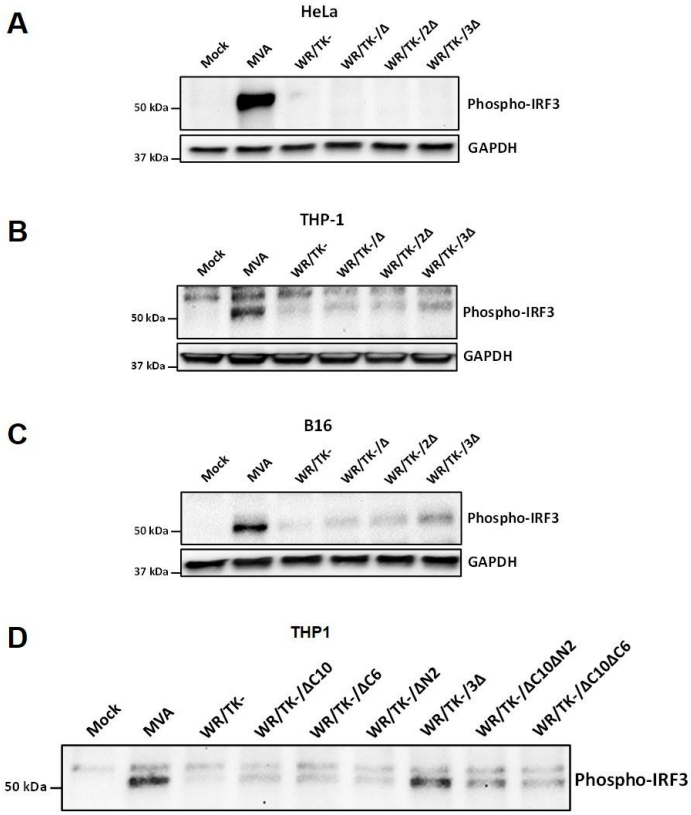


Figure 10. **Activation of the IRF3 pathway by candidate oncolytic VACV due to accumulation of genomic deletions**.

Deletion of viral genes interfering in the IRF3 pathway leads to IRF3 phosphorylation. HeLa (A+E), THP-1 (B+D), and B16 (C) cells were infected with the indicated viruses at a MOI of 10 and, 5 hours after infection, cells were lysed and Western Blot analysis was performed using a monoclonal antibody against phospho-IRF3. The non-replicating VACV MVA (Modified Vaccinia virus Ankara) served as a positive control and GAPDH-specific immunoblotting as a loading control.

IRF3 activation was confirmed by RT-PCR of Interferon-β in all cell lines tested. We detected increased levels of Interferon-β mRNA upon infection with the WR/TK-/3Δ virus (**Figure 11**

). Of note, this finding includes infections of HeLa cells, where increased levels of phosphorylated IRF3 protein could not be detected by immunoblot

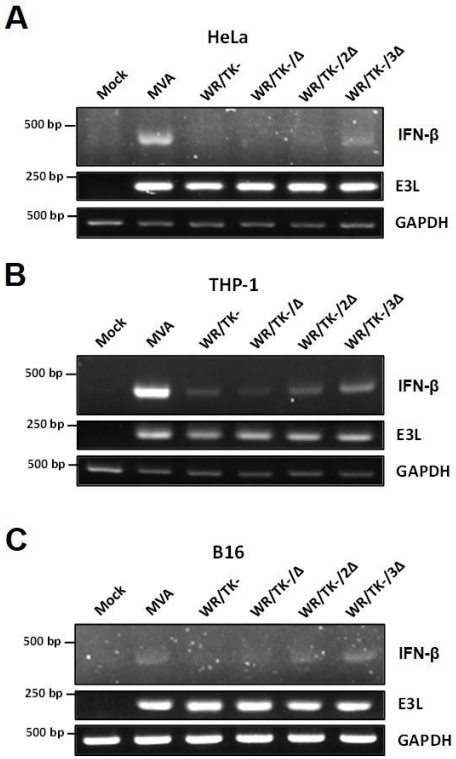


Figure 11. **Detection of IFN-β mRNA by RT-PCR**.

analysis.

HeLa (A), THP-1 (B), and B16 (C) cells were infected at a MOI of 5. At 6 hours after infection, total RNA was obtained and indicated the mRNAs of indicated genes were amplified by RT-PCR. The detection of VACV E3L mRNA was used as an infection control and GAPDH mRNA as a loading control.

4. *In vivo* evaluation of oncolytic VACV with deletions in the TLR3-IRF3 pathway

4.1. Replication of deletion mutant viruses is not impaired in mouse tumor models

To ensure that virus replication remains unimpaired *in vivo*, we injected mice bearing Renca tumors (mouse renal adenocarcinoma) intratumorally with the candidate deletion-mutant viruses and 4 days after virus injection tumors were harvested and viral growth was evaluated. Taking advantage of mCherry co-expression, fluorescence emitting from tumor tissues was quantified (**Figure 12A+B**). In addition, we titrated the virus loads within tumors (**Figure 12C**). Both methodological approaches illustrated that deletion mutant viruses and the parental virus WR/TK- replicated to very similar levels in tumor tissues. This indicates that deletion of up to three genes interfering with the IRF3 pathway does not hinder effective VACV replication, both *in vitro* and *in vivo*.

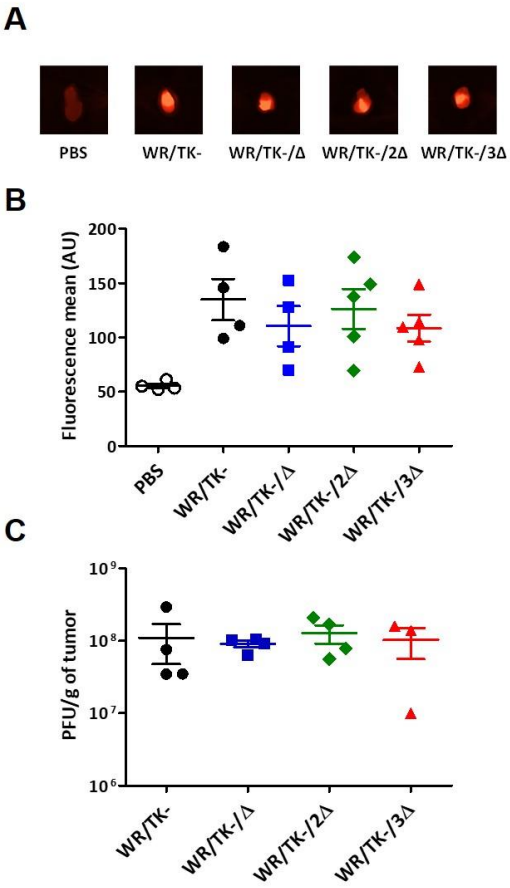


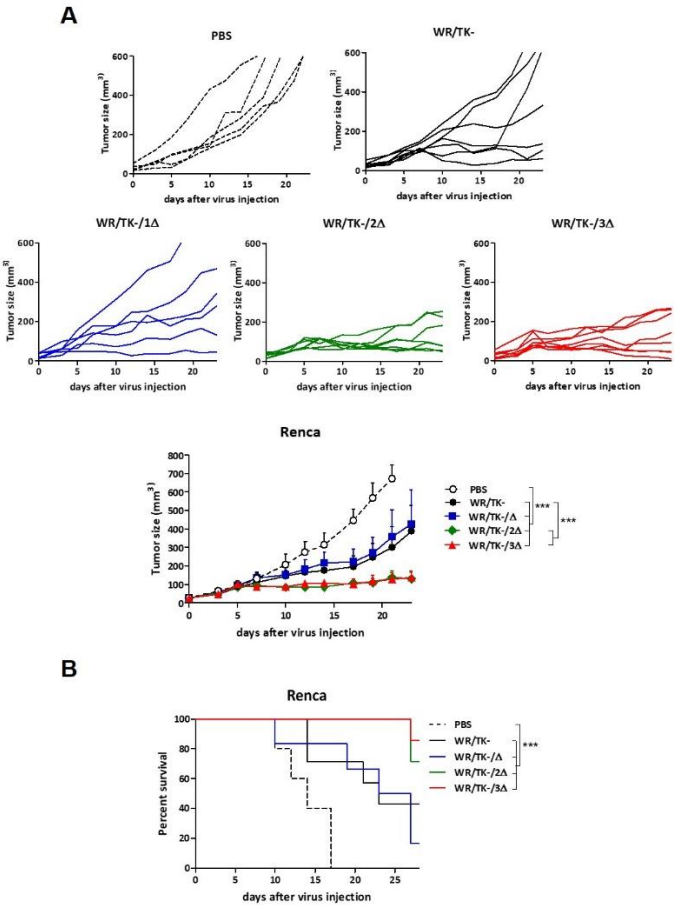
Figure 12. Replication of deletion mutant VACV in tumor models in vivo.

5 x 10^5 Renca cells were subcutaneously implanted on the flank of 6-8 week old Balb/C mice (n = 4 to 5). At day 0, a dose of 1x107 PFU was intratumorally injected and, 4 days later, mice were sacrificed and tumors were harvested. (A) Images of representative tumors showing mCherry-specific fluorescence. (B) Tumor fluorescence quantified using a MacroImaging system. Fluorescence of individual tumors and group means +SD are shown. (C) Viral titers determined by plaque assay after tumor homogenization. Titers obtained from each independent tumor and means +SD are depicted.

V RESULTS

4.2. Improved antitumor activity of oncolytic candidate VACV

As a next step, we evaluated the antitumor efficacy of the deletion mutant VACV *in vivo* using intratumoral virus delivery in two syngeneic mouse tumor models: BALB/C mice bearing Renca tumors and C57/BL6 mice bearing B16 tumors. In the Renca model, the injection of WR/TK-/2Δ or WR/TK-/3Δ viruses resulted in a strong significant reduction of tumor growth in comparison to the therapeutic effect observed with the parental WR/TK- (**Figure 13A**). Additionally, we also observed an increased survival time of mice injected with double and triple deletion mutant VACV (**Figure 13B**). When tested in the mouse melanoma tumor model B16, the WR/TK-/3Δ virus also induced a significant reduction in tumor growth (**Figure 13C**), but not the WR/TK-/2Δ virus. The survival time of this model is shown in **Figure 13D**.


A
PBS
WR/TK-
WR/TK-/1Δ
WR/TK-/2Δ
WR/TK-/3Δ
Tumor size (mm³)
days after virus injection
Renca
PBS
WR/TK-
WR/TK-/Δ
WR/TK-/2Δ
WR/TK-/3Δ
***
***
B
Renca
Percent survival
days after virus injection
PBS
WR/TK-
WR/TK-/Δ
WR/TK-/2Δ
WR/TK-/3Δ
***

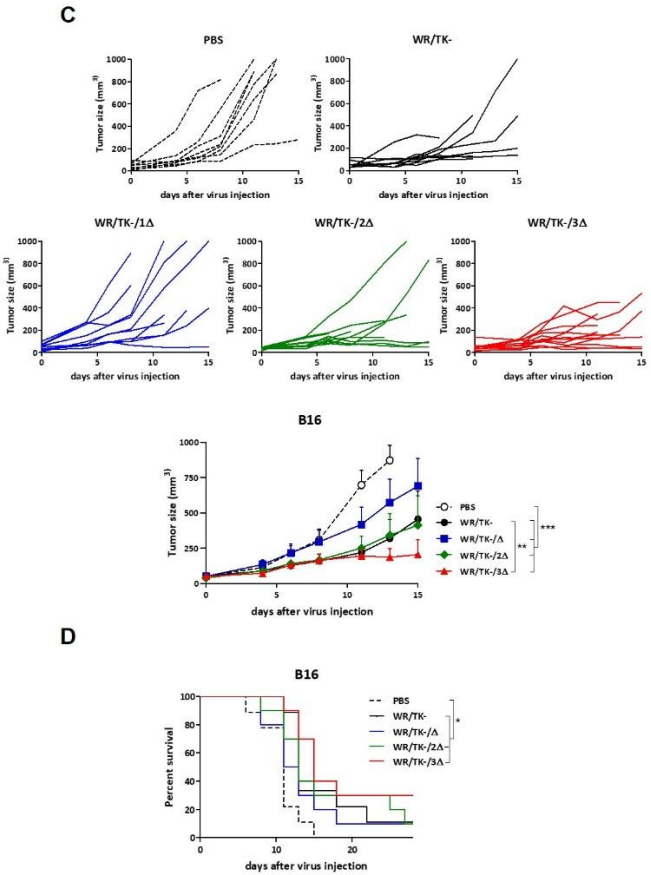


Figure 13. **Increased in vivo antitumor activity of candidate oncolytic VACV with combination of gene deletions rescuing IRF3 activation**.

$5x10^5$ tumor cells were subcutaneously implanted at day -9 on the flank of 6-8 week old BalbC mice (Renca tumors, a-b) or C57Bl/6 (B16 tumors, c-d), and viruses were intratumorally administered at days 0 and 4 at a dose of $1x10^7$ pfu/injection. PBS injected mice served as controls. For monitoring tumor growth, the tumors were measured 2-3 times per week until termination criteria were reached. Tumor volume (A, C) and overall survival (B, D) are plotted for 7-9 mice per group +SEM; (E-F) Renca (E) and B16 (F) tumor growth curves of individual animals treated with candidate oncolytic VACV*, $p<0.05$; **, $p<0.01$, ***, $p<0.001$.

4.3. Induction of tumor-specific cellular immune response by deleted VACV viruses

Our hypothesis was that the increased antitumor activity of deleted VACV is mediated by a more robust cellular antitumor immunity. Thus, we evaluated the tumor epitope-specific T cell responses established following virus administration in the B16 tumor model. ELISpot assays were performed to determine the T cell response directed against the virus (immunodominant VACV-specific $B8R_{20-27}$ peptide epitope) (Volz et al. 2018), a non-mutated gp100 tumor associated antigen epitope (Hanada et al. 2019), and the tumor neoepitope B16-M30 (Kreiter et al. 2015). Injection of WR/TK-/3Δ increased T cell reactivity to all the three epitopes (**Figure 14**), but, of note, we found clearly increased levels of epitope specific IFN-γ-producing T cells directed against the tumor antigens (gp100 and B16-M30) compared to treatments with the parental virus WR/TK-.

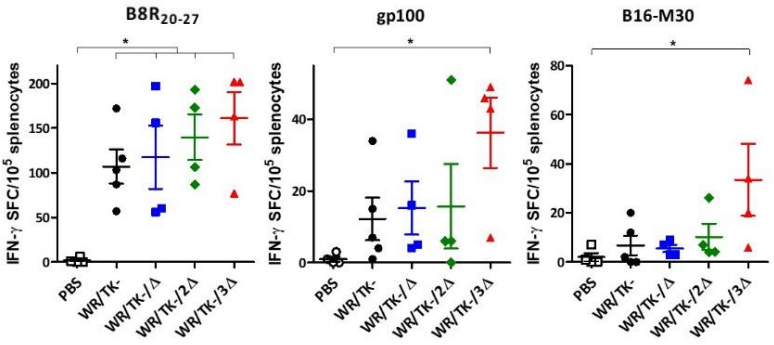


Figure 14. Intratumoral administration of deletion mutant VACV induces antitumor T cell responses directed against tumor neo-antigens.

C57BL/6 mice harboring B16 tumors were treated as indicated in Figure 5 and, 8 days after virus administration, splenocytes were prepared, *in vitro* stimulated with indicated peptides, and analyzed for IFN-γ producing cells by ELISPOT. Individual values of IFN-γ spot forming cells (SPC)/105 splenocytes in 4-5 mice/group and mean ±SD are plotted. *, p<0,05.

VI DISCUSSION

Although our understanding of cancer biology is constantly growing, cancer continues to be a leading cause of death worldwide. Despite the important advancements in treating many types of cancer, further research on cancer treatments is essential to improve the outcome of cancer patients and to find safe and effective therapies for all patients affected by the disease. Cancer immunotherapies emerged as a new form of cancer treatment that mobilizes the immune system to recognize and attack cancer cells. Several types of immunotherapies are used to treat cancer, including immune checkpoint inhibitors, T-cell transfer therapy, cancer vaccines, and oncolytic viruses. Oncolytic viruses are one form of immunotherapy that utilizes viruses to infect and destroy cancer cells. Either naturally or due to genetic modifications oncolytic viruses infect, replicate in and kill cancer cells without harming healthy cells. Importantly, the virus infection alarms the immune system and, if paired with the virus-mediated release of tumor antigens, can generate an immune response directed against tumor epitopes. Vaccinia virus (VACV), part of the poxvirus family, has served as backbone for the successful generation of promising candidates for oncolytic virotherapy (Moehler et al. 2019). However, candidate VACV viruses tested to date in patients demonstrated a suboptimal capacity to establish antitumor immunities (Harrington et al. 2019).

Why is further research in cancer immunotherapies necessary?

The immune system is a complex and powerful biological orchestra that not only fights off infections but also protects the body from mutated cells. Nevertheless, there is a fine balance between burden of cellular mutations and the capability of the immune system to destroy such cells, that at some point in tumor progression gets lost and the tumor overwhelms the immune system (DuPage et al. 2012). Tumors are able to recruit certain kind of

immune cells to help tumor progression and metastasis, like myeloid-derived suppressive cells (MDSCs) (Ouzounova et al. 2017) or tumor-associated macrophages (TAMs). TAMs are found in large numbers in breast tumors, for example, and are able to actively promote growth and metastasis (Williams et al. 2016). However, other immune cells found in the tumors, such as tumor-infiltrating lymphocytes (TILs), are a sign that the immune system is responding to the tumor and trying to destroy it (Lawson et al. 2020). A large number of TILs in patients' tumors is prognostically a good sign (Idos et al. 2020; Bremnes et al. 2016; Lee et al. 2008). Despite the effort of the immune system to prevent or slow down cancer growth, tumors develop mechanisms to avoid either recognition or destruction by the immune system (Seliger et al. 2017). Immunotherapies are developed for preventing these immune-evading mechanisms, and they demonstrated that the capability of the immune system to fight back tumor cells could be restored. These therapies focus on stimulating the immune system to better fight and recognize tumor cells rather than destroying them directly, like chemo- or radiotherapy. One of the main advantages of boosting the immune system compared to classical cancer therapy is the prevention of a relapse due to the memory function of the adaptive immune system.

One of the more successful immunotherapies so far are the so-called immune checkpoint inhibitors. They harness pre-existing but ineffective immune cells and achieve remarkable clinical success across different tumor types, including melanoma, non-small-cell lung cancer, urothelial carcinoma and Hodgkin's lymphoma with durable objective responses (Rosenberg et al. 2016; Garon et al. 2015; Ansell et al. 2014). Remarkable clinical results could also be seen in patients suffering from hematologic malignancies treated with chimeric antigen receptor (CAR) T-cells. In studies on acute lymphoblastic leukemia, complete response (CR) rates of 70% to 90% could be achieved. Regardless this success in the clinic, there are still questions and challenges that need to be addressed concerning

toxicity, efficacy, and targeting (Sambi et al. 2019). In the case of CAR T-cells, for example, the difficulty to find a specific antigen can lead to serious side effects after CAR-T cell therapy, including cytokine release syndrome (CRS), neurological toxicity, or the attack of non-tumor tissue with different severities of side effects (Hartmann et al. 2017; Makita et al. 2017). Furthermore, the complex structure of the immunosuppressive tumor microenvironment represents a major obstacle for immunotherapies. Up to today, they only work on a small subset of cancers and their efficacy highly varies between patients (Yang 2015; Nakamura and Smyth 2017). This indicates that therapies or combination of therapies that activates more robustly antitumor immune responses are needed. The success, but also the obstacles, of novel anticancer immunotherapies underlines the importance of understanding basic tumor immunology, the complex interaction of tumor cells and immune cells and emphasize the power the immune system has in the battle against cancer as well as the possibility for novel therapeutic options.

Benefits of oncolytic viruses as immunotherapeutic agents and the advantages of poxviruses

The use of oncolytic viruses (OVs) demonstrated encouraging clinical results and to be promising as an immunotherapeutic approach in the field of cancer treatment. An appealing feature of OVs is their tumor-specificity, supported by the fact that tumor cells favor viral replication due to apoptosis resistance, growth suppression and immune evasion strategies, and defects in antiviral signaling pathways (Hanahan and Weinberg 2011). What makes oncolytic viruses even more attractive is their capability not only to destroy tumor cells directly because of viral replication, but also to activate both the innate and the adaptive immunity. Oncolytic virus infection triggers a signaling cascade culminating in the release of damage-associated molecular patterns (DAMPs), as well as pathogen-associated molecular

pattern (PAMPs), which is able to overcome the immunosuppressive milieu in the tumor microenvironment (Marelli et al. 2018; Filley and Dey 2017; Davola and Mossman 2019). This, together with the release of tumor-associated antigens (TAAs) due to tumor cell lysis, leads to the activation of an innate and adaptive anti-tumor immune response, which is the key in oncolytic virotherapy. With increasing recognition of the immune system in OV efficacy, “arming” OVs with immunostimulating transgenes is a common modification. For example, the addition of granulocyte macrophage colony-stimulating factor (GM-CSF) to the viral genome, a potent inducer of antitumor immunity (Dranoff 2002), recruits antigen-presenting cells (APCs) and promotes their maturation while stimulating antitumor immunity (Andtbacka et al. 2015; Kemp et al. 2019).

In the last 20 years, 97 independent clinical trials investigating OVs were reported with 11 different viruses, being poxviruses one of the most common (12.4%) (Macedo et al. 2020). Within poxviruses, vaccinia virus (VACV) is the widest used due to several unique features, which makes it particularly attractive for the design of oncolytic viruses: (i) It has a rapid and lytic replication cycle and a high degree of tissue destruction (Wein et al. 2003; Zeh and Bartlett 2002); (ii) VACV’s excessive use as a live vaccine in the smallpox eradication campaign helped to a profound knowledge of VACV biology and pathogenesis, as well as to well defined contraindications and how to counteract side effects (Fenner et al. 1988; Cono et al. 2003). (iii) It is possible to insert large amount of foreign DNA (up to 25 kb) into the large double-stranded DNA genome, what makes VACV a promising candidate to modulate the TME and enhance its antitumor activity (Smith and Moss 1983). (iv) The cytoplasmic replicative cycle of VACV prevents integration of viral genome into host genome (Moss 2013) (v) Important for the systemic delivery, VACV can efficiently spread through the bloodstream and between tumors (Kirn et al. 2008; Downs-Canner et al. 2016). (vi) Furthermore, VACV infections are highly immunogenic, inducing both humoral and CD4+and CD8+T-cell responses, making it attractive for its use as a vaccine vector

(Walsh and Dolin 2011). By deleting selected VACV genes it is even possible to further increase their immunogenicity (Albarnaz et al. 2018) (vii) Finally, oncolytic VACV can replicate selectively in tumor-associated endothelial cells, which leads to tumor vessels destruction and vascular collapse of the tumor (Hou et al. 2014).

Several trials investigating oncolytic VACV are currently ongoing (National Institute of Health. The clinical trials databases. Available from: www.clinicaltrials.gov (accessed:12.02.2021)). Pexastimogene devacirepvec (Pexa-Vec, a thymidine kinase-deleted VACV expressing GM-CSF) is currently the most advanced oncolytic poxvirus candidate, and it has been administrated alone or in combination with sorafenib or immune checkpoint inhibitors; it is mainly being assessed for the treatment of hepatocellular carcinoma (HCC) in later phase trials. In a randomized phase II study of intratumoral Pexa-Vec, it demonstrated a favorable safety profile, with the most common side effects being flu-like symptoms. A response rate of 62% could be achieved after a high dose of Pexa-Vec and was associated with improved overall survival when compared with the low-dose group (Breitbach et al. 2015). Although these promising results in Phase II, an oncolytic VACV candidate has not reached yet approval.

How can the anti-tumor response induced by VACV be improved?

Despite the tremendous progress made in the design and generation of oncolytic viruses, durable clinical responses are rare. In order to revert that, further research is ongoing to exploit the potential of VACV as antitumor agents. Due to the importance of an anti-tumor immune response, one strategy followed to enhance their efficacy is the insertion of transgenes encoding for immunostimulatory chemokines or cytokines, being the most prominent example GM-CSF or different interleukins (Wang et al. 2017; Stephenson et al. 2012). Liu et al demonstrated that arming a tumor-selective oncolytic vaccinia virus with CXCL11 resulted in an increased

number of local CD8 T-cell and the induction of a systemic anti-tumor immunity, as well as a decrease in different immunosuppression factors, leading to an improved therapeutic effect (Liu et al. 2015c).

Another strategy followed with oncolytic VACV targets the immunosuppressive TME. An oncolytic vaccinia virus expressing the PGE_2 inactivating enzyme HPGD was able to significantly reduce levels of suppressive MDSC within the tumor and to re-sensitize tumors to viral therapy (Hou et al. 2016). As metabolic insufficiencies induced by the tumor microenvironment are a barrier due to their inhibition of the effector function of T-cells, Rivadeneira et al. demonstrated that the improvement of T-cell metabolic function in the tumor microenvironment translates into a better therapeutic response. Therefore, they engineered an oncolytic VACV delivering the adipokine Leptin, which metabolically reprogrammed T cells in order to support antitumor responses (Rivadeneira et al. 2019).

In our group, we have also attempted a different and novel strategy: activation of immunogenic cell death (ICD) after infection of cancer cells. Classical mechanisms of cell death, such as apoptosis or autophagy, are described as tolerogenic and do not attract or activate immune cells for the recognition of antigens codified within the dying cell (Curtin and Cotter 2003, Green et al. 2009). Differently, activation of ICD pathways, such as necroptosis or pyroptosis, leads to the release of different damage-associated molecular patterns (DAMPs), which recruit and activate DCs. The induction of tumor ICD subsequently leads to a stepwise induction of an anti-tumor immune response by enhancing cross-priming of CD8 T-cells (Galluzzi et al. 2017). The expression of the necroptosis executioner MLKL demonstrated to induce potent antitumor T cell responses directed against tumor neo-antigens, which translated into an outstanding antitumor activity (Van Hoecke et al. 2020).

As discussed, oncolytic viral replication within the tumor has beneficial effect

in terms of transiently overcoming the immunosuppressive TME. Yet, data from clinical trials make evident that OV-monotherapy is rarely curative, and every day it is clearer that tackling cancer from different fronts could be the deciding path to success in defeating cancer. The rational combination with other forms of cancer treatment like chemotherapy (Ranki et al. 2016), radiation therapy (Wilkinson et al. 2016), or other immunotherapies (Ottolino-Perry et al. 2015; Ottolino-Perry et al. 2010; Sampath and Thorne 2015) may be the key for an enhanced immunological effect and durable therapeutic responses (Zhang and Cheng 2020). For such combinatory immunotherapies, oncolytic VACV are preferential candidates due to their low toxicity and combinatory potential, as they have demonstrated synergistic effects when combined with several immunotherapies.

Immune checkpoint inhibitors (ICIs) block the so-called immune checkpoints that are expressed by certain types of immune and cancer cells. In regards to cancer, those immune checkpoints prevent T cells from killing cancer cells. The blockade of these immune checkpoints leads to a re-activation of TILs and demonstrated outstanding results in the clinic (Overman et al. 2017). However, the effect of ICIs depends on the number of immune cells within the tumor, and patients that do not respond to ICI normally present “cold” tumors, which are characterized by minimal CD8 infiltration (Herbst et al. 2014). Oncolytic viruses (OVs) can induce efficient immune infiltration and therefore promote the efficacy of ICIs (Sivanandam et al. 2019; Zamarin et al. 2018; Liu et al. 2017). Chesney et al evaluated in a randomized phase 2 study the effect of anti-CTLA4 antibody alone or in combination with T-Vec in patients with advanced melanoma. They observed an objective response of 39% to the combinational treatment, compared to 18% of ICI alone (Chesney et al. 2018). Similar results could be seen when T-Vec was combined with an anti-PD1 antibody. The objective response rate (ORR) was even 62% and those patients showed an increased level of CD8+T cells with elevated PD-L1 protein expression (Ribas et al. 2017). Nevertheless,

one has to consider that the effect of ICIs can diminish viral replication, and that the success of this combinational therapy depends on the choice of the antibody, the viral strain, and, more importantly, the timing of the treatments (Rojas et al. 2015). The simultaneous administration of OV and ICI leads to an early induction of anti-viral immunity and a decrease of oncolytic activity, although this can be solved by a sequential administration of the treatments (Rojas et al. 2015).

The OVs capability to turn “cold” tumors into “hot” tumors can also help to overcome the obstacles of CAR T-cell therapy in solid tumors. Combination with OV induces synergistic effect thanks to the virus-driven release of DAMPs, that can enhance tumor infiltration and persistence of CAR-T cells (Ajina and Maher 2017; Ajina and Maher 2019; Rosewell Shaw and Suzuki 2018). At the end of last year, the first-in-human Phase I trial investigating CAR T and OV combination started (NCT03740256).

Despite the potential of OV in general and VACV in particular, further development of novel candidates with increased capacity to robustly activate antitumor immune response is needed for exploiting all the potential that these agents have for the treatment of nowadays incurables cancers.

Generation of oncolytic VACV with deletions in immunomodulatory proteins inhibiting IRF3 activation in order to increase their potency to activate cellular antitumor immunity

The goal of this work was to obtain a replication-efficient oncolytic VACV with improved capacity to activate antitumor T cell responses. The replication of oncolytic VACV in cancer cells leads to the release of danger associated molecular patterns (DAMPs) together with a multitude of tumor-specific antigens, turning “cold” tumors into “hot” tumors for more efficacious immunotherapy (Shi et al. 2020). The strategy that we followed in this work for improving antitumor T cell responses was based on the observation that

poly(I:C), when used as an adjuvant in cancer vaccination, leads to a Th1 polarization of the immune response and increases the amount of anti-tumor CTLs (Kano et al. 2016), which directly correlates with robust antitumor immunity in the clinic (Mikhaylova et al. 2018). As poly(I:C) selectively activates TLR3, we attempted to construct an oncolytic vector virus with the capacity to activate the TLR3-IRF3 pathway after infection.

In line with its outstanding capacity to evade antiviral innate immunity, VACV encodes for several immunomodulatory proteins directly interfering with the host TLR3-IRF3 innate response pathway. To promote the activation of this pathway after infection, a series of oncolytic VACV were constructed combining the deletion of the thymidine kinase gene (to achieve selective replication in cancer cells) with targeted inactivation of selected genes interfering with IRF3 pathway activation. The following target proteins were chosen due to their important inhibitory mechanisms at different levels in the pathway: C10 (also known as C16 due to its nomenclature in the Western Reserve strain) prevents dsDNA recognition by DNA-PK (Peters et al. 2013; Scutts et al. 2018); N2 interferes by yet unknown mechanisms the downstream of phosphorylated IRF3 and its nuclear translocation (Ferguson et al. 2013); and C6 interacts with NAP1, TANK, and SINTBAD, the scaffold adaptor proteins for the kinases TBK1 and IKKε, that lead to IRF3 activation (Unterholzner et al. 2011; Smith 2018). The inactivation of such immunomodulatory genes of VACV demonstrated in the past to improve the level of CD8 T-cells in the context of vaccination strategies (Sumner et al. 2013; García-Arriaza et al. 2014). All the possible mutant VACV combining deletions in up to three genes were constructed, but one single-, one double-, and the triple-deleted mutant viruses were selected for complete testing; the selection of the candidates for complete testing was performed based on the lack of loss in cytotoxicity and in replicative-capacity *in vitro* (Figure 9A, 10). Deletions included in the final candidate oncolytic VACV are depicted in Figure 6. As shown in Figure 9 and Figure 10, inclusion of up to

three of these mutations does not impair the capacity of VACV to replicate in cancer cells nor their cancer cell-killing efficacy *in vitro*.

Combination of deletions in C10L, N2L, and C6L genes results in activation of the TLR3-IRF3 pathway as demonstrated by detection of phosphorylated IRF3 and interferon-β mRNA (Figure 11, 12). Increasing levels were detected by the introduction of deletions in genes interfering with the TLR3-IRF3 pathway to the genome of the control virus WR/TK-, and Figure 11D demonstrates that this activation is mediated by accumulation of such deletions rather than by any of the single deletions. In mouse models, this TLR3-IRF3 pathway activation translates into improved T cell responses, both directed against the virus and the tumor (Figure 15). Importantly, anti-tumor T cells are directed against tumor associated-antigens (gp100), but also against tumor neo-epitopes (B16-M30), and T cell activities elicited by the WR/TK-/3Δ are significantly higher than responses obtained with the non-treated group. Finally, these enhanced tumor-directed immune responses are associated with an improved antitumor activity in two syngeneic mouse tumor models (Figure 14), strongly suggesting the feasibility and the efficacy of the proposed strategy.

Previously, an oncolytic VACV expressing TRIF (the main adaptor in the TLR3-IRF3 signaling pathway) also explored the strategy of activating the TLR3-IRF3 pathway after infection of tumor cells (Rojas et al. 2016). This virus demonstrated a switch from a Th2- to a Th1-skewed response and displayed enhanced therapeutic activity in mouse models. However, replication of the virus was strongly hindered within tumors (using the Renca model) due to massive pathway activation; on the contrary, our novel strategy of accumulating up to three deletions in VACV genes interfering with the TLR3-IRF3 pathway fully conserved the replication capacity in Renca tumors (Figure 13). Previously, the importance of VACV replication for activating an antitumor immune response was demonstrated (Van

Hoecke et al. 2020), which is in discrepancy to some previous reports (Dai et al. 2017). Yet, virus replication leads to tumor cell lysis and release of tumor antigens and danger signals, in addition to amplify the initial dose administrate. Thus, maintaining an efficient replication in tumor cells is a key factor for the outcome of oncolytic therapies and should be an important feature when developing a candidate for clinical evaluation.

Although able to improve antitumor immune responses, levels of phosphorylated IRF3 and IFN-β mRNA detected after infection with the WR/TK-/3Δ do not reach the levels observed after infection with MVA (Figures 11, 12). Previously, VACV incorporating single deletions in the C6L, the N2L, or the C10L gene demonstrated enhanced immunogenicity and highly reduced virulence in mice (Ferguson et al. 2013; Sumner et al. 2013; Fahy et al. 2008). Yet, after infection with any of these three single deleted VACV, we did not detect TLR3-IRF3 pathway activation *in vitro* (Figure 11D). We hypothesize that the activation detected in *in vitro* assays may not properly reflect levels of activation *in vivo* in animal models, and lack of activation in tumor cell cultures may not be predictive for stronger activation of antitumor immune responses within tumors due to the complexity of tumor microenvironment and the diversity of cells present in tumors.

Future perspectives

Together, the data here presented demonstrate that it is possible to generate an oncolytic VACV with the ability to activate the TLR3-IRF3 pathway while maintaining full capacity to productively replicate in cancer cells. Importantly, the combination of these features translates into an improved antitumor immunity and antitumor efficacy of the oncolytic vector virus. To test the possibility to improve this activation, further deletions could be incorporated into the WR/TK-/3Δ candidate virus. One possible candidate gene is D10R, a de-capping protein, whose deletion has also been described to activate IRF3 through phosphorylation of PKR and eIF2α (Liu et al. 2015a). We

constructed a VACV derived from the WR/TK- strain and incorporating a single deletion in such gene to test their capacities as an oncolytic. *In vitro*, such virus produced 10 times less progeny virus than its WR/TK- counterpart and presented an important reduction in the size of plaques (unpublished data). Apparently, the combined deletion of the thymidine kinase and the D10R gene compromise the ability of this deletion mutant virus to efficiently replicate in cancer cells. Thus, we discarded the D10R deletion for the construction of candidate oncolytic viruses due to the reduction of replicative capacity in cancer cells. Additional VACV regulatory proteins with inhibitory functions in the activation of the TLR3-IRF3 signaling pathway that are considered for incorporation into our candidate virus include A46, which interacts with TRIF (Fedosyuk et al. 2016), K7, which binds the DEAD-box RNA helicase 3 (DDX3) (Schröder et al. 2008), B19 (also known as B18 due to its nomenclature in the Western Reserve strain), which is a soluble type I interferon receptor (Alcamí et al. 2000), and B2, which encodes a viral nuclease with cGAMP-specific activity (Eaglesham et al. 2019). However, further deletions incorporated to the WR/TK-/3Δ virus may compromise the ability of these deletion mutant viruses to efficiently replicate in cancer cells, as demonstrated by the growth deficiency of the natural highly-deleted mutant virus MVA, which is unable to replicate in mammalian cells. An appropriate balance between the activation of danger signaling pathways and virus replication must be found for optimizing oncolytic VACV immunotherapies.

One further advantage of our strategy to activate more robust antitumor immunities is its capacity to be combined with other genetic modifications. Our candidate deleted VACV can serve as a backbone for incorporating transgenes to further improve antitumor immune responses or other aspects of the therapy. These transgenes, as discussed earlier, can include cytokines or chemokines, genes that target the tumor microenvironment, or genes that activate ICD. Our candidate VACV allows cloning of several

transgenes simultaneously as deleted genes can serve as insertion *loci*; expression of more than one transgene at the same time can maximize the therapy and attack the tumor on different fronts. In addition, the candidate VACV can be combined with classical antitumor therapies or novel immunotherapies to produce robust responses in patients suffering from a variety of solid tumors.

VII SUMMARY

Characterization of candidate oncolytic vaccinia viruses with deletions in viral genes blocking the activation of interferon regulatory factor 3 immune signaling.

Over the last two decades, the understanding of the relationship between cancer and the immune system has considerably changed and implemented the role of the immune system controlling tumorigenesis and tumor progression. Cancer immunotherapies aim to mobilize the immune system to kill cancer cells and represent a major progress in cancer therapeutics.

Robust anti-tumor CTL-responses have demonstrated to play a key role in the successful treatment of cancer. In cancer vaccination, the use of Poly I:C, a TLR3 agonist, as an adjuvant has demonstrated to increase the number of CTLs targeting tumor antigens. TLR3 signaling culminates in IRF3 phosphorylation and consequently expression of type I interferons (IFN). As type I IFNs also play a crucial role in anti-VACV defense, VACV encode several proteins (including C10, N2 or C6) that antagonize the TRL3-IRF3 signaling pathway at different levels, efficiently inhibiting phosphorylation of IRF3. However, deletions in some of these genes, such as C6 or N2, demonstrated to improve CD8 T-cell responses in vaccination. MVA (Modified Vaccinia virus Ankara), a highly attenuated strain of VACV with genomic mutations and deletions that inactivate many immunomodulatory genes, can robustly induce the secretion of type I IFN after infection. Nevertheless, due to MVA's defective replication in mammalian cells, its capacity for usage as an oncolytic agent is greatly reduced. Thus, the generation of oncolytic VACV combining the capacity to activate the TLR3-IRF3 pathway with an efficient replication in cancer cells represents a major step towards an efficient VACV-based oncolytic therapy.

In this work, we constructed a battery of oncolytic VACV that combine

deletions in key VACV genes involved in the inhibition of IRF3 activation. We evaluated their replication competence as well as their ability to elicit T-cell responses against tumor neo-antigens and their antitumor activity. The removal of up to three key genes (C10L, N2L, and C6L) from VACV genome did not reduce the strength of viral replication, both *in vitro* and *in vivo*, but resulted in the rescue of IRF3 phosphorylation upon infection of cancer cells. Importantly, when tested in syngeneic mouse tumor models, this activation translated into enhanced CTL responses directed against tumor associated antigens and neo-epitopes, and a greatly improved antitumor activity. We demonstrated the feasibility to obtain replication efficient VACV with increased capacity to activate the IRF3 pathway. Moreover, the candidate triple deletion-mutant virus represents an excellent basis for future preclinical and potential clinical studies in cancer virotherapy. Hereby, development and application of such oncolytic VACV to treat cancers in clinical veterinary medicine appear promising to further assess the potential of virotherapy in human medicine.

VIII ZUSAMMENFASSUNG

Charakterisierung onkolytischer Vacciniaviren mit Deletionen in viralen Genen zur Blockade der Aktivierung des Interferon Regulatory Factor 3 Signalweges

In den letzten 20 Jahren hat sich das Verständnis der Beziehung zwischen dem Immunsystem und Krebs grundlegend geändert und dem Immunsystem wurde eine neue Rolle in Bezug auf Tumorgenese und Tumorprogression zugesprochen. Krebsimmuntherapien zielen darauf ab das Immunsystem zu mobilisieren, um Krebszellen zu zerstören und stellen eine Revolution in der Krebsbehandlung dar.

Robuste anti-tumor T-Zell-Antworten haben sich als Schlüsselrolle in der erfolgreichen Behandlung von Tumoren erwiesen. Bei der Krebsimpfung hat die Verwendung des Toll-Like-Rezeptor 3 (TLR3)-Agonisten Poly-I:C als Adjuvant zu einem Anstieg der Zahl der gegen Tumorantigene gerichteten zytotoxischen T-Zellen (CTL) geführt. Eine TLR3-Aktivierung führt zu einer Verstärkung der Expression von Typ 1 Interferonen (IFN), welche direkt mit der Zahl der CTL korreliert. VACV kodieren für verschiedene Proteine (einschließlich C10, N2 oder C6), die diesen TLR3-IFN-Signalweg auf mehreren Ebenen antagonisieren und so eine Phosphorylierung und Aktivierung des zentralen Transkriptionsfaktors Interferon Regulatory Factor 3 (IRF3) verhindern. Die Herstellung und Untersuchung von VACV Deletionsmutanten mit der Fähigkeit den TLR3-IRF3 Signalweg zu aktivieren und sich gleichzeitig effizient in Krebszellen zu vermehren, stellt einen vielversprechenden Schritt in Richtung einer wirksamen VACV-basierenden onkolytischen Therapie dar.

In dieser Arbeit wurden eine Reihe onkolytischer Testviren konstruiert, indem im VACV-Genom Deletionen in Schlüsselgenen, welche eine IRF3-Aktivierung verhindern, eingefügt und kombiniert wurden. Die

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Charakterisierung der Viren beinhaltet die Überprüfung der Replikationskompetenz in Krebszellen, die Fähigkeit eine gegen Tumor-Neoantigene gerichtete T-Zellantwort auszulösen und deren Antitumoraktivität im präklinischen Modell. Eine Deletion von bis zu drei Schlüsselgene (C10L, N2L und C6L) aus dem Genom von VACV führte nicht zu einer reduzierten Replikationsfähigkeit, weder *in vitro* noch *in vivo*, jedoch aber zur Phosphorylierung und Aktivierung des Transkriptionsfaktors IRF3 bei der Infektion von Krebszellen. In syngenen Maus-Tumormodellen konnte eine verstärkten CTL Antwort induziert werden, die gegen tumorassoziierte Antigene und Neoepitope gerichtet war und mit einer erheblich verbesserten Antitumoraktivität assoziiert war. Damit konnte gezeigt werden, dass es möglich ist, ein vollständig vermehrungsfähiges und den IRF3 Signalweg aktivierendes VACV herzustellen und erfolgreich einzusetzen. Das in dieser Arbeit beschriebene neue Testvirus mit dreifacher Deletion bietet nun eine hervorragende Grundlage für zukünftige präklinische und potenzielle klinische Studien in der experimentellen Krebstherapie dar. Dabei erscheint die Entwicklung und Anwendung eines solchen onkolytischen VACV zur Behandlung von Krebserkrankungen in der klinischen Veterinärmedizin besonders vielversprechend, um das Potenzial einer Virotherapie in der Humanmedizin noch besser bewerten zu können.

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1. Consumables/plasticware, reagents, chemicals

Describtion	Supplier
6-/24-/96-well flat bottom plates	Sarstedt, Nümbrecht, Germany
Cell culture flasks (25/75/175 cm2)	Sarstedt, Nümbrecht, Germany
Serological pipettes (5/10/25 ml)	Sarstedt, Nümbrecht, Germany
Reagent reservoir	Fisher Scientific, Waltham, USA
Micro tubes 1,5ml	Sarstedt, Nümbrecht, Germany
Micro tubes, safe seal, 2ml	Sarstedt, Nümbrecht, Germany
Falcon (10ml/50ml)	Sarstedt, Nümbrecht, Germany
ep Dualfilter T.I.P.S.®, 10µl/100µl	Eppendorf AG, Hamburg, Germany
Biosphere® Filter Tips (20µl/100µl/200µl/1000µl)	Sarstedt, Nümbrecht, Germany
Eppendorf PCR tubes	Eppendorf AG, Hamburg, Germany
TUBES UC 1 X 3-1/2	Beckman Coulter, Brea, USA
Petri dishes	SIGMA-ALDRICH, St. Louis, USA
Luer eccentric tip	VWR, Radnor, USA
Falcon cell strainer, Falcon®	A Corning Brand, Corning, USA
Spectrum Labs™ MICROKROS HOLLOW FIBER FILTER MODULE 1XFL PS 0.05	Fisher Scientific, Waltham, USA
Syringe PP/PE, without needle	SIGMA-ALDRICH, St. Louis, USA
Disposable syringe Omnican	Fisher Scientific, Waltham, USA
Blotting-Membranen, Amersham™ Protran™ Premium 0.2µm NC	GE Healthcare Life Science, Chicago, USA
Western Blotting Filter Paper	Fisher Scientific, Waltham, USA
Mini-PROTEAN® TGX™ Gels	Bio-Rad, Hercules, USA
BSA	SIGMA-ALDRICH, St. Louis, USA
Precision Plus Protein™ Dual Color Standard	Bio-Rad, Hercules, USA
1 kb DNA ladder	New England Biolabs, Ipswich, USA
GelRed	VWR, Radnor, USA

OneTaq® 2x Master Mix	New England Biolabs, Ipswich, USA
Lipofectamine 2000	Fisher Scientific, Waltham, USA
4X Laemli sample buffer	Bio-Rad, Hercules, USA
Blue Juice Gel loading buffer	Life Technologies, Carlsbad, USA
Red Blood Cell Lysis buffer	SIGMA-ALDRICH, St. Louis, USA
Phorbol myristate acetat	SIGMA-ALDRICH, St. Louis, USA
Ionomycin	SIGMA-ALDRICH, St. Louis, USA
TAE buffer 50X	Fisher Scientific, Waltham, USA
10X Tris/Glycine/SDS	Bio-Rad, Hercules, USA
Trypan Blue	SIGMA-ALDRICH, St. Louis, USA
OneShot™Top10	Fisher Scientific, Waltham, USA
Ampicillin, sodium salt	Life Technologies, Carlsbad, USA
Crystal violet	SIGMA-ALDRICH, St. Louis, USA
Benzonase	VWR, Radnor, USA
Carboxymethylcellulose sodium salt, low viscosity	SIGMA-ALDRICH, St. Louis, USA
Sucrose	SIGMA-ALDRICH, St. Louis, USA
RIPA buffer	Abcam, Cambridge, UK
Half™ Protease & Phosphatase Inhibitor Cocktail	Fisher Scientific, Waltham, USA
Isofluran CP®	cp-pharma, Burgdorf, Germany
Tween®20	Promega, Madison, USA
LE Agarose	Biozym, Hessisch Oldendorf, Germany
2-Mercaptoethanol	Carl-Roth GmbH, Karlsruhe, Germany
Hydrochloride acid (HCl)	Carl-Roth GmbH, Karlsruhe, Germany
Ethanol, 70%/96% (C_2H_6O)	Carl-Roth GmbH, Karlsruhe,

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	Germany
Methanol (CH_4O)	Carl-Roth GmbH, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl-Roth GmbH, Karlsruhe, Germany
Tris ultrapure ($C_4H_{11}NO_3$)	AppliChem, Darmstadt, Germany
Glycin ($C_2H_5NO_2$)	AppliChem, Darmstadt, Germany
PBS	In-house production, LMU, München, Germany
Distilled water	In-house production, LMU, München, Germany

2. Buffers, solutions

Buffers, solutions	**Conditions**
TBS 10X (pH 7,4)	200 mM Tris base 1.4 M NaCl distilled water
Transfer buffer 1X	25 mM Tris base 200 mM glycine 20% methanol distilled water
1M Tris-HCl (pH 8)	1 M Tris base distilled water

3. Commercial kits

Describtion	**Supplier**
Plasmid Plus Maxi Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAquick PCR purification Kit	Qiagen, Hilden, Germany
QIAamp DNA Mini Kit	Qiagen, Hilden, Germany
RNeasy Plus Mini Kit	Qiagen, Hilden, Germany
Omniscript RT Kit	Qiagen, Hilden, Germany
Spin Miniprep Kit	Qiagen, Hilden, Germany
Quick Ligation Kit	New England Biolabs, Ipswich, USA
Pierce BCA Protein Assay Kit	Fisher Scientific, Waltham, USA

Pierce ECL Plus Western Blotting substrate	Fisher Scientific, Waltham, USA
CellTiter96® Aqueous Non-radioactive cell proliferation assay	Promega, Madison, USA
Mouse IFN-γ ELISPOT	MABTECH, Stockholm, Sweden

4. Laboratory equipment and software

Equipment	**Supplier**
Eppendorf Research Plus (10µl/100µl/1000µl/12-channel 300µl)	Eppendorf AG, Hamburg, Germany
Eppendorf Reference 2	Eppendorf AG, Hamburg, Germany
Thermo Scientific ™ Pipet filler S1	Fisher Scientific, Waltham, USA
Safety Work Bench BDK-SK 1200	BDK, Sonnenbühl, Germany
Heraeus HERAsafe	Heraeus, Hanau, Germany
Heraeus Kendro HeraCell 150 CO2 Inkubator	Heraeus, Hanau, Germany
C24 Incubator Shaker	New Brunswick Scientific, Edison, USA
Neubauer chamber, improved BLAUBRAND®	BRAND GMBH + CO KG, Wertheim, Germany
Mini Vortex Mixer	Fisher Scientific, Waltham, USA
Corning® LSE™ single block	SIGMA-ALDRICH, St. Louis, USA
Sonoplus	Bandelin electronics, Berlin, Germany
Olympus CKX41	Olympus Life Sciences, Hamburg, Germany
Inverted microscope MBL3200	Krüss, Hamburg, Germany
Mini-PROTEAN® Tetra vertical electrophoresis cell	Bio-Rad, Hercules, USA
Power Pac 200	Bio-Rad, Hercules, USA
Trans Blot® Turbo™ Transfer System	Bio-Rad, Hercules, USA
PeqSTAR 2x thermocycler	PEQLAB Biotechnology GmbH,

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	Erlangen, Germany
ChemiDoc™MP	Bio-Rad, Hercules, USA
Sunrise™	Tecan Trading AG, Männedorf, Switzerland
A.EL.VIS Eli.Scan	A.EL.VIS, Hannover, Germany
Mupid®-One Electrophoresis Unit	Mupid, Dubai
TissueLyser II	Quiagen, Hilden, Germany
Hettich Zentrifuge EBA 12R	Hettich, Tuttlingen, Germany
Eppendorf Centrifuge 5810R	Eppendorf AG, Hamburg, Germany
OptimaTM LE-80K Ultracentrifuge	Beckman Coulter, Brea, USA
NanoDrop® ND-1000	PEQLAB Biotechnology GmbH, Erlangen, Germany
UV Transilluminator	UVP, Upland, USA
Hanna Checker® pH meter	SIGMA-ALDRICH, St. Louis, USA
Navigator™	OHaus, Parsippany, USA
Caliper, stainless steel	Fisher Scientific, Waltham, USA
Aesculap clipper Isis	B. Braun, Melsungen, Germany
Isoflurane Vaporiser IsoFlo	Eickemeyer, Sunbury-on-Thames, UK

XI DANKSAGUNG

Mein besonderer Dank gilt meinem Doktorvater Herrn Prof. Dr. Gerd Sutter, der mir die Möglichkeit gab hier am Institut diese Arbeit anfertigen zu können, für die freundliche fachliche und immerwährende Unterstützung, sowie das Vertrauen in mich und meine Fähigkeiten und den Freiraum beim Anfertigen dieser Arbeit.

Mein herzlichster Dank geht an meinen Betreuer, Dr. Juan Rojas, der mich von Tag 1 betreut hat, eingearbeitet hat und mit dem Enthusiasmus für diesen Fachbereich angesteckt hat. Sein umfangsreiches Fachwissen war immer eine große Hilfe und Inspiration. Vielen Dank für die immerwährende Unterstützung und das offene Ohr, und für das ausdauernde und geduldige Lesen und Korrigieren meiner Arbeiten. Es war mir eine Ehre mit dir zu arbeiten!

My warmest thanks goes to my supervisor, Dr. Juan Rojas, who took me under his wing from day 1, trained me and infected me with the enthusiasm for this field of research. His broad expertise and expertise was always helpful and inspiring. Thank you for the constant support and the open ear. And thanks a lot for the constant and patient proofreading and correction of my written work. It was an honor working with you!

Ein großes Dankeschön geht an meine lieben (ehemaligen) Doktorandenkolleginnen und –kollegen Alina Tscherne, Hendrik Schwarz, Dr. Svenja Veit, Dr. Daniela Arndt, Dr. Dominik Forster, die mit mir diesen Weg beschritten haben, für die Zusammenarbeit und den mentalen Support. Wie sagt man so schön, geteiltes Leid ist halbes Leid. Es war mir eine Ehre mit euch dieses Leid geteilt zu haben!

Des Weiteren möchte ich mich bei **allen** Mitarbeiterinnen und Mitarbeiter

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des gesamten Lehrstuhls für Virologie und den Tierhaus-Mitarbeiterinnen und -Mitarbeitern für die allgegenwärtige Hilfsbereitschaft, Freundlichkeit und die angenehme Arbeitsatmosphäre bedanken. Mein besonderer Dank gilt Dr. Robert Fux, Dr. Michael Lehmann, Marlowe Peters, Christine Brandmüller, Sylvia Jany, Dr. Ellen Link und Nina Hechtberger, für die immerwährende fachliche und moralische Unterstützung. Auch möchte ich mich bei Prof. Dr. Asisa Volz für ihre tatkräftige Unterstützung beim Tierversuchsantrag bedanken.

Mein größter Dank gilt meiner Familie, die mich während dieser Zeit immer und überall unterstützt hat und an mich geglaubt hat. Mama, Papa, Philipp, Sunny vielen Dank für alles! Und Dani, vielen Dank, dass du mir immer und bedingungslos zu Seite stehst, für den ermutigenden Zuspruch in allen kleinen oder größeren Sinnkrisen und Nervenzusammenbrüchen. Ohne dich würde es diese Arbeit wahrscheinlich heute so nicht geben!