CHARACTERIZATION OF VACCINIA VIRUS MVA CANDIDATE VACCINES MUTATED IN VIRAL GENES MODULATING INFLAMMASOME ACTIVATION

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Für Mama und Papa.

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LIST OF ABBREVIATIONS

AA	Aminoacids
APAF-1	Apoptosis protease-activating factor 1
Bcl2	B-cell lymphoma 2 family
CEF	Chicken embryo fibroblasts
CTL	Cytotoxic T-lymphocyte
CVA	Chorioallantois Vaccinia virus Ankara
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
Del	Deletion site
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EV	Enveloped virion
FACS	Fluorescence-activated cell sorting
FADD	Fas associated via death domain
FCS	Fetal calf serum
gpt	*Escherichia coli* guanine phosphoribosyltransferase
GSDMD	Gasdermin D
HIV	Human immunodeficiency virus
hpi	Hours post infection
IFN	Interferon
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
IL-18bp	Interleukin 18 binding protein

IL-18R	Interleukin 18 receptor
IL-18Rα	Interleukin 18 receptor α chain
IL-18Rβ	Interleukin 18 receptor β chain
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	IL-1 receptor accessory protein
IL-1RI	IL-1 receptor Type I
IL-1RII	IL-1 receptor Type II
IRAKs	IL-1 receptor–associated kinases
kb	Kilobase
kDa	Kilodalton
MEM	Minimum Essential Medium Eagle
MHC	Major histocompatibility complex
MLKL	Mixed lineage kinase domain-like
MMP	Mitochondrial membrane permeabilization
MOI	Multiplicity of infection
MV	Mature virion
MVA	Modified Vaccinia virus Ankara
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
ORF	Open reading frame
OVA	Ovalbumin
p.i.	Post infection
p.v.	Post vaccination
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PFU	Plaque-forming units
PmH5	Modified promoter H5
PRR	Pattern recognition receptor
SEM	Standard error of mean
TAA	Tumor associated antigen
T_h1	Type 1 helper T cell
TIR	Toll-IL-1 receptor
TNF	Tumor necrosis factor
TRAF-6	TNF receptor associated factor 6
TSA	Tumor specific antigen
VACV	Vaccinia virus
vIL-18bp	Viral Interleukin-18 binding protein
vIL-1βR	Viral Interleukin 1β receptor
WHO	World Health Organization

I. INTRODUCTION

Today, cancer is still among the leading causes of death. In 2015 alone, an estimated 8.8 million people died from cancer worldwide. Thus, cancer still represents one of the major burdens for public health. Despite intensive research on new alternative treatment methods, most patients still rely on classical treatment options comprised of chemotherapy, radiotherapy and surgical intervention.

While many strategies are being pursuit, viral immunotherapy on the basis of viral-vectors delivering tumor-associated antigens is on the rise. In this context, poxviruses serve as a promising platform for the development and production of therapeutic cancer vaccines. Among others, the Modified Vaccinia virus Ankara (MVA), a member of the *Poxviridae* family, is being investigated as a potential vector candidate. MVA has already been successfully tested as a vector vaccine for various infectious diseases in several clinical trials. It is a highly attenuated vaccinia virus strain with an exceptional safety profile due to its inability to productively replicate in human and most mammalian cells. At the same time, MVA is able to accept large inserts of foreign DNA and express high levels of recombinant proteins. In addition, MVA does not need additional adjuvant application. Yet, it is able to induce humoral and cellular immune responses, especially high levels of antigen-specific T cells, which is an important feature for use as a therapeutic cancer vaccine.

The aim of this study was to investigate new approaches for enhancement of the efficacy of MVA as a viral vector vaccine against cancer. Therefore, two distinct, genetically modified recombinant MVA viruses were constructed and tested as candidate vaccines. These vector viruses are inactivated in two respectively three genes that encode for viral immune evasion proteins. For evaluation of vaccine immunogenicity and efficacy chicken ovalbumin (OVA) served as a model antigen.

II. LITERATURE REVIEW

1. Immunity to tumors

In general, cancer arises from the body's own cells that go into abnormal uncontrolled proliferation resulting in a tumor, yet not every tumor classifies as cancer. While some tumors stay local and are classified as non-cancerous or benign, malignant tumors are determined by their rapid proliferative capacity along with their ability to invade surrounding tissue and eventually metastasize to distant sites (HANAHAN & WEINBERG, 2011).

Since the beginning of cancer research the possibility of cancer eradication by a specific immune response has always been of interest. In the 1960's Frank Macfarlane Burnet firstly proposed the idea of immune surveillance in which he stated that the body's immune system is not only able to detect and destroy invading pathogens but also cells that become cancerous (BURNET, 1967). Today, the existence of such a surveillance system is an established concept but its importance in tumor formation is still being controversially discussed (CORTHAY, 2014).

1.1. Immune response to tumors

For a long time, tumors were believed to be non-immunogenic and unrecognizable for the immune system. Only the investigation of transplantable tumors in mice led to the discovery, that immune recognition of tumor cells is not impossible. In the key experiments of these studies, mice were firstly immunized with irradiated tumor cells. Upon injection of viable tumor cells of the same type, no tumor development was observed. In contrast, mice that were injected with viable tumor cells of a different kind quickly developed tumors. These findings indicated for the first time, that tumors can express antigens that become the target of the immune system and were therefore described as tumor rejection antigens (JAFFEE & PARDOLL, 1996; MURPHY et al., 2008b).

Today, it is widely accepted that tumor cells, although they are derived from host cells, are in fact immunogenic and elicit an adaptive immune response

(DISIS, 2010). Some tumors even regress spontaneously (e.g. melanomas) which is believed to be caused by an immunological response (PRINTZ, 2001). The immunogenicity of tumors implies that tumor cells can express one or more antigens which the adaptive immune system is able to detect and recognize as foreign. Therefore, those tumor antigens play a key role in the development of cancer immunotherapy and have become a focus in cancer research (PARISH, 2003).

1.2. Tumor antigens

Our immune system has developed a sophisticated system to differentiate between “self” and “non-self” structures. Ideally, everything non-self-discovered by the immune system triggers an immunological response that leads to elimination, while self-tolerance prevents destruction of the body’s own structures. Pathogens, such as bacteria, present a rich repertoire of proteins, lipids and carbohydrates on their cell surface, all of which are potential antigens for immune recognition. Thus, they are relatively easy detectable by immune cells. The problem that arises with cancer cells is that cancer derives from mutations that occur in the body’s own cells and these cells are protected from destruction by self-tolerance. However, these somatic mutations can also cause alteration of so far self-proteins presented of the cell surface. If those proteins were altered in a way that they can be detected by T-cells, they become immunogenic tumor antigens and can trigger an immunological response. At the same time, cancer cells sometimes also start to overexpress certain self-proteins, and this overexpression also makes them tumor-antigens that can be recognized by the immune system. Against this background, two types of tumor antigen can be differentiated: tumor-specific antigens (TSAs) caused by mutations and tumor-associated antigens (TAAs) caused by overexpression of self-proteins (SRINIVASAN & WOLCHOK, 2004; SCHIETINGER et al., 2008).

In theory, TSAs represent the ideal targets for cancer immunotherapy because they are highly specific, solely expressed by cancerous cells and often also play an important role in tumorigenicity (SCHIETINGER et al., 2008). However, because of their high specificity they cannot be used in a universal approach but immunotherapeutics would have to be custom made for each patient or at least small subgroups of patients, which is extremely

time-consuming and very expensive. Therefore, TAAs have become the main focus in the development of cancer immunotherapies and there is an ongoing intensive search for ever new antigens to be used. (NELLER et al., 2008; SCHIETINGER et al., 2008).

2. Immunotherapy and cancer vaccines

Over the last decades, intensive research on immunology and the biology of cancer and its development has given valuable new insights into how tumors develop and the way out immune systems reacts to them. Many studies have underlined the importance of tumor cell-specific cytolytic T-lymphocytes (CTLs) to mediate an efficient anti-tumoral response after successful recognition of TAAs (KENNEDY & CELIS, 2008). That is, because CTLs have been proven to recognize TAAs through MHC class I molecules and directly kill tumor cells via lysis (GARCIA-LORA et al., 2003; VIGNERON, 2015). In mice, tumor specific CTLs were shown to be able to mediate tumor regression (ROBBINS & KAWAKAMI, 1996). Consequently, efforts were made to gain more knowledge on the complex mechanisms involved in TAA recognition and T-cell activation. These new insights were used in the development of a number of novel cancer treatments that focus on the activation of immune response and most importantly activation of tumor-specific CTLs.

Among many different approaches is the broad field of cancer immunotherapy. Immunotherapy relies on the use of immune cells or the entire immune system to fight cancer cells (PARDOLL & DRAKE, 2012). Surprisingly, the idea of cancer immunotherapy goes back to the 19th century. Already in the 1890s, surgeon William Coley observed complete remission of cancer in a cancer patient infected with Streptococcus pyogenes (St. pyogenes). He then began injecting cancer patients with St. pyogenes (which became known as “Coley’s toxin”) and observed a cure rate of over 10% (COLEY, 1991; WIEMANN & STARNES, 1994). In the years that followed, attitude towards cancer immunotherapy kept changing from favor to disfavor and back again (PARISH, 2003). Today, there is little doubt that the immune system can detect and even eliminate tumor cells

and that immunotherapy is a promising concept in the field of novel cancer therapies. A first peak was reached in 2013, when it was declared breakthrough of the year by Science magazine (COUZIN-FRANKEL, 2013).

In general, two types of immunotherapy exist: active and passive immunotherapy and both approaches can either be specific or non-specific. While non-specific therapy only induces a general immune response, specific immunotherapy is able to elicit a tumor-specific immune response. Passive immunotherapy relies on the immunization with components of the immune system (e.g. monoclonal antibodies) that were created *ex vivo*. There is an immediate anti-tumoral effect, however it is only temporary and relies on repetitive administration. As on the other side, active immunotherapy activates the immune system to induce its own lasting response and recognize respectively attack tumor cells (SCHUSTER et al., 2006). Recognition of tumor cells is achieved through specific TAAs introduced to the immune system in various ways. One way possible is the introduction of TAAs via therapeutic vaccines based on viral vectors.

2.1. Vector-based delivery systems

The general aim of every therapeutic cancer vaccine is to adequately generate an efficient immune response against a specific TAA. In 1994, it was already shown that such TAAs are recognized by both CD4+ and CD8+ T-cells. In addition, activated CD8+ T-cells were reported to be able to directly lyse TAA-presenting tumor cells (BOON et al., 1994). Out of the several ways of how to introduce TAAs to the immune system, the vector-mediated presentation of TAA was shown to be highly efficient and presents some advantages. It was described to lead to an increase in frequency and avidity of TAA specific cytotoxic CD8+ T-cells (YANG et al., 2005). Furthermore, a vector-mediated presentation of antigens to the immune system was shown to be more immunogenic than when antigens were administered as a whole protein with adjuvant (KASS et al., 1999). Taken together it becomes clear that viral-vector based delivery platforms have a high potential in the development of therapeutic cancer vaccines. In this context, poxviruses have emerged as promising candidates for the development of safe and efficacious vaccines for both infectious diseases and cancer (SANCHEZ-SAMPEDRO et al., 2015).

Although there are many different viral vectors being investigated which are very diverse, one important feature of all of them is their replication competence in humans. Vectors that cannot only infect cells but are also able to replicate and spread are being investigated for use in oncolytic virotherapy. The term oncolytic refers to the property of these viruses to selectively replicate in cancer cells and by doing so, killing them through lysis and spreading to uninfected tumor cells (FUKUHARA et al., 2016)

At the same time, lysis not only actively destroys tumor cells but also triggers an immune response against the tumor, likely due to the release of tumor antigens (LAROCCA & SCHLOM, 2011). In addition, they are mostly engineered to also express one or more TAAs to elicit an additional anti-tumoral immune response. That way, oncolytic viruses are able to fight cancer cells at two fronts. In this context, Vaccinia virus (VACV) has emerged as a promising oncolytic agent that is being engineered to express various TAAs and has entered numerous clinical trials (RUSSELL et al., 2012). However, the replication capacity of oncolytic vectors is advantage and disadvantage at the same time. While healthy individuals usually show good responses and tolerate the vector quite well, immunosuppressed people are at great risk to experience serious adverse effects, even with fatal outcome, due to replication of the vector beyond control (KELLY & RUSSELL, 2007). That is of course of major concern in cancer patients, especially when oncolytic viruses are used as follow-up treatment after radiation and/or chemotherapy. Because of that, more and more replication deficient viruses are being investigated as therapeutic cancer vaccines. They can be engineered to express TAAs and elicit a strong anti-tumor immune response, but due their replication deficient phenotype, they can safely be administered to immunocompromised people and can serve as a save alternative to replicating oncolytic viruses. In this context, Modified Vaccinia virus Ankara has evolved as a promising candidate for the development of TAA expressing therapeutic cancer vaccines (KREIJTZ et al., 2013).

3. Modified Vaccinia virus Ankara

3.1. MVA: a member of the Poxvirus family

Modified Vaccinia virus Ankara (MVA) is a highly attenuated strain of VACV, a member of the family *Poxviridae*. The family is divided into two subfamilies, the vertebrae specific *Chordopoxvirinae* and insect specific *Entomopoxvirinae*. The *Chordopoxvirinae* subfamily is further divided into nine genera, one of them being the genus *Orthopoxvirus*. VACV is the prototype member of the genus *Orthopoxvirus* along with one of its most famous members, variola virus, the causative agent of smallpox.

Poxviruses are exceptionally large enveloped viruses with brick-shaped geometry (ca. 250 nm x 360 nm). They contain a large (130-300 kilobase pais) s-shaped genome of double stranded DNA (dsDNA) with single stranded terminal hairpin loops (MOSS et al., 1996). The central region of the genome is highly conserved among poxviruses and mostly consists of open reading frames (ORFs) associated with replication while the terminal regions are more diverse and dedicated to host range and immune evasion (MOSS, 2007; WERDEN et al., 2008). Poxviruses present two distinct forms of infectious particles: a mature virion (MV) and an extracellular enveloped virion (EV). Their structure is very similar with a dsDNA containing Core, two lateral bodies and an outer membrane. The EV additionally possesses another outer protein containing lipid membrane, the EV envelope (MOSS, 2007) (Fig. 1). While MVs represent the more frequent particle, EVs are essential for an efficient cell-to-cell and long-range spread (BLASCO & MOSS, 1992).

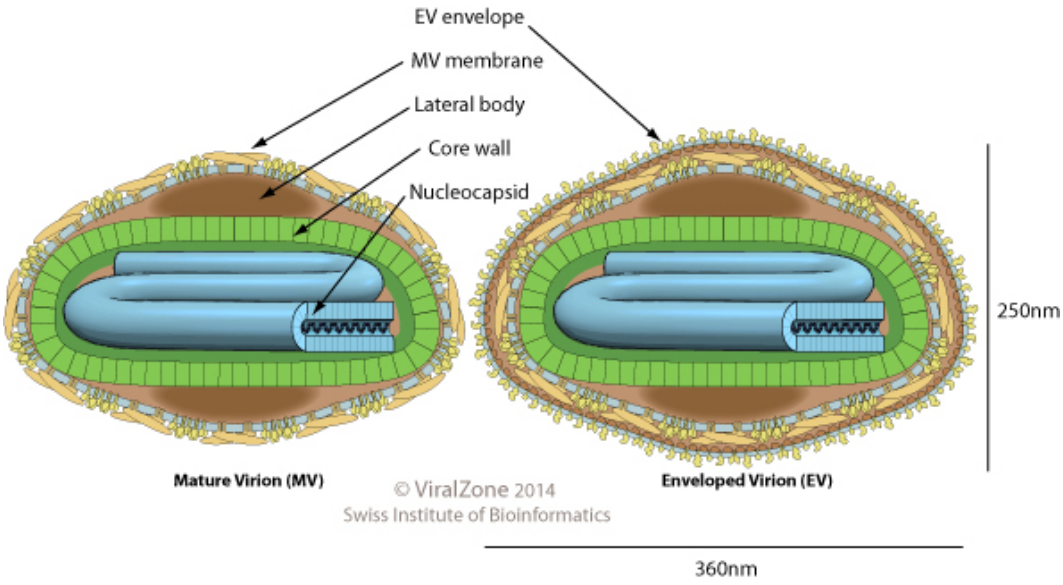

Fig. 1: Poxvirus morphology

(Source: ViralZone; www.expasy.org/viralzone; SIB Swiss Institute of Bioinformatics, with permission)

3.2. Poxviruses as viral vectors

Despite the fact that many poxviruses are the cause of serious disease, some even with zoonotic potential (BAXBY, 1988) they also have a long history in the development of potent vaccines (SANCHEZ-SAMPEDRO et al., 2015). Most famously, VACV was used as the active component of the smallpox vaccine that was used in the worldwide smallpox eradication program with over one billion people vaccinated. The program was declared a success in 1979, which made smallpox the first infectious disease to have ever been eradicated (FENNER et al., 1988). Post-eradication, the study of VACV was perpetuated and focused on the use of VACV for gene expression and viral vector based vaccines (MACKETT et al., 1982; PANICALI & PAOLETTI, 1982). Especially in terms of vector-based vaccine development, poxviruses in general promise many advantages. They have been proven to make stable recombinants along with successful processing of foreign proteins (SUTTER & MOSS, 1992). Furthermore, due to their own large genome, they are not only capable of accepting large inserts of foreign DNA (SMITH & MOSS, 1983) but can even accommodate multiple genes at once, which enables them to express more than one antigen (PERKUS et al., 1985). Furthermore, it was shown, that the expressed antigen is

processed and then presented by both major histocompatibility complex (MHC) class I and II molecules, thus activating both CD4+ and CD8+ T-lymphocytes (TSANG et al., 1995). In addition, an antigen-specific humoral response has also been described along with the induction of protective immunity in mice (SUTTER et al., 1994). Regarding the safety profile of poxviruses it is of advantage, that their replication is limited to the cell's cytoplasm, thus no risk of insertional mutagenesis exists (ROBERTS & SMITH, 2008). Furthermore, poxviruses do not persist in the host after infection. However, some side effects have also been observed, partly severe, after VACV inoculation, mostly attributed to its replicative capacity. Particularly, immunocompromised people were at risk, which led to the search for safer poxvirus alternatives. The search came to an end with the discovery of second-generation VACV derived vectors, such as replication deficient MVA (SUTTER & MOSS, 1992).

3.3. MVA as an expression vector

MVA was derived from Chorioallantois Vaccinia virus Ankara (CVA) after CVA was passaged more than 500 times in primary chicken embryo fibroblasts (CEF) in an attempt to generate a VACV with reduced virulence (MAYR & MUNZ, 1964; MAYR et al., 1975). During passaging, MVA lost about 15% of genomic information compared to CVA, including six major deletion sites and numerous other small deletions, most of which affect virulence and immune evasion, drastically reducing pathogenicity (MEYER et al., 1991; ANTOINE et al., 1998). As a result, MVA was found to be no longer able to replicate in human and most other mammalian cells but its propagation was limited to avian cells, making it an exceptionally safe vaccine candiate (MEYER et al., 1991; DREXLER et al., 1998). MVA's safety profile was confirmed after more than 100,000 individuals in Bavaria received MVA as a smallpox vaccine and only little side effects were observed (STICKL et al., 1974; MAYR et al., 1978). Moreover, MVA has also been extensively studied as an expression vector for the construction of vaccines against different infectious dieseases and cancer (SUTTER & STAIB, 2003; KREIJTZ et al., 2013; VOLZ & SUTTER, 2017). Although MVA lacks replicative capacity, it can still infect mammalian cells with no

negative effect on early, intermediate and late gene expression, making way for the successful expression of foreign antigens (SUTTER & MOSS, 1992). Remarkably, when comparing replication competent VACV to MVA expressing the same antigen in terms of immunogenicity and protective capacity, MVA was shown to be equally effective if not slightly better (SUTTER et al., 1994; CARROLL et al., 1997). Concerning immunogenicity, many studies have proven MVA's capacity to potently stimulate innate immunity, including the induction of IL-1β (WAIBLER et al., 2007; DELALOYE et al., 2009; LEHMANN et al., 2009). Furthermore it was shown, that no adjuvants are required in an MVA based vaccine compared to non-vector based vaccines, adding to its good safety profile (KREIJTZ et al., 2013). To further assess safety, MVA was tested in immunosupressed monkeys and no severe side effects were observed, confirming once again that MVA in contrast to VACV can be administered to immunocompromised people (STITTELAAR et al., 2001).

One concern often expressed for the use of viral vectors is the induction of neutralizing antibodies against the vector itself, limiting their use to a one-time-only application. Despite MVA-neutralizing antibodies being detectable after immunization, several studies have shown that strong antigen-specific immune responses can be boosted repeatedly upon multiple administrations of recombinant MVA (HARROP et al., 2010; GOEPFERT et al., 2011; KREIJTZ et al., 2014). Thus, MVA was found to be an efficacious, immunogenic alternative to VACV that holds many advantages and presents a very high safety profile for clinical use.

Today, MVA is a well studied viral vector in both preclinical and clinical research. Many recombinant MVAs have been developed as vaccine candidates against different infectious diseases, e.g. influenza, west nile, HIV and also bacterial and intracellular pathogens like malaria and tuberculosis. In more recent years, MVA has also become increasingly interesting for the construction of various therapeutic cancer vaccines with many of these candidates having already entered clinical trials. (GILBERT, 2013; KREIJTZ et al., 2013; ALTENBURG et al., 2014; GOEPFERT et al., 2014; KREIJTZ et al., 2014; SHEEHAN et al., 2015; SEBASTIAN &

GILBERT, 2016; VOLZ & SUTTER, 2017).

3.4. Immunomodulation by MVA

In theory, virus infection of a cell is detected by pattern recognition receptors (PRRs) and leads to an immune response, which interrupts replication, stops spreading and eventually clears the infection while possibly inducing immunity. In response, most viruses, including poxviruses, have evolved many strategies to either avoid detection or directly interfere with the host's immune response (i.e. immunomodulation) (MURPHY et al., 2008b). For example, such immunomodulatory strategies interrupt signaling pathways that would lead to cell death, production of interferons (IFNs), cytokines and chemokines as well as the activation of cytotoxic T-lymphocytes (CTLs) and natural killer cells (NKs) (SEET et al., 2003; HAGA & BOWIE, 2005). As a result of extensive studies conducted on virus-host-interaction in VACV as a prototype poxvirus, many immunomodulatory genes have been identified and described over the years and it is estimated that VACV dedicates about one half of its genome to encode virulence, host-range and immunomodulatory proteins (SMITH et al., 2013). In general, immunomodulatory proteins found in poxviruses can be subdivided into three different functional classes: virostealth, viromimicry and virotransduction. Virostealth describes the ability to block the presentation of antigen to immune cells, thus down regulating antigen recognition. Viromimicry means the production of viral proteins that mimic cytokines, chemokines or their receptors and virotransduction is the inhibition of innate antiviral responses such as the induction of apoptosis in infected cells (JOHNSTON & MCFADDEN, 2003).

However, the majority of immunomodulators known in VACV were depleted in MVA during passaging in CEF cells. Nevertheless, few immunomodulators are still present in MVA, some of which have been identified, studied and were shown to influence both, the innate as well as the acquired immune response (GARCIA-ARRIAZA & ESTEBAN, 2014).

The study of MVA and its interaction with the host's immune response is an especially important aspect when it comes to the use of MVA as a viral vector for therapeutic cancer vaccines. That is because immunomodulators

interfere with the fundamental principle of any given vaccine: to provoke an adequate and strong immune response. Therefore, depletion of certain immunomodulatory genes in MVA can be an attractive approach to further enhance MVA's immunotherapeutic effects and strengthen its capacity to induce strong immunity against the desired antigen(s).

3.4.1. Interleukin-1β

Interleukin 1β (IL-1β) is a well-known member of the interleukin-1-superfamily. Originally only comprised of IL-1α and IL-1β, today the family includes a total of 11 cytokines. Stronger than any other cytokine family, the IL-1 family is primarily linked to inflammation, both acute and chronic. Most of its members including IL-1β show strong pro-inflammatory properties while only one (IL-37) is known to be anti-inflammatory (DINARELLO, 2011). Structurally, IL-1β is a monomer, consists of 153AA and is primarily produced by activated macrophages (MURPHY et al., 2008a). It is synthesized in form of an inactive 31kDa proenzyme and activation occurs upon cleavage of pro-IL-1β into mature 18kDa IL-1β by activated Caspase-1 (formerly known as the Interleukin-1 converting enzyme, ICE) in response to various danger signals (DINARELLO, 2009; LOPEZ-CASTEJON & BROUGH, 2011). IL-1β is known to activate T-cells, the vascular endothelium and to lead to local tissue destruction at the side of release. Systemically, it is a very potent endogenous pyrogen inducing fever and the major pyrogen in a poxvirus infection (ALCAMI & SMITH, 1996). In addition, IL-1β activates hepatocytes to synthesize and release acute-phase proteins, which then act as opsonins (MURPHY et al., 2008c). In contrast to VACV, IL-1β is one of the pro-inflammatory cytokines that was shown to be induced during MVA infection (DELALOYE et al., 2009).

Il-1β and IL-1α both share the same receptors which belong to the IL-1 receptor (IL-1R) superfamily: The Type I IL-1 receptor (IL-1RI, CD121a) and the Type II IL-1 receptor (IL-1RII, CD121b), both of which are able to bind mature IL-1β. To initiate a signal, recruitment of a coreceptor is required, the IL-1 receptor accessory protein (IL-1RAcP). However, only binding to the IL-1RI can activate a signal transduction (Fig. 2a) since IL-1RII lacks the needed cytoplasmatic domain, thus serves as a decoy receptor to regulate levels of IL-1β (Fig. 2b). Besides the occurrence of a decoy receptor, a

receptor antagonist additionally exists for the regulation of IL-1β secretion: the Interleukin-1 receptor antagonist (IL-1Ra) is able to bind to IL-1RI and by doing so blocking it for IL-1β (DINARELLO, 2009).

As shown in figure 2a, mature IL-1β first binds to the IL-1RI which is followed by the recruitment of IL-1RAcP, forming a heterodimeric complex. The signal is then initiated through the recruitment of the adaptor protein MyD88 by the two Toll-IL-1 receptor (TIR) domains of both receptors. This leads to phosphorylation of IRAKs followed by IKKβ, eventually resulting in the translocation of NF-κB into the nucleus and initiation of pro-inflammatory signals. At the same time, recruitment of MyD88 also stimulates the transcription and translation of IL-1β-mRNA in a way that mature IL-1β up regulates itself (DINARELLO, 2009). In addition to activation by Caspase-1, non-caspase dependent mechanisms have also been identified that are able to generate active IL-1β (FANTUZZI et al., 1997).

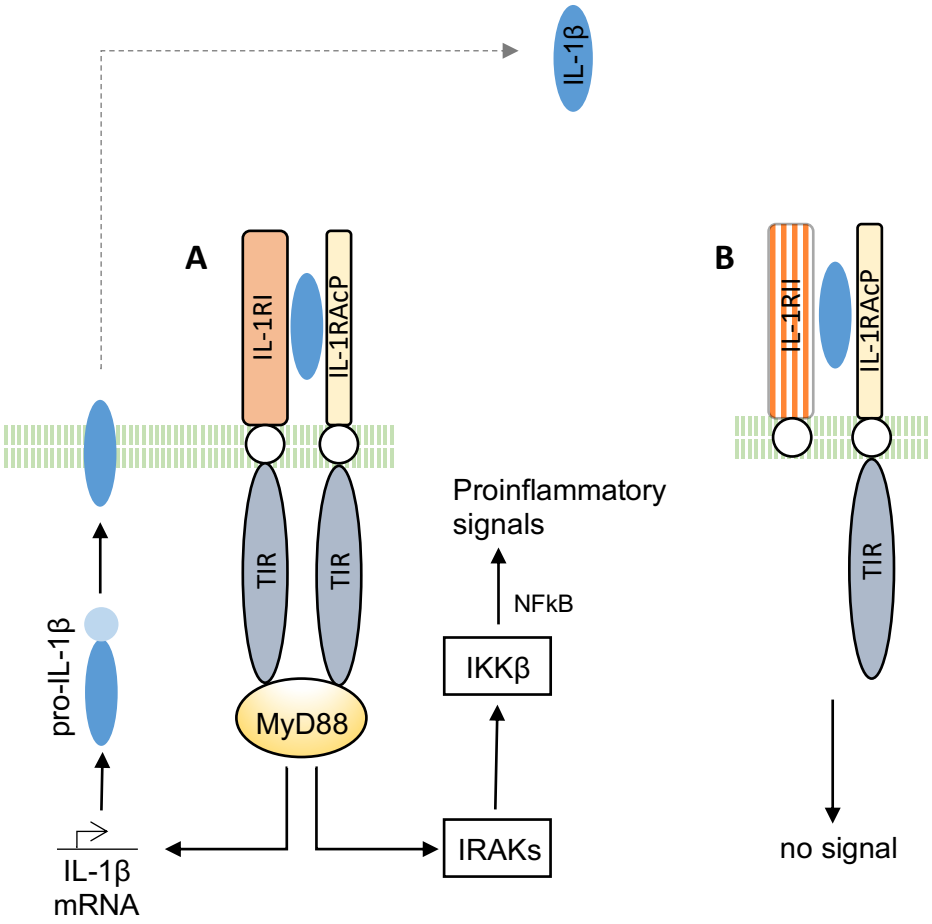

Fig. 2: IL-1β pathway. (A) Only binding to IL-1RI can initiate a signal that leads to pro-inflammatory stimuli and up regulation of IL-1β. (B) IL-1RII is missing a cytosolic TIR domain and serves as a decoy receptor.

3.4.2. MVA's IL-1β receptor homolog

One of the immunomodulatory proteins still conserved in MVA is the viral IL-1β receptor (vIL-1βR) homolog, which is encoded by the 927bp open reading frame (ORF) 184R. The secreted soluble protein is 326AA in length (MEISINGER-HENSCHEL et al., 2007) and was shown to be expressed late during the life cycle, which is consistent with the viral IL-1βR homolog described in VACV (ZIMMERLING et al., 2013). Furthermore, it was also shown to be highly specific for binding mature IL-1β whereas it is unable to bind Interleukin-1α (ALCAMI & SMITH, 1992; BLANCHARD et al., 1998). Thus, MVA's vIL-1βR mimics the host cell's own IL-1RI and is an example of viromimicry as described before (JOHNSTON & MCFADDEN, 2003).

In 1996, the vIL-1βR was published as the first example of how viruses are able to inhibit fever to promote virus survival (ALCAMI & SMITH, 1996).

While the deletion of the homolog B15R ORF in VACV was demonstrated to clearly enhance severity of illness and overall pathogenicity in mice (ALCAMI & SMITH, 1992), it was reported for MVA that its avirulent phenotype remained intact. However, depletion of the gene in MVA was shown to lead to an enhanced memory CD8+ T-cell response in mice towards the inserted antigens, resulting in a better protection against a lethal challenge (STAIB et al., 2005; ZIMMERLING et al., 2013).

3.4.3. Interleukin-18

Interleukin 18 (IL-18) is a pro-inflammatory cytokine produced by activated macrophages and dendritic cells and was originally identified in 1989 as the "IFN-γ inducing factor" (NAKAMURA et al., 1989; OKAMURA et al., 1995; WAWROCKI et al., 2016). It is produced as a precursor protein and is structurally related to the IL-1 family, which is resembled by the fact that premature IL-18 is also activated by caspase-1 (GU et al., 1997). In addition, the IL-18 receptor (IL-18R) shares sequence homology to the IL-1R superfamily (BORN et al., 1998; THOMASSEN et al., 1998). Therefore, it is currently considered a full member of the IL-1 superfamily. In contrast to IL-1β, the IL-18 precursor protein is continuously expressed in several cells including epithelial cells throughout the gastrointestinal tract (DINARELLO et al., 2013). Concerning its function, IL-18 plays an important role in the regulation of both the innate and adaptive immune response. It stimulates IFN-γ production in natural killer cells (NKs) and T-cells. Furthermore, it promotes NK cell cytotoxicity and stimulates T-cell proliferation (BORN et al., 2000; READING & SMITH, 2003). IL-18 also acts synergistically with IL-12 and together they promote a CD4+ T-cell response (T_h1), increasing IFN-γ production and cell-mediated immunity, defending the cell against intracellular microbes (READING & SMITH, 2003; MURPHY et al., 2008c). The antiviral effect of IL-18 in poxvirus infections was shown when in mice, which were intravenously inoculated with VACV, development of poxvirus legions was suppressed by treatment with IL-18 (TANAKA-KATAOKA et al., 1999).

IL-18 binding proteins (IL-18bps) exist and have been described in both humans and mice. Although they share no significant homology to neither subunit of the IL-18R, they can bind and neutralize mature IL-18 and

supposedly play an important regulatory role and can modulate the T_h1 response (NOVICK et al., 1999).

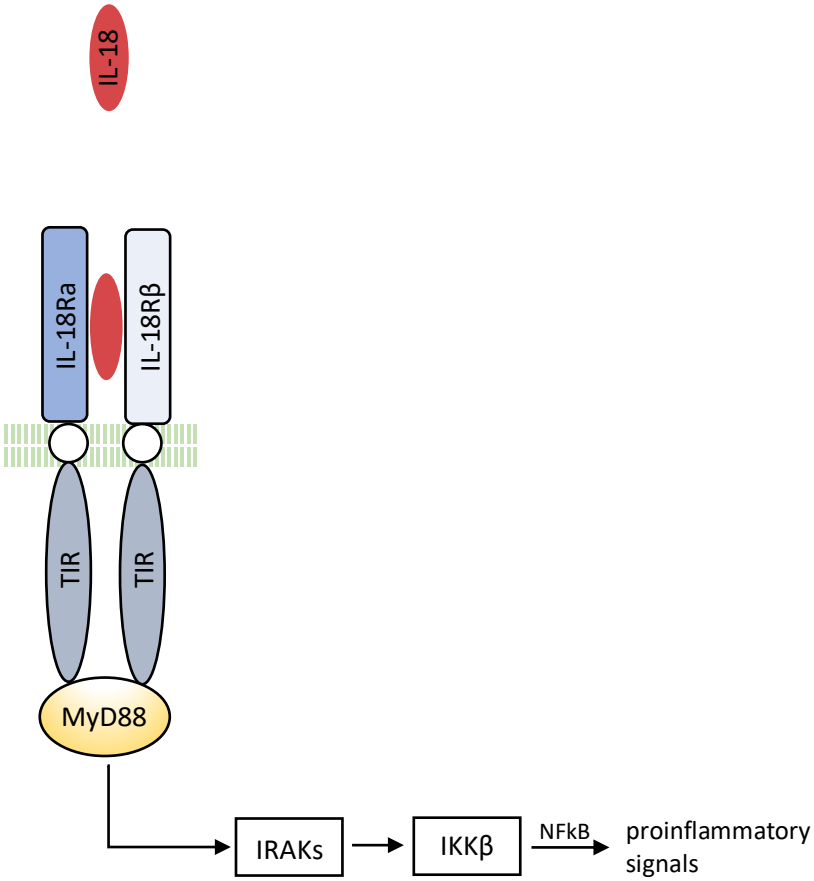

Fig. 3: IL-18 signal transduction. Mature IL-18, IL-18Rα and IL-18Rβ form a high affinity complex, leading to the approximation of both receptor TIR domains followed by recruitment of MyD88, phosphorylation of the four IRAKs and TRAF-6, degradation of IKKβ and release of NFκB. (modified according to (DINARELLO & FANTUZZI, 2003), with permission).

The IL-18R is composed of two subunits, the IL-18Rα chain and the IL-18Rβ chain. The α chain is considered the ligand binding chain for mature IL-18 while the β chain is considered a co-receptor needed for a full signal transduction similar to IL-1RAcP. Thus, only cells expressing both chains are able to signal. Firstly, mature IL-18 forms a complex of low affinity with IL-18Rα. Only after additional recruitment of IL-18Rβ, a high affinity complex in form of a heterodimer is formed, followed by an approximation of two TIR domains. What follows is a cascade of recruitment of MyD88, phosphorylation of the four IRAKs and TRAF-6, degradation of IKKβ and

finally the release of NFκB as a broad proinflammatory stimulus (Fig.3) (WAWROCKI et al., 2016). In contrast to IL-1, where very low concentrations are sufficient to trigger a signal, IL-18 requires much higher levels (LEE et al., 2004).

3.4.4. MVA's viral IL-18 binding protein

Another VACV immunoregulatory protein that is still conserved in MVA is the viral IL-18 binding protein (vIL-18bp). It is encoded by the 008L ORF (C12L in VACV), which is 360 bp in length (MEISINGER-HENSCHEL et al., 2007). The mature protein is approximately 13kDa in VACV and is secreted as a soluble protein from infected cells that is able to bind mature IL-18 (BORN et al., 2000; SYMONS et al., 2002). The vIL-18pb is widely distributed among orthopoxviruses and other poxvirus genera and its amino acid sequence was shown to be highly homologous to IL-18 binding proteins found in humans and mice (NOVICK et al., 1999; SYMONS et al., 2002). Several deletion mutants have been constructed and tested concerning virulence and immunogenicity. Deletion of the C12L gene from VACV strain Western Reserve (WR) led to virus attenuation (SYMONS et al., 2002). Deletion of the corresponding ORF in ectromelia virus was associated with higher levels of IFN-γ and increased cytotoxic activity of NK cells (BORN et al., 2000).Deletion of the vIL-18bp from MVA was shown to improve the vector immunogenicity by increasing magnitude and quality of the specific cellular response (FALIVENE et al., 2012).

3.4.5. Cell death and immunity

Regulated cell death is a physiological property of all somatic cells and occurs in response to a change in either intra- or extracellular environment (ELMORE, 2007). It is a highly regulated and finely controlled mechanism to remove damaged or infected cells and considered a powerful anti-viral tool (HENGARTNER, 2000). One reason for that is that cell death inevitably leads to the complete shut-down of the cell including the entire intracellular machinery. Viruses however rely on a functional intracellular environment to productively replicate before spreading to other cells or tissues and continue the chain of infection (COLLINS, 1995; RAZVI & WELSH, 1995). Hence, cell death interrupts the virus replicative cycle and if it occurs early

enough during infection, it will stop further spreading (HAGA & BOWIE, 2005). Furthermore, regulated cell death can potentially alert neighboring cells to the threat of infection via the release of immunostimulatory molecules (TAYLOR et al., 2008). Thus, it is of no surprise that many viruses, including poxviruses, encode different anti-apoptotic proteins to cope with this defense strategy, preserve cell viability and prolong virus survival (ROULSTON et al., 1999; TAYLOR & BARRY, 2006; GALLUZZI et al., 2008). Cells on the other hand present a rich repertoire of different types of cell death and versatile signaling ways to induce them. In principle, three different types of cell death can be differentiated, i.e. apoptosis, pyroptosis and necroptosis (JORGENSEN et al., 2017).

3.4.5.1. Apoptosis

Apoptosis, or programmed cell death, is characterized by the disassembly of the cell from within while the plasma membrane remains intact. One distinct event of apoptotic cell death is karyorrhexis, i.e. the destructive fragmentation of the cell's nucleus, which leads to irregular distribution of chromatin throughout the cytoplasm (ZAMZAMI & KROEMER, 1999). But because the plasma membrane remains intact, no intracellular components potentially harmful to neighboring cells and surrounding tissue are released (TAYLOR et al., 2008). What is left is so called apoptotic bodies, which are eliminated via phagocytosis by dendritic cells (DC's) (GREINER et al., 2006). Once the DC's pick-up the apoptotic bodies, they are processed and foreign antigens are presented to T- lymphocytes via MHC class I molecules, enhancing the immune response. This process is known as cross-presentation and allows for apoptosis to be of immunogenic importance via the activation of CD8+ cytotoxic T- lymphocytes (CTLs) (ALBERT et al., 1998). Furthermore, it was also shown that cells undergoing apoptosis send signals, which induce the migration of phagocytes (LAUBER et al., 2003).

Apoptosis and its initiation are highly regulated by the cell, primarily because once activated, apoptosis cannot be stopped and inevitably leads to cell death (BOHM & SCHILD, 2003). In principle, it can be triggered by two different pathways, the intrinsic or mitochondrial pathway and the extrinsic pathway, both of which culminate in the activation of terminal caspases (i.e.

caspase 3 and 7), which activate nucleases and lead to apoptosis (GALLUZZI et al., 2008) (Fig. 4).

The extrinsic pathway

The extrinsic pathway is a death-receptor mediated cascade triggered by death ligands such as Fas ligand (FAS) and tumor necrosis factor α (TNFα) that bind to the tumor necrosis factor receptor (TNFR) on the cell surface. Upon binding, oligomerization of ligand and receptor occurs and induces the intracellular assembly of the so-called death-inducing signaling complex (DISC). This complex is essential to create yet another complex, the ripoptosome (complex IIa), consisting of receptor interacting protein kinase 1 (RIPK1), Fas associated via death domain (FADD) and pro-caspase-8 (TENEV et al., 2011). The ripoptosome activates caspase 8 through cleavage of pro-caspase-8. Mature Caspase-8 is then biologically active and activates the terminal caspases (i.e. caspase-3, -6 and -7) and nucleases, finally causing apoptosis (Fig. 4) (GALLUZZI et al., 2008).

The intrinsic pathway

The intrinsic pathway on the other hand is controlled by the mitochondrium, which serves as a control unit for incoming competing pro-apoptotic and anti-apoptotic signals. Apoptosis is initiated once the pro-apoptotic proteins overcome the anti-apoptotic proteins. Both groups belong to the B-cell lymphoma 2 family (Bcl-2) that includes Bcl-2 and Bcl-2-like proteins. While anti-apoptotic proteins share similarity in four Bcl-2-homology (BH) domains, pro-apoptotic proteins only share homology to the third domain (BH3) (WILLIS & ADAMS, 2005; CHIPUK et al., 2010). Anti-apoptotic proteins prevent cell death by maintaining mitochondrial integrity through the inhibition of effectors Bax and Bak (CORY & ADAMS, 2002; YOULE & STRASSER, 2008). In contrast, pro-apoptotic proteins such as Bid and Bim are able to activate Bax and Bak (REN et al., 2010). Upon activation, Bax and Bak oligomerize and form pores in the mitochondrial membrane, referred to as mitochondrial membrane permeabilization (MMP) (KORSMEYER et al., 2000; DEGLI ESPOSTI & DIVE, 2003). MMP leads to

the release of the apoptogenic molecule cytochrome c into the cytosol (HENGARTNER, 2000). Once inside the cytosol, cytochrome c enables the assembly of the so called apoptosome complex, consisting of pro-caspase-9 and the apoptosis protease-activating factor 1 (APAF-1). The apoptosome activates caspase-9, which then again activates terminal caspases and causes apoptosis. At the same time, the intrinsic pathway can additionally be activated by the extrinsic pathway, because mature caspase 8 can not only activate terminal caspases but also the pro-apoptotic BH3-only protein Bid (Fig. 4) (SHIMIZU et al., 1999; GALLUZZI et al., 2006; GALLUZZI et al., 2008).

3.4.5.2. Pyroptosis

Pyroptosis is a more recently described form of cell death and unlike apoptosis it is characterized by lysis of the cell and the release of intracellular pro-inflammatory content (COOKSON & BRENNAN, 2001). Like apoptosis, it is caspase dependent but it is solely induced by caspase-1 (MIAO et al., 2011). Thus, caspase-1 deficient cells are unable to perform pyroptosis and usually undergo apoptosis when triggered (FINK & COOKSON, 2005). Caspase-1 can proteolytically activate caspase 7, one of the terminal caspases of apoptosis, showing a cross link between apoptosis and pyroptosis (MIAO et al., 2011).

Caspase-1 is present in the cytosol of phagocytic cells in form of an inactive proenzyme termed pro-Caspase-1. Stimulation by various danger signals leads to the formation of the inflammasome, a multi protein complex consisting of an adaptor molecule (ASC), a cytosolic PRR (e.g. NLR) and pro-Caspase-1. Formation of the inflammasome facilitates cleavage of pro-Caspase-1 and subsequent release of active Caspase-1 (FRANCHI et al., 2009; SCHRODER & TSCHOPP, 2010). Caspase-1 is considered a highly pro-inflammatory caspase because it proteolytically cleaves the precursor proteins of pro-inflammatory cytokines IL-1β and IL-18 into active proteins (FANTUZZI & DINARELLO, 1999). However, recent data has shown that Caspase-1 also cleaves the gasdermin D (GSDMD) protein. GSDMD cleavage then results in an N-terminal product, GSDMD-NT, which was

proven to trigger pyroptosis (HE et al., 2015; LIU et al., 2016). It mediates the formation of plasma-membrane pores, allowing influx of water into the cell that leads to swelling, membrane rupture and lysis, explaining the distinct morphological differences of pyroptosis and apoptosis (LIU et al., 2016).

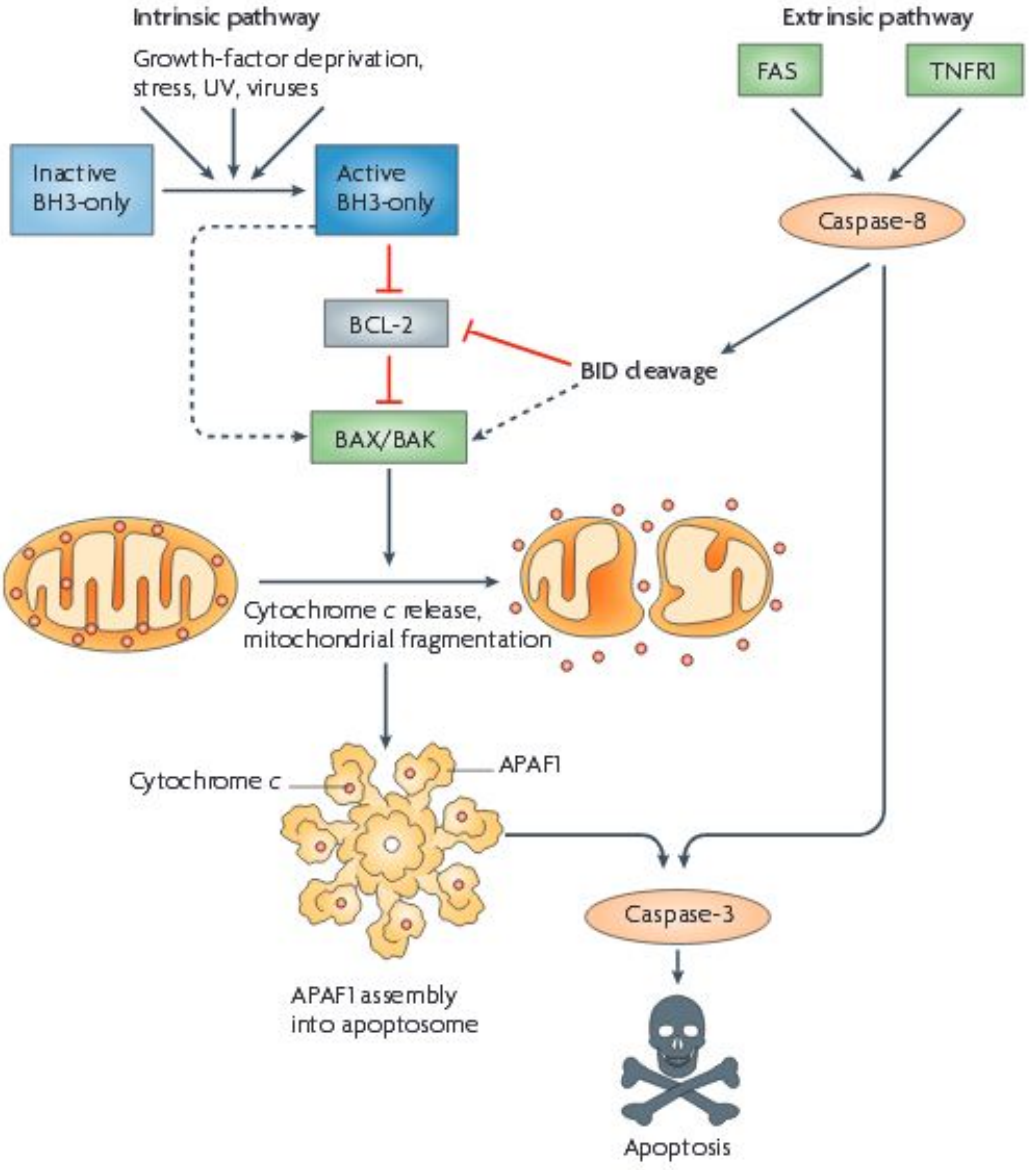

Fig. 4: The intrinsic and extrinsic pathways of apoptosis. While the intrinsic pathway is orchestrated by the mitochondrium, the extrinsic pathway is a death-receptor mediated cascade (YOULE & STRASSER, 2008, with permission).

3.4.5.3. Necroptosis

Necroptosis, also referred to as programmed necrosis, is yet another form of programmed cell death. Like the extrinsic apoptotic pathway, necroptosis is triggered by TNF. The difference between both ways is that necroptosis is caspase-8 independent (VERCAMMEN et al., 1998). Thus, it occurs when the extrinsic pathway of apoptosis is triggered but caspase-8 activity is compromised (CHAN et al., 2015). In this case, the ripoptosome (complex IIa) is formed but is either lacking caspase-8 completely or contains an inactive form of caspase-8 (e.g. due to caspase-8 inactivating infectious agents or certain pharmaceuticals). This leads to the stabilization of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and RIPK3 and the conversion of the ripoptosome into the necrosome (complex IIb) (CHAN et al., 2015). This new complex activates mixed lineage kinase domain-like (MLKL), which is phosphorylated, oligomerized and eventually translocated into the cell membrane, where it causes membrane leakage and the release of damage-associated molecular patterns (DAMPs) (SUN et al., 2012; CAI et al., 2014; SUN & WANG, 2014; CHAN et al., 2015).

3.4.6. MVA's F1 protein

Another immunomodulatory protein still conserved in MVA is the F1 protein, encoded by the F1L ORF. The mature protein is 26 kDa in size and was shown to be expressed early during the viral life cycle (POSTIGO et al., 2006). It has been identified as a viral Bcl-2 homologous protein in VACV that inhibits the mitochondrial apoptotic pathway (WASILENKO et al., 2003). Although homology between F1 and Bcl-2 is rare with no obvious sequence semblance, F1 was shown to possess a C-terminal transmembrane domain, which serves as an anchor and enables mitochondrial localization necessary for anti-apoptotic activity (WASILENKO et al., 2003; STEWART et al., 2005; CAMPBELL et al., 2010).

The anti-apoptotic activity is exerted by F1 through the prevention of release of cytochrome c into the cytosol. F1 was shown to directly interact with Bak as well as the BH3 domain of Bim. By binding to Bak, F1 prevents oligomerization of Bak with activated Bax, disabling pore formation (WASILENKO et al., 2005; POSTIGO et al., 2006; CAMPBELL et al., 2010).

At the same time activation of Bax is also restricted by inhibition of Bim (TAYLOR et al., 2006) by F1 with the same effect. Additionally, F1 was also shown to bind to and inhibit already activated caspase-9. Accountable for this is the N-terminal region of the F1 protein (ZHAI et al., 2010). Thus, the F1 protein enables VACV to interrupt the intrinsic apoptotic pathway of infected cells in two sequential steps (WASILENKO et al., 2001; ZHAI et al., 2010) (Fig. 5)

Besides inhibition of the mitochondrial pathway of apoptosis, F1 has recently been reported to also interfere with pyroptosis. It was shown to inhibit the formation of the inflammasomes by targeting proteins of the nucleotide-binding domain, leucine-rich repeat and pyrin domain containing protein (NLRP) family. Inhibition of the inflammsomes prevents activation of caspase-1, hindering gasdermin D cleavage and thus preventing pyroptosis (GERLIC et al., 2013). At the same time, prevention of caspase-1 activation also reduces levels of pro-inflammatory cytokines IL-18 and IL-1β which need caspase-1 cleavage for activation (Fig. 5).

For MVA, it was shown that expression of F1 prohibited cells from undergoing apoptosis. At the same, absence of the F1L ORF in MVA led to apoptosis induction and that this induction relies on Bak and Bax (FISCHER et al., 2006). Furthermore, deletion of the F1L ORF was reported to not only induce apoptosis *in vitro* but enhance antigen-specific immunogenicity *in vivo*, with significantly higher antigen-specific CD8+ T cell responses (PERDIGUERO et al., 2012a; HOLGADO et al., 2016).

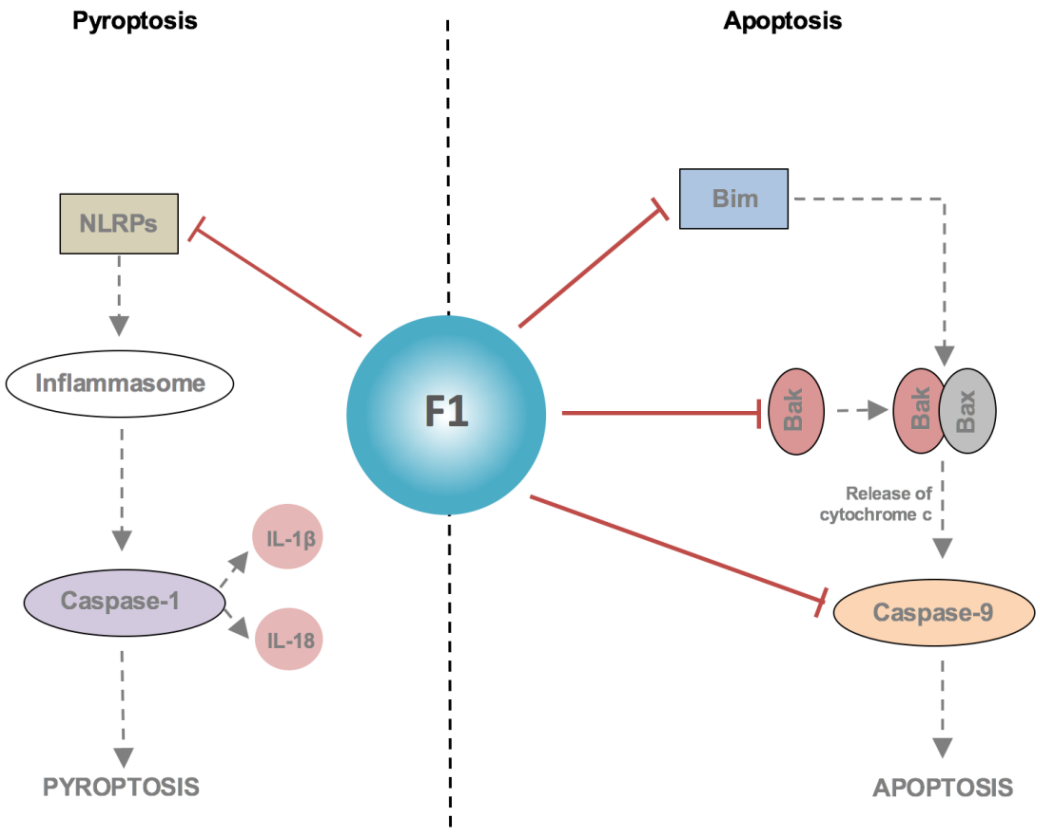

Fig. 5: Inhibition of cell death by MVA's F1protein.

3.5. MVA as a therapeutic cancer vaccine

Several recombinant MVAs have been constructed as immunotherapeutic vaccines, some of which have entered clinical trials for different types of cancer. One promising candidate is TG4010, a recombinant MVA expressing mucin-1 (MUC1) as a TAA along with human interleukin-2 (IL-2). Previous studies have suggested, that in addition to expressing TAAs, the expression of cytokines such as IL-2 can enhance the vaccines efficacy by increasing T-cell avidity (KUDO-SAITO et al., 2007b; KUDO-SAITO et al., 2007a). TG4010 has entered numerous phase II clinical studies for various types of cancer (e.g. prostate cancer, renal cell carcinoma) as single and combination treatment and has recently entered a phase IIB/III trial for non-small-cell lung cancer (NSCLC) (RAMLAU et al., 2008; DREICER et al., 2009; OUDARD et al., 2011; QUOIX et al., 2011; QUOIX et al., 2016).

A second MVA-based candidate vaccine is OXB-301, commercially referred to as TroVax®. It consists of MVA delivering 5T4 as a TAA, which is a human oncofetal glycoprotein rarely detected in normal tissue but highly expressed

in many carcinomas (SOUTHALL et al., 1990). TroVax® is currently being developed and tested by Oxford BioMedica. It has undergone multiple phase II clinical studies and one phase III study with promising results (HARROP et al., 2006; HARROP et al., 2007; AMATO et al., 2008b; AMATO et al., 2008a; ELKORD et al., 2008; HARROP et al., 2008; AMATO et al., 2009; KAUFMAN et al., 2009; AMATO et al., 2010; HARROP et al., 2013).

Fortunately, all these clinical studies showed that all the recombinant MVAs were always immunogenic, well tolerated and safe with only little adverse effects. However, none of the MVAs were successful as monotherapies. Nevertheless, when administered as combinational therapy along with the standard of care for the specific cancer types, clinical outcome was generally improved. Especially a combinational approach of therapeutic cancer vaccines with conventional cytotoxic chemotherapy seems promising. While chemotherapy can be used to debulk the tumor mass, follow-up treatment with a vaccine induces immunity, which can be used to control micrometastatic disease (DRAKE, 2012). Thus, improving MVA as a vector itself by increasing its potential to elicit a stronger immune response can be used in an approach to further improve MVA-based cancer immunotherapy.

4. Ovalbumin as a model antigen

The use of model antigens to investigate genetically modified MVAs in terms of immunogenicity is a well-established concept. There are several model antigens that are commonly used for that purpose, such as green fluorescent protein (GFP), β-Galactosidase and ovalbumin (OVA). Particularly OVA is a well characterized model antigen has been used in many studies with modified MVAs to evaluate the strength of the immune response, especially to assess T-cell responses (BAUR et al., 2010; BECKER et al., 2014). OVA is a chicken egg white protein and was classified as a member of the serpin superfamily when it was first discovered (HUNT & DAYHOFF, 1980). Originally, members of the serpin family were all considered serine protease inhibitors. However, OVA was later shown to lack protease inhibitory activity, yet remained part of the family and is now

a member of a sub-group known as ovalbumin-related serpins (ov-serpins). Although its main function is still unknown, it is believed to be a storage protein (BENARAFA & REMOLD-O'DONNELL, 2005). Today, OVA is being used for both *in vitro* and *in vivo* studies. Because it is so widely used, OVA epitopes are well known and many reliable read-out systems have been established, facilitating data collection and interpretation. *In vitro* it was shown for OVA-expressing recombinant MVA viruses that OVA is constantly secreted into the cell supernatant, allowing analysis of protein expression without affecting the cells. In terms of *in vivo* use, OVA has been established as a model antigen in various setting. Due to its well-studied epitopes, determination of OVA-specific T-cell responses is well established. At the same time, different OVA-expressing tumor cell lines exist (e.g. E.G7-OVA, B16 OVA). These tumor models, most of which are commercially available, enable efficacy testing of cancer vaccines for established tumors in mice (VIANELLO et al., 2006; FOTIN-MLECZEK et al., 2014).

E.G7-OVA for example is a mouse T cell lymphoma cell line, that is stably expressing OVA epitopes. It was originally established as a tool to investigate antigen presentation and processing via MHC class I pathway and was derived from the mouse lymphoma cell line EL4 (MOORE et al., 1988). Like most tumor cell lines, the E.G7-OVA is often used as a subcutaneous tumor model for therapy evaluation.

5. Objectives

MVA is a well-established vector platform with an excellent safety profile and potent immune stimulatory capacity. However, the expression of several remaining immunomodulatory proteins might hamper the use of the vector's full potential. The aim of this study was to investigate if inactivation of multiple immunomodulatory gene functions still allows for the construction of stable recombinant MVA vaccines and at the same time may lead to an improvement of the vector vaccine's immunogenicity and efficacy. For that purpose, we targeted three candidate immunomodulatory proteins still produced by MVA and constructed two genetically modified candidate vector vaccines (MVAΔΔ-OVA and MVAΔΔΔ-OVA) in which two respectively all three of the chosen immunomodulatory genes are simultaneously deleted. This study describes:

(i) the generation of the MVAΔΔ-OVA and MVAΔΔΔ-OVA candidate vector vaccines

(ii) the genetic and functional analysis of MVAΔΔ-OVA and MVAΔΔΔ-OVA *in vitro* in comparison to conventional recombinant MVA-OVA

(iii) the testing of immunogenicity and efficacy of MVAΔΔ-OVA and MVAΔΔΔ-OVA *in vivo* in comparison to conventional recombinant MVA-OVA

III. MATERIAL UND METHODS

1. Antibodies

Tab. 1: Primary Antibodies used for Western blot analysis

Antibody	Dilution	Company
mouse anti-GAPDH	1:10,000	Millipore, Darmstadt, Germany
mouse anti-GAPDH	1:1,000	Thermo Fisher Scientific, Rockford, USA
rabbit anti-Caspase 3	1:1,000	Cell Signaling Technology, Danvers, USA
rabbit anti-chicken Ovalbumin	1:10,000	Aviva Systems Biology Corporation, San Diego, USA
rat anti-VACV C7	1:200	Hybridoma culture supernatant (BACKES et al., 2010)

Tab. 2: Secondary Antibodies used for Western blot analysis

Antibody	Dilution	Company
Horseradish peroxidase (HRP) conjugated goat anti-rat polyclonal IgG	1:20,000	BioLegend, San Diego, USA
Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG/IgM	1:20,000	Jackson ImmunoResearch, West Grove, USA
anti-mouse MFP 488	1:200	Mobitec, Berkheim, Germany
anti-rat MFP 631	1:200	
Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG	1:2,000	Cell Signaling Technology, Danvers, USA

Tab. 3: Antibodies used for Immunostaining

Antibody	Dilution	Company
rabbit anti-VACV	1:2,000	Acris GmbH, Arnbruck, Germany
goat anti-rabbit	1:5,000	Jackson ImmunoResearch, West Grove, USA

Tab. 4: Antibodies used for ELISA

Antibody	Dilution	Company
Mouse IL-1β ELISA MAX™ Capture Antibody	1:200	BioLegend, San Diego, USA
Mouse IL-1β ELISA MAX™ Detection Antibody	1:200	BioLegend, San Diego, USA
Mouse IFN-γ ELISA MAX™ Capture Antibody	1:200	BioLegend, San Diego, USA
Mouse IFN-γ ELISA MAX™ Detection Antibody	1:200	BioLegend, San Diego, USA

2. Oligonucleotide primers

Tab. 5: MVA specific oligonucleotide primers used for appropriate polymerase chain reactions

Primers	Sequence	Size (bp)
Del 1-F*	5‘-CTT TCG CAG CAT AAG TAG TAT GTC-3‘	291
Del 1-R*	5’-CAT TAC CGC TTC ATT CTT ATA TTC-3’	
Del 2-F*	5’-GGG TAA AAT TGT AGC ATC ATA TAC C-3’	354
Del 2-R*	5‘-AAA GCT TTC TCT CTA GCA AAG ATG-3‘	

Del 3-F*	5‘-GAT GAG TGT AGA TGC TGT TAT TTT G-3‘	446
Del 3-R*	5‘-GCA GCT AAA AGA ATA ATG GAA TTG-3‘	
Del 4-F*	5‘-AGA TAG TGG AAG ATA CAA CTG TTA CG-3‘	502
Del 4-R*	5’-TCT CTA TCG GTG AGA TAC AAA TAC C-3’	
Del 5-F*	5‘-CGT GTA TAA CAT CTT TGA TAG AAT CAG-3‘	603
Del 5-R*	5‘-AAC ATA GCG GTG TAC TAA TTG ATT T-3‘	
Del 6-F*	5’-CGT CAT CGA TAA CTG TAG TCT TG-3’	702
Del 6-R*	5‘-TAC CCT TCG AAT AAA TAA AGA CG-3‘	
008L-F	5‘-AAA GTT TAA TTT GTT GAC GAC GTA TG-3‘	597 (wt)
008L-R	5‘-CAT CAA ATA CAA AAT ATT CGA GCA AC-3‘	450 (del)
029L-F	5’-TCC ACT TCC AGA AAA TAT GG-3’	838 (wt)
029L-R	5‘-GCG AAG GAG ACC ACT ACA TC-3‘	553 (del)
184R-F	5‘-ATA TTC CGG CGT ATG AAT TG-3‘	1163 (wt)
184R-R	5‘-TTC GTC AAT TGT TTG TTG GAA G-3‘	398 (del)

wt: wildtype MVA, del: MVA deletion mutant; *(KREMER et al., 2012)

3. Media, additives and cell culture

Tab. 6: media and additives used for cell culture maintenance and infection

Media, additives and cell culture	Supplier
HEPES buffer (1 M)	Sigma-Aldrich, St. Louis, USA
Fetal calf serum (FCS)	Gibco Invitrogen, Karlsruhe, Germany
Fetal bovine serum (FBS), endotoxinfree	Biochrom GmbH, Berlin, Germany
Minimum Essential Medium Eagle (MEM)	Sigma-Aldrich, St. Louis, USA
MEM Non-essential amino acid solution	Sigma-Aldrich, St. Louis, USA
PBS Dulbecco (w/o Mg^{2+})	PAN Biotech, Aidenbach, Germany
Penicillin, Streptomycin (10,000U/ml, 10mg/ml)	Sigma-Aldrich, St. Louis, USA
RPMI-1640	Gibco Invitrogen, Karlsruhe, Germany
Trypsin-EDTA	Sigma-Aldrich, St. Louis, USA
VLE Dulbecco's MEM (DMEM)	Biochrom GmbH, Berlin, Germany
Pathogen-free chicken eggs	Charles River Laboratories, Massachusetts, USA VALO Biomedia GmbH, Osterholz-Schambeck, Germany

4. Viruses

4.1. Viruses used in this study

The following viruses were used for this study:

(i) MVA-OVA

(ii) MVA∆184∆008-OVA (referred to as MVA∆∆-OVA)

(iii) MVA∆184∆008∆029-OVA (referred to as MVA∆∆∆-OVA)

All recombinant, mutant and non-recombinant MVA viruses used in this study were based on the MVA clonal isolate F6. MVA F6 has been clonally isolated in limiting dilution passage experiments on chicken embryo fibroblasts (CEF) grown in 96-well tissue culture plates by Professor Gerd Sutter at LMU Munich in 1988. MVA F6 demonstrates clonal genetic homogeneity in comparison to its ancestor MVA stock virus as confirmed by analysis of viral DNA (Sutter 1990 LMU thesis; (MEYER et al., 1991).

Recombinant MVA expressing chicken ovalbumin (MVA-OVA) was generated prior to the start of this study and served as standard recombinant MVA vector vaccine for control purposes (BRANDMÜLLER, LEHMANN, SUTTER, unpublished results).

Deletion mutant MVA∆184∆008 was generated prior to the start of this study (ZARNIKO, STAIB, SUTTER, unpublished results) and served as starting material for the generation of the double/triple mutant-recombinant viruses MVA∆∆-OVA and MVA∆∆∆-OVA.

ORF	Length (AA)*	Gene product
008L	120	Interleukin-18 binding protein (vIL-18bp)
029L	222	F1 protein
184R	326	Interleukin-1β receptor (IL-1βR)

Tab. 7: Modulatory genes in MVA analyzed in this study

* (MEISINGER-HENSCHEL et al., 2007)

4.2. Virus amplification, purification and handling

All viruses were amplified on CEF in large (T175) flasks. Cell monolayers were infected and incubated for 3-4 days at 37° C until extensive cytopathic effects could be observed; then flasks were frozen at -20° C until further processing.

For purification, cells were subjected to three freeze-thaw cycles before being centrifuged at 15,000 rpm for three hours at 4° C (Avanti J-26XP, Beckman Coulter). The supernatant was discarded completely and the resulting pellets were altogether resuspended in 30 ml Tris-buffer (10 mM Tris-HCl, pH 9.0). The pellet suspension was then sonicated three times for 15 seconds and vortexed in between. The suspension was centrifuged at

1,200 rpm for five minutes at 4° C and the supernatant was carefully collected. The remaining pellet was once again resuspended in five ml Tris-buffer and the process was repeated for 4-5 times. The collected supernatant was then purified by 36% sucrose gradient centrifugation. In plastic tubes, 15 ml sucrose was carefully overlaid with 20 ml supernatant. Tubes were centrifuged at 15,000 rpm for 90 minutes at 4° C (Optima^{TM} LE-80K Ultracentrifuge, Beckman Coulter).

The supernatant was discarded completely and the remaining pellets were carefully resuspended in 2-3 ml Tris-buffer (depending on the size of the pellets), aliquoted and stocks were long-term stored at -80° C. Before use, virus stocks were thawed on ice and sonicated three times for one minute each time.

4.3. Virus titration and immunostaining

To determine an accurate virus titer, all viruses were titrated three times in parallel. Titration was performed in 6-well plates on confluent CEF cell monolayers. Tenfold serial dilutions (10^{-1} to 10^{-9}) were prepared with MEM/2% FCS/1% P/S and used to infect CEF cells in duplicate. Infected cells were incubated at 37°C for two hours, gently shook every 15 minutes. Thereafter, cells were washed once with PBS, fresh MEM/2% FCS/1% P/S was added and plates were incubated for 48 hours at 37° C.

To fix the cell monolayer and permeabilize cells ice-cold acetone-methanol (1:1) was added and incubated at room temperature for five minutes. Upon removal, plates were air dried and blocked with PBS+3%FCS for 30 minutes at room temperature.

To visualize plaques, plates were first incubated with primary rabbit anti-VACV diluted 1:2,000 in PBS+3%FCS for one hour at room temperature on a rocking platform to ensure even antibody distribution. Plates were then washed three times with PBS before adding the secondary goat anti-rabbit diluted 1:5000. Plates were again incubated for one hour at room temperature on a rocking platform. They were then washed three times with PBS before adding 0.5 ml True Blue^{TM} Peroxidase per well and incubated for 5-15 min at room temperature until stained foci became visible. Virus titer was determined by counting wells with 20-100 visible foci. Titer was

expressed as plaque forming units per milliliter (PFU/ml) by multiplying the number of counted foci by its dilution.

4.4. Multi-step analysis of virus growth

To determine virus growth, CEF and HaCat monolayers were infected with viruses at a multiplicity of infection (MOI) of 0.01 and incubated at 4°C for 30 minutes (cold start). Thereafter, plates were washed once with cold PBS before adding warm medium and incubating them at 37°C. Subsequently, plates were frozen at different time points after infection (0, 4, 12, 24, 48, 72h p.i.). Upon collection of all samples, they were subjected to three freeze-thaw cycles and titrated on CEF cells according to section III.4.3. Two multi-step growth curves were assembled for virus growth on permissive (CEF) and non-permissive (HaCat) cell lines.

5. Cell culture

5.1. Cultivation of permanent cells

All permanent cell cultures were kept in a humidified incubator at 37° C with 5% CO_2. The proper culture medium was mixed with the amount of fetal calf serum (FCS) suitable for the cells plus 1% Penicillin/Streptomycine (P/S). If required, 1% HEPES and/or 1% non-essential amino acid solution (NEA) was also added. Cell cultures were split 1-2 times per week when dense, for which they were detached with Trypsin EDTA. For infection, cells were kept in their proper medium supplemented with only 2% FCS.

Cell line	**Culture medium**
Human keratinocyte cell line (HaCat)	VLE Dulbecco's MEM (DMEM) + 7% FCS + 1% P/S + 1% HEPES
Mouse embryo fibroblast cell line (NIH3T3)	VLE Dulbecco's MEM (DMEM) + 10% FCS + 1% P/S
Human cervical carcinoma cell line (HeLa)	Minimum essential eagle (MEM) medium + 7% FCS + 1% P/S + 1% NEA

5.2. Isolation and cultivation of primary cells

5.2.1. Murine splenocytes

Murine splenocytes were isolated from the spleens of C57BL/6 mice. After extraction, the spleens were kept in PBS to keep them from drying out, then homogenized by pressing them though a 70µm filter into a 50ml falcon tube. Subsequently, the cell suspension was centrifuged at 11,000 rpm for 5 minutes at room temperature. The supernatant was discarded and the remaining cell pellet was resuspended in 3ml “red blood lysing buffer” to remove erythrocytes and incubated for 5 minutes at room temperature. After lysis, 7ml of PBS was added and the cell suspension was once again centrifuged at 11,000 rpm for 5 minutes at room temperature. The supernatant discarded and the cell pellet was resuspended in RPMI-1640 medium supplemented with 10% FCS and the number of cells was determined in a Neubauer chamber. For cultivation and stimulation, splenocytes were seeded into cell culture plates at the desired density.

5.2.2. Chicken embryo fibroblasts (CEF)

CEF cells were freshly prepared once a week from pathogen-free chicken eggs that had been incubated for 11 days at 37° C prior to preparation. They were then maintained in Minimum essential eagle (MEM) medium supplemented with 10% FCS and 1% P/S.

5.3. Cell count

Cells were trypsinized, diluted 1:10 and stained with Trypan blue solution. Stained cells were counted in a Neubauer chamber.

6. Polymerase chain reaction (PCR)

To obtain PCR samples CEF monolayers were infected at an MOI of 5 and incubated for 1-2 days. Cells were harvested, centrifuged at 13,000 rpm for 15 seconds and resuspended in 200 µl supernatant. The rest of the supernatant was discarded. Viral DNA was extracted and purified using the QIAmp DNA Mini Kit (Qiagen) following the enclosed protocol.

Polymerase chain reaction (PCR) was performed with 50ng of DNA (2µl)

per sample and 23 µl of a PCR Master Mix.

PCR Master mix composition:

18.8 µl	distilled water
2.5 µl	buffer (10x)
0.5 µl	dNTP's
0.5 µl	forward oligonucleotide primer
0.5 µl	reverse oligonucleotide primer
0.2 µl	Dynazyme II

Specific oligonucleotide primers (as listed in Tab. 5) were used for the correspondent PCR reaction. The following thermocycling conditions were applied using the peqSTAR 2x thermocycler (PEQLAB Biotechnology GmbH):

Del I-VI PCR

Step	Temperature	Time
Initial denaturation	95°C	3 minutes
30 cycles	95°C 57°C 72°C	30 seconds 45 seconds 45 seconds
Final extension	72°C	5 minutes
Store	4°C	forever

184R/008L PCR

Step	Temperature	Time
Initial denaturation	95°C	3 minutes
30 cycles	95°C 57°C 72°C	30 seconds 45 seconds 75 seconds
Final extension	72°C	5 minutes
Store	4°C	forever

029L PCR

Step	Temperature	Time
Initial denaturation	95°C	3 minutes
30 cycles	95°C 54°C 72°C	30 seconds 45 seconds 45 seconds
Final extension	72°C	5 minutes
Store	4°C	forever

After PCR, loading buffer (6x) was added to PCR products in the appropriate amount and samples were either analyzed via gel electrophoresis or stored at -20°C for later analysis.

7. Gel electrophoresis

PCR products were separated by size via gel electrophoresis. To visualize nucleic acid, Gel Red™ was added to the 1% agarose gel. DNA samples were loaded onto the gel together with an appropriate molecular weight marker. 1x TAE buffer was used as running buffer and nucleic acid was

visualized with ChemiDoc™MP Imaging system (Bio-Rad). Composition of buffers is listed in the appendix.

8. Western blot analysis

8.1. Verification of OVA expression

To obtain samples for western blot analysis NIH3T3 cell monolayers were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 5. 24h post infection, samples were collected and centrifuged at 13,000 rpm for 30 seconds. Supernatants were discarded; the remaining cell pellets were resuspended in SDS-lysis-buffer and heated to 95°C for five minutes, then kept on ice. Samples were loaded onto a 10% SDS-gel and the Color Protein Standard broad range (New England BioLabs) was used as a molecular weight marker. Protein electrophoresis was performed in 1x Tris/Glycine/SDS buffer at 120V for 90 minutes (Power Ease 500, Invitrogen life technologies). Proteins were then transferred to a 0.2 µm nitrocellulose blotting membrane (GE Healthcare Life Science) with 1x transfer buffer using the Trans Blot Turbo system (Bio-Rad). The membrane was blocked in Tris-buffered saline 0.1% Tween20 (TBS/T) supplemented with 5% nonfat dried milk powder overnight at 4°C. Thereafter, the blot was washed three times for 10 minutes each in TBS/T.

After washing, the blot was simultaneously incubated with rabbit anti-OVA (1:10,000) and mouse anti-GAPDH (1:10,000) in TBS/T+2.5% milk for one hour at room temperature. The blot was washed three times for 10 minutes each in TBS/T before it was incubated with goat anti-rabbit HRP (1:2,000) and goat anti-mouse (1:20,000) in TBS/T+2.5% milk for one hour at room temperature. The blot was again washed three times for 10 minutes each in TBS/T before it was incubated with Clarity™ ECL Western Blotting substrate and analyzed using the ChemiDoc™MP Imaging System (Bio-Rad).

8.2. Kinetic analysis of OVA expression

CEF cells and NIH3T3 cells were infected with MVA-OVA at an MOI of 5. Samples were taken at different time points after infection (2, 4, 8, 12 and 24h p.i.). As control, cells were also infected with MVA F6 at an MOI of 5

and sample was taken 24h post infection. Additionally, a mock control was also taken at 24h post infection. All samples were centrifuged at 13,000 rpm for 30 seconds, the supernatant was discarded and the cell pellet was resuspended in SDS-lysis-buffer and heated to 95°C for five minutes. Samples were stored at -80°C. Samples were loaded onto a 10% SDS-gel and the Color Protein Standard broad range (New England BioLabs) was used as a molecular weight marker.

CEF and NIH3T3 samples were loaded onto two separate gels but electrophoresis was performed simultaneously in the same chamber Protein-electrophoresis, blotting and blocking was performed as described in III.8.1.

Both blots were incubated with rabbit anti-OVA (1:10,000) and mouse anti-GAPDH (1:10,000) as primary and goat anti-rabbit HRP (1:2,000) and goat anti-mouse (1:20,000) as secondary antibodies following the same protocol described in III.8.1.

8.3. Examination of Caspase 3 activity

HeLa cells were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 10. Samples were taken 15 hours after infection. Cells were centrifuged, the supernatant was discarded and the cell pellet was resuspended in SDS-lysis-buffer and heated to 95°C for five minutes. After that, samples were kept on ice until loaded onto a 15% SDS-gel, the Color Protein Standard broad range (New England BioLabs) was used as a molecular weight marker. Protein electrophoresis was performed in 1x Tris/Glycine/SDS buffer at 120V for 90 minutes (Power Ease 500, Invitrogen life technologies). Proteins were then transferred to a 0.2 µm nitrocellulose blotting membrane (GE Healthcare Life Science) with 1x transfer buffer using the Trans Blot Turbo system (Bio-Rad). The membrane was blocked in Tris-buffered saline 0.1% Tween20 (TBS/T) supplemented with 5% bovine serum albumin (BSA) for one hour at room temperature. Thereafter, the blot was washed three times for 10 minutes each in TBS/T.

The blot was incubated with rabbit anti-Caspase 3 (1:1000) in TBS/T 5% milk overnight at 4°C. The blot was then washed three times for 10 minutes each in TBS/T before it was incubated with goat anti-rabbit HRP (1:2,000)

in TBS/T 2.5% milk for one hour at room temperature. The blot was then again washed three times for 10 minutes each in TBS/T before it was incubated with Clarity™ ECL Western Blotting substrate and analyzed using the ChemiDoc™MP Imaging System (Bio-Rad).

After that, the blot was washed again three times for 10 minutes each in TBS/T before it was incubated with mouse anti-GAPDH (1:20,000) and rat anti-C7 (1:200) diluted in 2.5% BSA for one hour at room temperature. The blot was washed three times for 10 minutes each in TBS/T and then incubated with anti-mouse MFP 488 (1:200) and anti-rat MFP 631 (1:200) diluted in 2.5% BSA for one hour at room temperature. Thereafter, the blot was washed three times for 10 minutes each in TBS/T and detected using the ChemiDoc™MP Imaging System (Bio-Rad).

9. Functional assays

9.1. IL-1β functional assay

NIH3T3 cells were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 5. After an incubation time of 30 minutes at 4°C (cold start), cells were washed once with cold PBS before warm medium was added. Supernatants were collected at different time points after infection (0, 4, 8 and 24h p.i.), treated with UV light (400 mJ, two minutes) and stored at -20°C until all samples had been collected.

Supernatants were incubated in duplicate with murine recombinant IL-1β (BioLegend) at a final concentration of 1000 pg/ml for one hour at 37°C. Afterwards, supernatant were analyzed with a murine IL-1β enzyme-linked immunosorbent assay (ELISA) kit from BioLegend according to the protocol described in III.9.1.1.

9.1.1. Mouse IL-1β ELISA

Nunc™ MaxiSorp™ ELISA plates were coated with 100 µl of Capture antibody (1:200) in 1x Coating buffer and incubated overnight at 4°C. Plates were washed four times with 300 µl PBS+0.05%Tween20 per well and then blocked by adding 200 µl assay diluent per well and incubating

them for one hour at room temperature on a rocking platform. After washing the plates four times with PBS+0.05%Tween20, 100 µl of supernatants and diluted mouse IL-1β standard was added per well and incubated for two hours on a rocking platform.

The mouse IL-1β standard was reconstituted in 1x assay diluent to a final stock concentration of 145 ng/ml. For the assay, the stock solution was diluted to a concentration of 2000 pg/ml and six two-fold dilutions were performed with 1x assay diluent.

Thereafter, plates were washed four times with 300 µl PBS+0.05%Tween20 before 100 µl detection antibody (1:200) diluted in 1x assay diluent was added per well and incubated for one hour at room temperature on a rocking platform. After washing the plates four times with 300 µl PBS+0.05%Tween20, 100 µl of diluted Avidin-HRP (1:1000) were added per well and incubated for 30 minutes at room temperature with shaking. Plates were washed five times, soaking for 30 seconds per wash, before 100 µl of substrate solution was added per well and incubated for 15 minutes in the dark. Thereafter, 100 µl of stop solution was added and plates were analyzed using the Tecan Sunrise™ microplate absorbance reader at 450 nm.

9.2. IL-18 functional assay

CEF cells were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 5. 24 hours after infection, supernatants were collected and treated with UV light (400 mJ) for two minutes. Afterwards, 100 µl of supernatants were preincubated in duplicate with 10ng/ml murine recombinant IL-18 for one hour at room temperature. Concanavalin A (Con A) was added to the preincubated supernatants at a concentration of 200 ng/ml and the mix was used to stimulate freshly seeded murine splenocytes. Stimulated splenocytes were incubated at 37°C and supernatants were collected at 16 and 24 hours after infection.

As a control, splenocytes were stimulated with Con A and cell supernatant without IL-18. Additionally, splenocytes were also stimulated with Con A and murine IL-18 without supernatant.

Supernatants from stimulated splenocytes (w/ and w/o IL-18) were analyzed

with a murine IFN- γ ELISA kit from BioLegend according to the protocol described in III.9.2.1.

9.2.1. Mouse IFN-γ ELISA

Nunc™ MaxiSorp™ ELISA plates were coated with 100 µl of Capture antibody (1:200) diluted in 1x Coating buffer and incubated overnight at 4°C.

Plates were washed four times with 300 µl PBS+0.05%Tween20 per well and then blocked by adding 200 µl assay diluent per well and incubating for one hour at room temperature on a rocking platform. After washing the plates four times with PBS+0.05%Tween20, 100 µl of supernatants and diluted mouse IL-1β standard was added per well and incubated for two hours on a rocking platform.

The mouse IFN-γ standard was reconstituted in 1x assay diluent to make the standard stock solution with a concentration of 110 ng/ml. For the assay, the stock solution was diluted to a concentration of 1000 pg/ml and six two-fold dilutions were performed with 1x assay diluent.

Thereafter, plates were washed four times with 300 µl PBS+0.05%Tween20 before 100 µl detection antibody (1:200) diluted in 1x assay diluent was added per well and incubated for one hour at room temperature on a rocking platform. After washing the plates four times with 300 µl PBS+0.05%Tween20, 100 µl of diluted Avidin-HRP (1:1000) were added per well and incubated for 30 minutes at room temperature with shaking. Plates were washed five times, soaking for 30 seconds per wash, before 100 µl of substrate solution was added per well and incubated for 15 minutes in the dark. Thereafter, 100 µl of stop solution was added and plates were analyzed using the Tecan Sunrise™ microplate absorbance reader at 450 nm.

9.3. Apoptosis assay

To monitor apoptosis upon infection, HeLa cells were infected at an MOI of 5 and analyzed via western blot analysis and fluorescent staining of fixed cells.

9.3.1. Caspase 3 western blot analysis

HeLa cells were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA

at an MOI of 5 in duplicate in a 24-well-plate. As a positive control, cells were treated with 1µM Staurosporine.

15 hours after infection western blot samples were collected and analyzed as described in III.8.3.

9.3.2. Fluorescent staining of fixed cells

HeLa cells were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 5 in a 96-well-plate. Uninfected cells served as mock control. 15 hours after infection, medium was removed and cells were fixed with 4% paraformaldehyde (50 µl per well) for 60 minutes on ice. After fixation, the plate was washed once with 200 µl PBS per well before cells were permeabilized with 0.5% TritonX-100 (100 µl per well) for 15 minutes at room temperature. The plate was washed again with PBS and then cells were incubated with 300 µM 4′,6-Diamidin-2-phenylindol (DAPI) in the dark for 7 minutes at room temperature on a rocking platform. Cells were washed twice with PBS and fluorescent cell nuclei were analyzed at 20x and 40x magnification using the KEYENCE fluorescence microscope.

10. *In vivo* experiments

All *in vivo* experiments were performed externally in cooperation with our collaborators from Boehringer Ingelheim at the research facility in Biberach under the guidance of Prof. Dr. Klaus Erb (efficacy study) and Dr. Aleksandra Kowalczyk (immunogenicity study).

IV. RESULTS

1. Construction of viruses

The first part of this study focuses on the construction of MVAΔΔ-OVA and MVAΔΔΔ-OVA on the basis of MVAΔ184Δ008.

1.1. Introduction of Ovalbumin

In a first step of construction, we introduced OVA into deletion III (Del III) of the double mutant MVAΔ184Δ008.

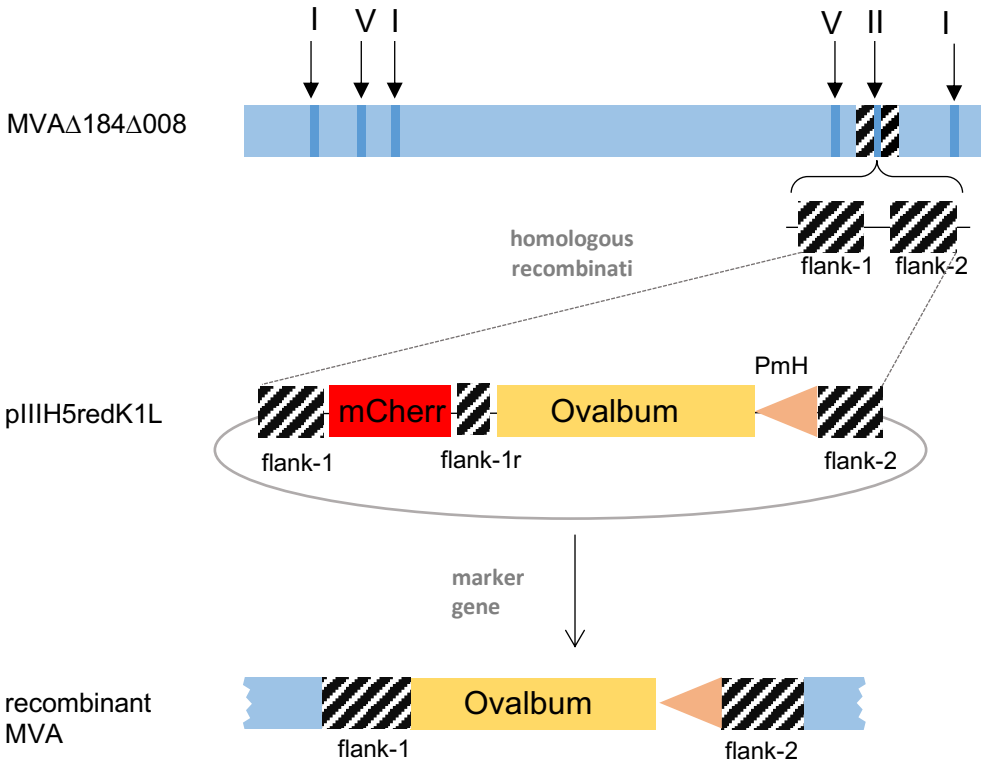

Fig. 6: Scheme of recombinant MVA generation. Locations of MVA's six major deletion sites (I-IV) are indicated. Ovalbumin (under transcriptional control of PmH5) was introduced into Del III via homologous recombination. flank-1/flank-2: nucleotide sequences flanking MVA's Del III, flank-1r: flank-1 repeat

We synthesized the gene encoding OVA and cloned it into our standard pIIIH5redK1L plasmid where it was put under transcriptional control of the strong early/late modified vaccinia promoter PmH5 (WYATT et al., 1996). We then introduced it into MVA's Del III via standard homologous

recombination using our established standard methodology (Fig. 6). Deletion of the marker gene mCherry occurred during plaque passaging (SHANER et al., 2004; KREMER et al., 2012).

1.2. Deletion of the 029L gene

Using the previously constructed recombinant MVAΔΔ-OVA as starting material, the second part of construction was to construct a triple mutant recombinant MVA virus, in which the 029L gene is additionally deleted.

For deletion of the 029L gene, we performed a transient dominant selection (TDS) (FALKNER & MOSS, 1990) on CEF cells as shown in Fig. 7. The plasmid contained a truncated version of the 029L gene along with the 029L gene's original flanking sites. For selection, *Escherichia coli* guanine phosphoribosyltransferase (gpt) was added to the plasmid as a dominant selectable marker, which enables resistance to mycophenolic acid (MPA) (FALKNER & MOSS, 1990).

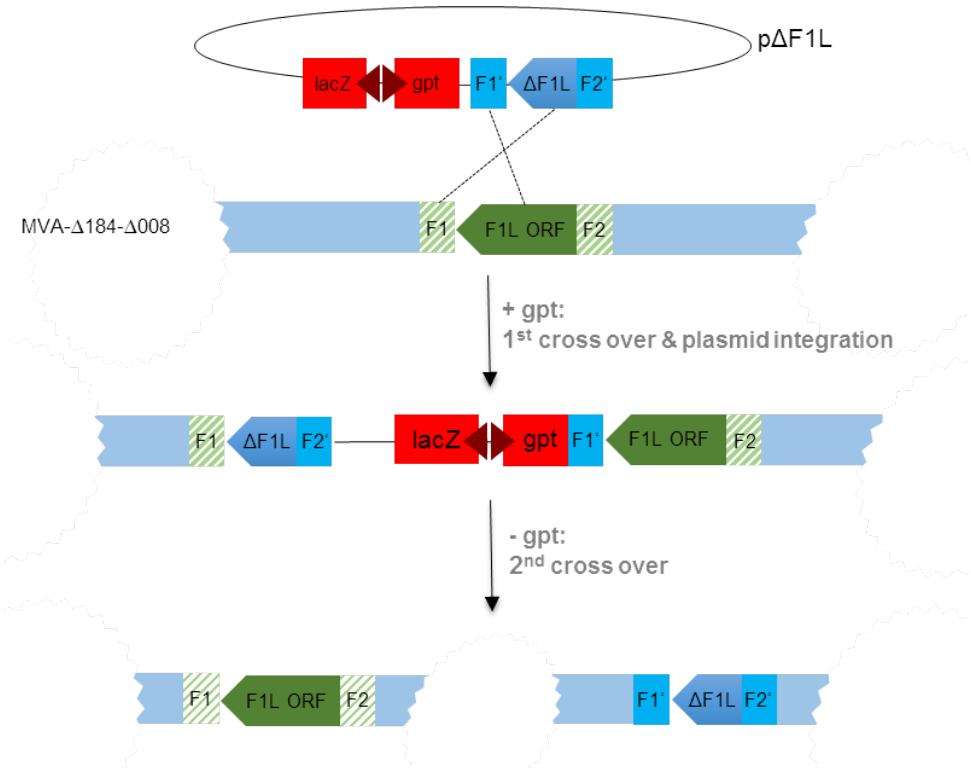

Fig. 7: Scheme of transient dominant selection (TDS). ORF: Open reading frame, ΔF1L: truncated F1L gene, F1/F1': flanking site 1, F2/F2': flanking site 2, gpt: *Escherichia coli* guanine phosphoribosyl-transferase

For selection, we induced selection pressure directly after transfection by supplementing MEM 2% FCS with gpt-selection medium consisting of 0.25% MPA (10mg/ml in 0.1 N NaOH), 2,5% xanthine (10mg/ml in 0.1 N NaOH) and 0.15% hypoxanthine (10mg/ml in 0.1 N NaOH), sterile filtered. After four rounds of consecutive plaque picking under selection pressure, we removed selection pressure, which results in a subdivision of the virus population into non-recombinant and recombinant MVA at a 50:50 ratio. 30 plaques were isolated, amplified and screened via Polymerase chain reaction (PCR) for the truncated 029L gene.

2. Genetic characterization of constructed viruses

The second part of this study focuses on the genetic characterization of our constructed recombinant mutant MVA viruses. A series of experiments was conducted to affirm correct insertion of OVA along with confirmation of genetic purity, successful gene deletion and backbone integrity

2.1. Verification of correct insertion of Ovalbumin gene

All three recombinant MVA viruses used in this study where generated via homologous recombination of an OVA encoding gene into Del III. To verify the correct insertion of the OVA gene into Del III, an oligonucleotide primer set specific for the deletion was used for a PCR analysis. This primer set was previously established and designed to flank the deletion sites and allow for their amplification with a distinct product size (KREMER et al., 2012).

The expected size of the amplified fragment was calculated by adding the length of the nucleotide sequence of OVA to the known size of the existing Del III.

The OVA specific fragment was detectable in all recombinant mutant and non-mutant MVA viruses (1736 bp) and confirmed correct insertion of OVA into Del III. MVA F6 served as a control and showed a fragment in size according to the Del III without insert (446 bp). The same fragment was absent in all recombinant MVAs, confirming genetic purity. A negative control was also used in which DNA was replaced by water, to ensure the

PCRs specificity (Fig. 8).

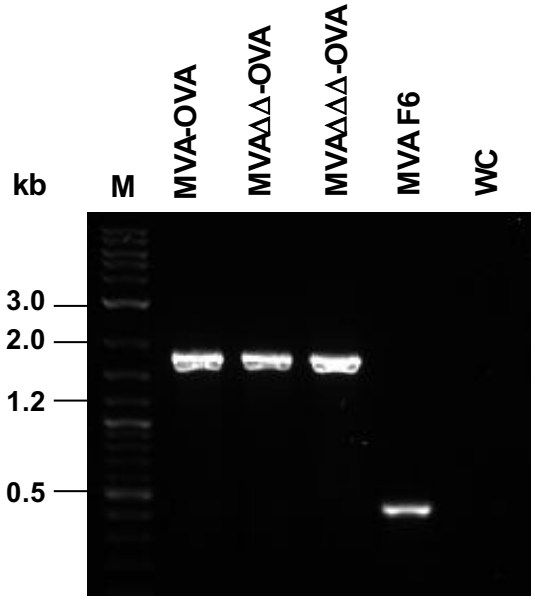

Fig. 8: PCR analysis to verify correct insertion of OVA into Del III. PCR reactions were performed with viral DNA and a nucleotide primer set specific for MVA's Del III. Expected sizes: Del III: 446 bp, Del III + OVA: 1736bp; Marker (M), water control without viral DNA (WC), sizes in kilobases (kb)

2.2. Verification of genetic modifications and MVA backbone integrity

Recombinant MVAs were also tested to verify the specific genetic modifications that were introduced into MVA's backbone genome. For that purpose, three sets of oligonucleotide primers were designed to flank the three genes in question (008L, 029L, 184R) and amplify a specific fragment with a predetermined distinct size. PCRs were performed for MVAΔΔ-OVA (Fig. 9A) and MVAΔΔΔ-OVA (Fig. 9B) and MVA-OVA was used as a control to verify correct size of the intact gene. The expected sizes of all fragments were calculated by subtracting the deleted nucleotide sequence from the size of the corresponding intact gene.

Presence of a predicted smaller fragment was detectable for all modified genes when expected and confirmed successful deletion of the genes. Water served as a negative control to ensure specificity.

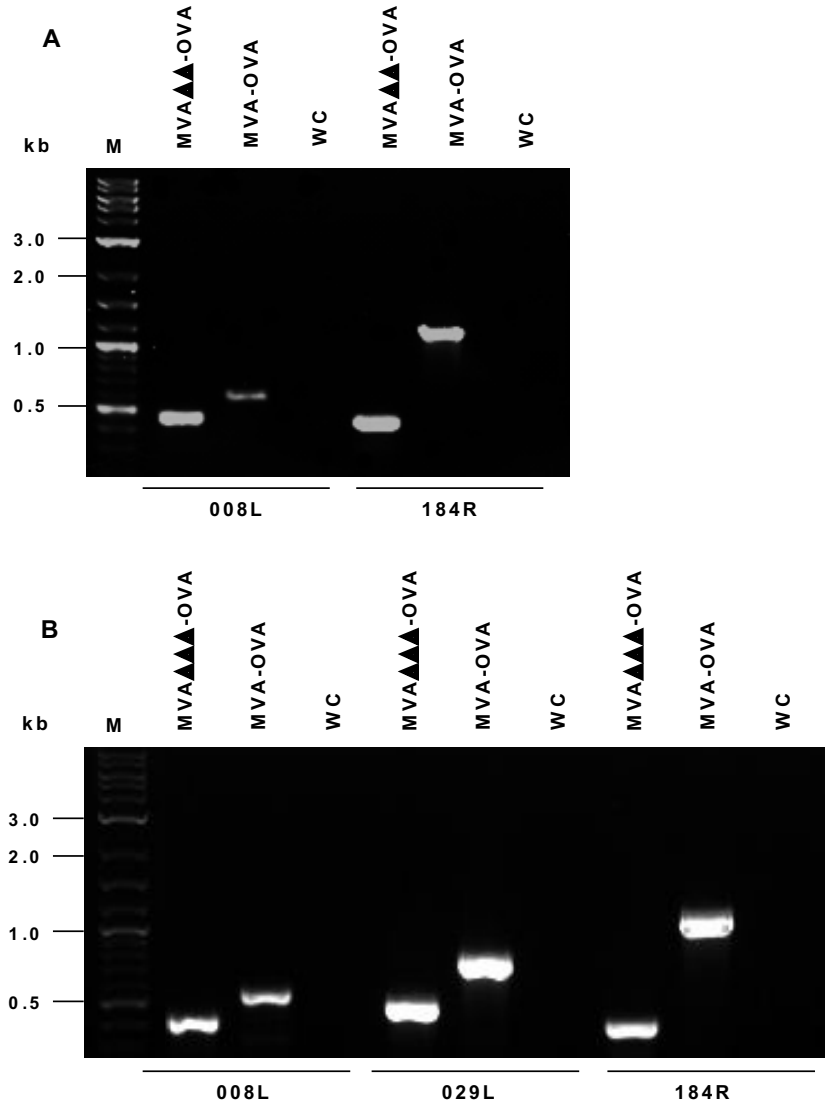

Fig. 9: PCR analysis to verify genetic modifications. PCR reactions were performed with oligonucleotide primer sets specific for the two respectively three modified genes and viral DNA of MVAΔΔ-OVA (A) and MVAΔΔΔ-OVA (B) Expected sizes: 008L: 597 bp, Δ008L: 450 bp, 029L: 838 bp, Δ029L: 553 bp, 184R: 1163 bp, Δ184R: 398 bp; Marker (M), water control without viral DNA (WC), sizes in kilobases (kb).

Furthermore, we tested our MVA viruses for genetic integrity of the MVA backbone. For that, primer sets specific for MVAs six major deletion sites (Del I-VI), which had previously been established by our lab, were used and PCRs were performed for all three recombinant MVAs and MVA F6 as a control. All primer sets were designed to flank the major deletion sites and each set amplifies one fragment with a distinct size in ascending order from Del I to Del VI (Fig. 10) (KREMER et al., 2012). All MVA viruses showed fragments in the expected sizes. All recombinant MVA viruses showed an

expected fragment bigger in size for Del III due to insertion of OVA. Additionally, MVAΔΔ-OVA and MVAΔΔΔ-OVA showed no fragment for Del II which was expected due to their MVA II new backbone (STAIB et al., 2003).

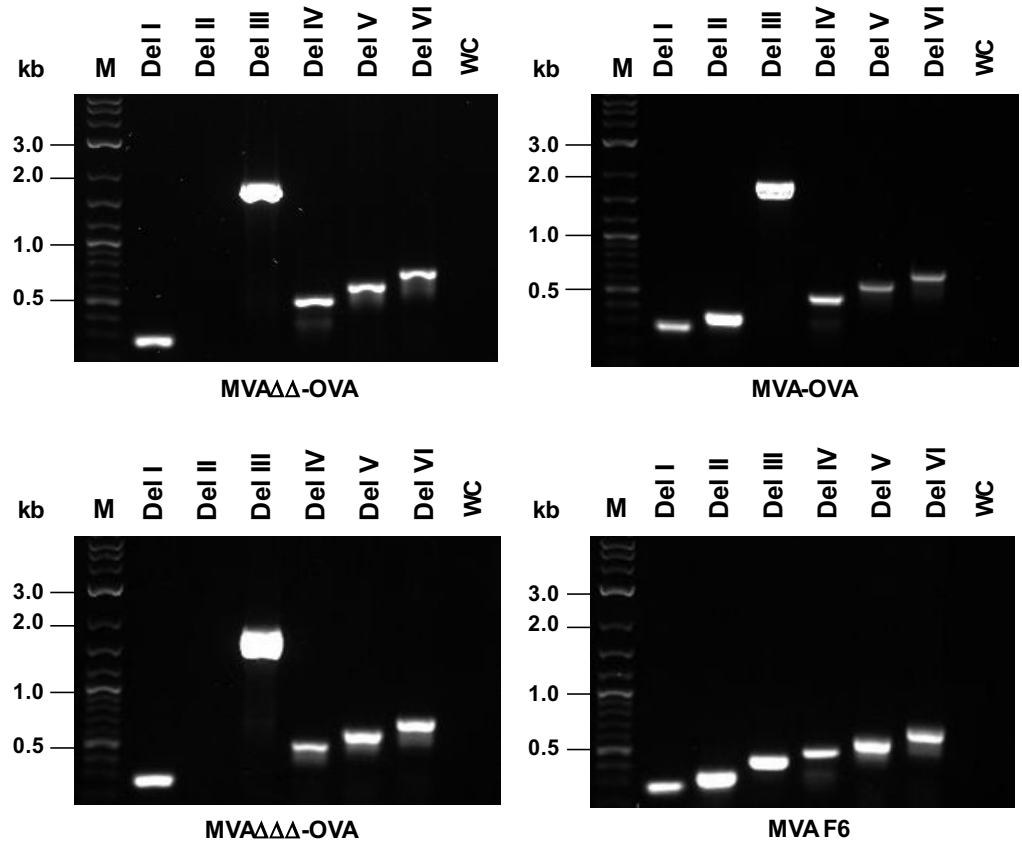

Fig. 10: Genetic integrity of recombinant MVAs. PCR reactions were performed with viral DNA and nucleotide primer sets specific for MVAs Del I-VI. Expected sizes: Del I: 291 bp, Del II: 354 bp, Del III: 446 bp/1736 bp, Del IV: 502 bp, Del V: 603 bp, Del VI: 702 bp; marker (M), control without viral DNA (WC), sizes in kilobases (kb).

2.3. Recombinant MVAs stably express OVA

After verification of successful insertion of OVA into Del III, we checked expression of the protein via Western blot analysis.

2.3.1. Comparative kinetic analysis of OVA expression in chicken and mouse cell line

To determine the time point after infection that is best to check OVA expression in cell culture, a kinetic analysis of OVA expression was

performed in CEF cells (Fig. 11A). Additionally, a comparative kinetic anaylsis was performed in NIH3T3 cells (Fig. 11B) to verify OVA expression in murine cells with regard to future experiments in mouse models.

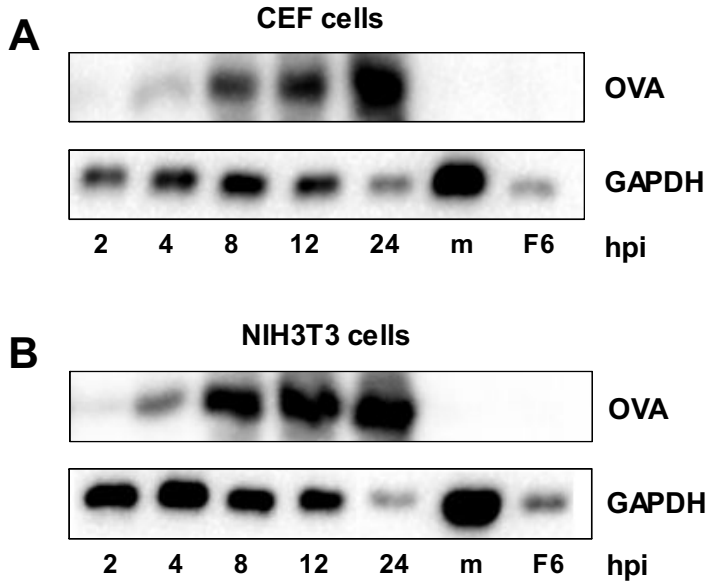

Fig. 11: Kinetic analysis of OVA expression in NIH3T3 and CEF cells. CEF (A) and NIH3T3 (B) were infected with MVA-OVA at an MOI of 5. Cells were harvested at the indicated time points (hours post infection, hpi) and lysates were analyzed for OVA and GAPDH expression. Mock (m) and MVA F6 (F6) infected cells served as controls and samples were obtained 24 hpi.

CEF cells respectively NIH3T3 cells were infected with MVA-OVA at an MOI of 5 and samples were taken at different time points post infection MVA-F6 infected cells and uninfected cells (mock) served as a negative control and both samples were taken 24h post infection. Lysates were analyzed by western blotting using anti-OVA and anti-GAPDH specific antibodies. OVA expression was confirmed (46 kDa) with a first signal detectable at 4 hours p.i. (Fig. 11) and continued with a steady increase over the course of infection and a strong signal 24 hours p.i.. Expression levels were comparable to slightly stronger in NIH3T3 cells (Fig. 11). GAPDH (38 kDa) was detectable in all samples and confirmed cellular input (Fig. 11). 24hpi was chosen as the time point for obtaining western blot samples in further experiments.

2.3.2. Western Blot analysis of OVA expression

After confirmation and kinetic analysis of OVA expression of MVA-OVA, the

next step was to confirm OVA expression in all our recombinant MVA viruses. CEF cells were infected with MVA-OVA, MVAΔΔ-OVA, MVAΔΔΔ-OVA and MVA F6 at an MOI of 5 and samples were obtained 24h p.i. according to the kinetic analysis. Lysates were analyzed by western blotting with anti-OVA and anti-GAPDH specific antibodies (Fig. 12).

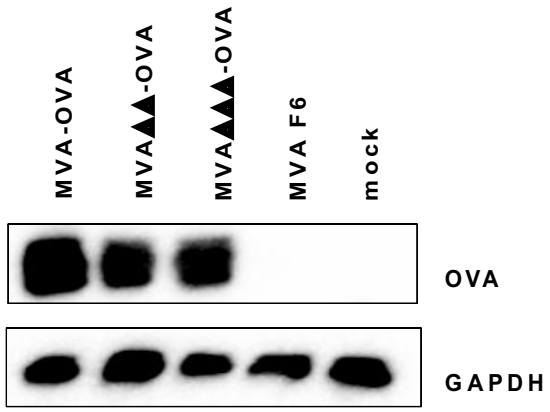

Fig. 12: Verification of OVA expression by recombinant MVAs. CEF cells were infected with the indicated viruses at an MOI of 5 and samples were taken 24h post infection. Lysates were analyzed by western blot for OVA (upper lane) and GAPDH (lower lane) expression. MVA-F6 and uninfected cells (mock) served as negative control.

We detected OVA-specific (46 kDa) bands in all samples infected with recombinant MVA viruses but not for MVA-F6 and mock controls, confirming OVA expression (Fig. 12, upper lane). GAPDH (38 kDa) was detectable in all samples and confirmed cellular input (Fig. 12, lower lane).

2.4. Multi-step growth kinetic of recombinant MVAs

To confirm MVA's safety profile after genetic modifications and insertion of OVA, its replication capacity and limited host range was determined in cell culture. This confirmation is important to verify that recombinant MVA viruses can indeed be handled under biosafety level 1 conditions like non-recombinant MVA (ZKBS, 2002).

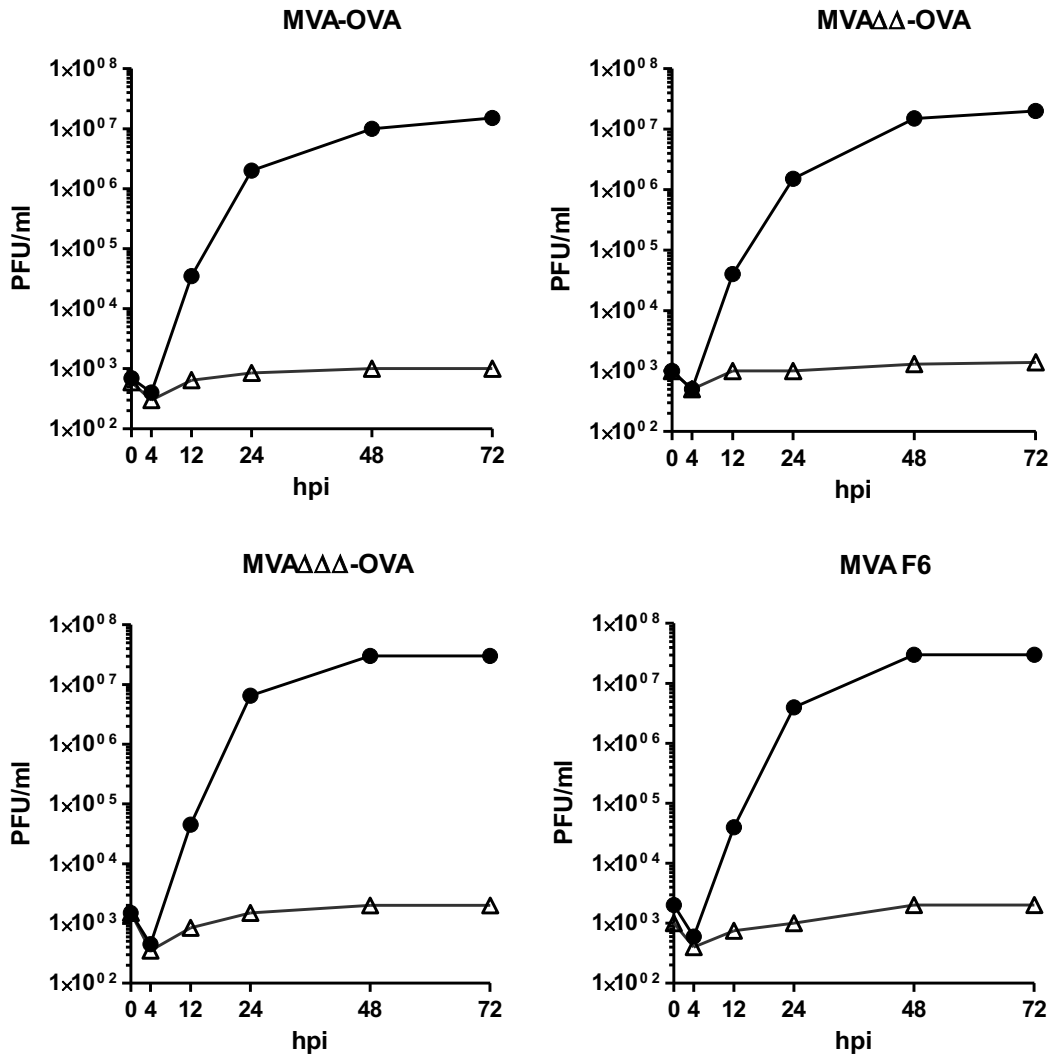

Fig. 13: Multi-step growth analysis of recombinant MVAs. Virus growth was evaluated over the course of 72h under permissive (CEF ●) and non-permissive (HaCat Δ) conditions. MVA F6 was used as wildtype control virus.

In this context, the inability to productively replicate in cells of human origin is an important feature of MVA (MAYR & MUNZ, 1964; SUTTER & MOSS, 1992; BLANCHARD et al., 1998). Therefore, a multi-step analysis of virus growth was performed for all recombinant MVA viruses along with MVA F6 in HaCat cells, which is an established laboratory human cell line. During a period of 72h of infection, none of the tested viruses was able to productively replicate in HaCat cells (Fig. 13) At the same time, virus growth was also analyzed in CEF cells, which are permissive for MVA infection. Here, all viruses tested were able to productively infect CEF cells and titers steadily

increased during the course of infection with a maximum of 10.000-fold increase after 72 hours (Fig. 13).

3. Functional characterization of constructed viruses

The previously conducted molecular characterization confirmed genetic purity, successful gene deletion and backbone integrity along with successful and efficient expression of OVA. Furthermore, all tested recombinant MVA viruses were shown to be unable to productively propagate on human HaCat cells. The third part of this study focuses of the functional characterization of the constructed viruses.

3.1. Mutant MVA viruses lack vIL-1βR expression

To confirm the deletion of the vIL1βR on a functional level, the missing binding activity of the vIL-1βR in the modified viruses used for this study was assessed in the supernatant of infected cells. For that, NIH3T3 cells were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 5 and supernatants of infected cell cultures were collected at 4h, 8h and 24h post infection. To investigate the presence respectively absence of the vIL-1βR, murine IL-1β (mIL-1β) was added (1000 pg/ml) to the supernatants and incubated for one hour at 37° C. After that, supernatants were subjected to a mIL1β-specific ELISA, which is only able to recognize unbound mIL-1β, whereas receptor-bound mIL-1β is no longer detectable. Therefore, presence of vIL-1βR and its binding to mIL-1β will decrease levels of detectable mIL-1β in the supernatant.

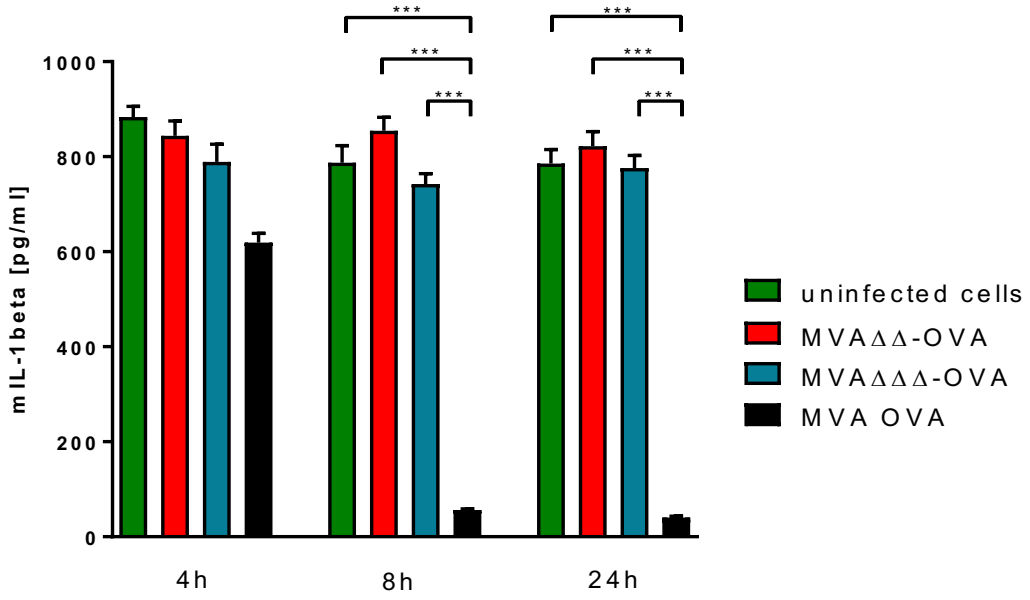

Fig. 14: Expression and binding activity of vIL-1βR after infection of NIH3T3 cells. NIH3T3 cells were infected with the indicated viruses at MOI of 5. Supernatants were harvested at the indicated time points post infection and incubated with mIL-1β. Binding activity was analyzed by ELISA. The supernatant of uninfected cells was used as a control. *** P < 0,001

All analyzed samples showed high levels of free mIL-1β at 4h post infection, thus no expression and binding activity of the receptor was detectable. However, in the samples taken 8h and 24h post infection, a significant decrease of free mIL-1β was observed in supernatants of MVA-OVA infected cells. In contrast, supernatants of cells infected with MVAΔΔ-OVA and MVAΔΔΔ-OVA maintained high levels of free mIL-1β (Fig. 14). comparable to mIL-1β levels detected in the mock control. This confirmed expression and binding activity of a vIL-1βR in MVA-OVA along with its late expression during the virus life cycle. Furthermore, functional absence of the receptor was confirmed for both mutant viruses, MVAΔΔ-OVA and MVAΔΔΔ-OVA.

3.2. Mutant MVA viruses lack vIL-18bp expression

To confirm the missing expression and study the missing binding activity of the vIL-18bp in the modified viruses used for this study, we conducted an analysis of supernatants of infected cells. For that, CEF cells were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 5 and supernatants were collected 24h p.i.. To check for the presence of the vIL-

18bp in the supernatants, they were then incubated with recombinant mouse IL-18 (10 ng/ml) for one hour, before stimulating murine spleoncytes with these supernatants in combination with Concanavalin A (Con A) (200 ng/ml). Unbound IL-18 in combination with Con A has previously been described to stimulate IFN-γ production in splenocytes (BOHN et al., 1998). Hence, the supernatants from stimulated spleconcytes were collected 16h (Fig. 15 A) and 24h (Fig. 15 B) post stimulation and analyzed for IFN-γ production via ELISA. Presence of vIL-18bp in the supernatant should bind to free IL-18 and significantly decrease levels of IFN-γ in stimulated murine splenocytes. To ensure specificity of IL-18 dependent stimulation, splenocytes were also stimulated with supernatants treated only with Con A but not with IL-18 (Fig. 15 A and B).

The supernatants of splenocytes stimulated with IL-18, Con A and supernatants from either MVAΔΔ-OVA or MVAΔΔΔ-OVA infected CEF cells show high levels of IFN-γ after 16h and 24h post stimulation (Fig. 15 A and B). The same was observed for the mock control, i.e. splenocytes stimulated with IL-18, Con A and supernatant from uninfected CEF cells. However, splenocytes stimulated with IL-18, Con A and supernatant from MVA-OVA infected CEF cells show significantly lower levels of IFN-γ in the supernatant after both 16h and 24h post stimulation (Fig. 15 A and B).

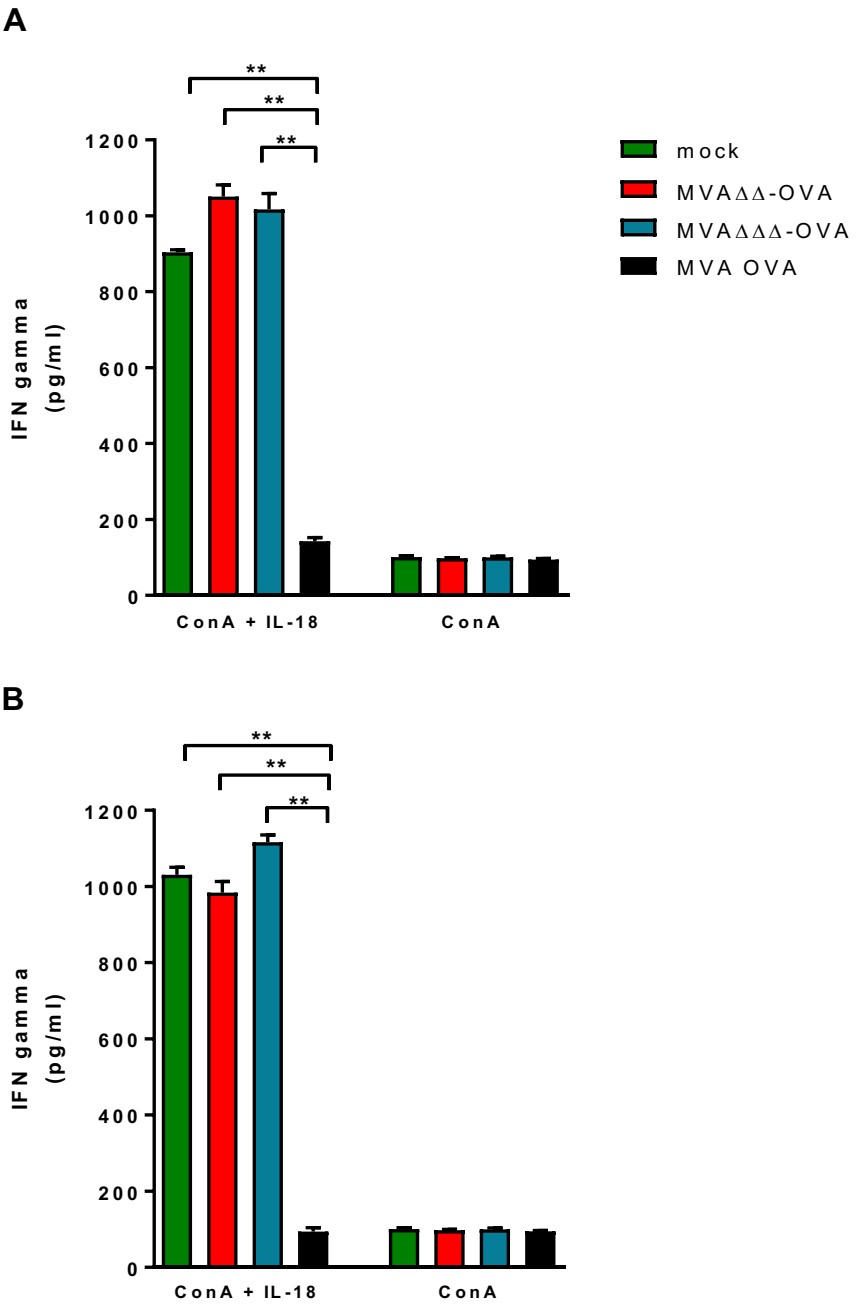

Fig. 15: Expression and binding activity of IL-18BP. CEF cells were infected with the indicated viruses at an MOI of 5 and supernatants were collected 24h p.i.. Collected supernatants were then incubated with mIL-18 and used to stimulate freshly prepared murine splenocytes in the presence of Con A. Supernatants from splenocytes were collected 16h (A) and 24h (B) after stimulation and subjected to IFN-γ ELISA. ** $P < 0.005$

Splenocytes that were only stimulated with supernatants and Con A showed only low levels of IFN-γ in the supernatant, ensuring that observed IFN-γ production relies on IL-18 stimulation (Fig. 15).

This experiment confirmed that MVA-OVA produces a soluble vIL-18bp and that that binding protein is able to bind mature IL-18. At the same time, IL-18 that is bound to vIL-18bp is no longer biologically active, thus no stimulation of splenocytes occurred. Both knock-out viruses however were unable to produce such a binding protein, allowing IL-18 to induce IFN-γ production in the presence of Con A at 16h and 24h post stimulation, confirming functional absence of the binding protein in both viruses.

3.3. MVAΔΔΔ-OVA induces apoptosis in infected cells

To confirm successful deletion of the F1 protein in MVAΔΔΔ-OVA on a functional level, we conducted two different experiments, focusing on the protein's known ability to block apoptotic cell death in MVA infected cells (WASILENKO et al., 2003).

3.3.1. Lack of F1 protein increases activation of Caspase-3 during MVA infection

Caspase-3 is one of three terminal caspases that upon activation lead to apoptotic cell death (Fig. 4). MVA's F1 protein however blocks activation of Caspase-3 by preventing cleavage of the pro-enzyme (pro-Casp-3) into its active form. To confirm that lack of F1 protein allows for cleavage and thus leads to activation of pro-Casp-3, HeLa cells were infected with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 5 and samples for western blot analysis were obtained 15h post infection. As a positive control, cells were stimulated with Staurosporine, a known inducer of apoptosis, whereas untreated cells served as negative control.

Samples from uninfected cells along with samples from cells infected with MVA-OVA and MVAΔΔ-OVA all showed strong signals for full-length pro-Casp-3 (35 kDa). Samples obtained from cells stimulated with Staurosporine or infected with MVAΔΔΔ-OVA on the other hand showed a clear reduction in signal strength for pro-Casp-3. At the same time, this was associated with the appearance of a much stronger signal for activated Caspase-3 (17 kDa) (Fig. 16). These results indicate that lack of F1 protein indeed leads to an activation of Casp-3 though cleavage of pro-Casp-3, making way for apoptotic cell death in infected cells.

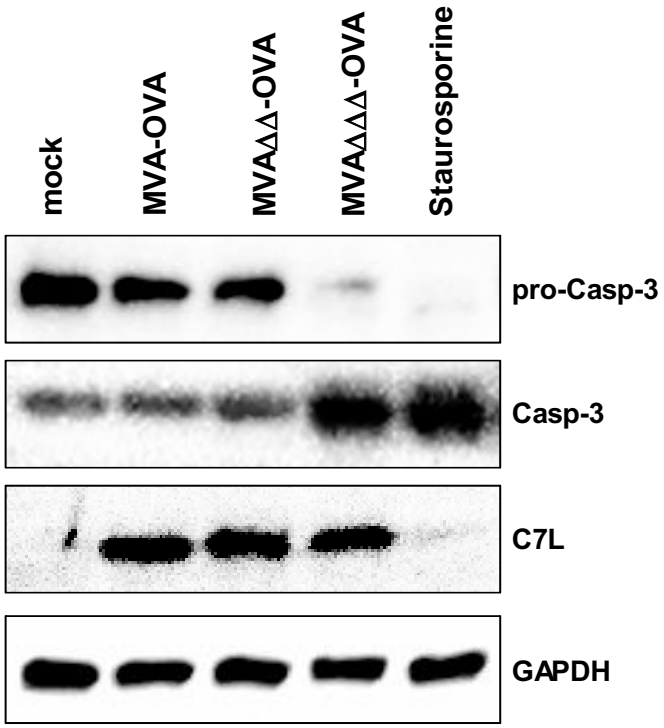

Fig. 16: Cleavage of pro-Casp-3 upon MVA infection. HeLa cells were infected with the indicated viruses at an MOI of 5. Samples were taken 15h p.i. and checked for cleavage of Caspase-3 by western blotting. Uninfected cells (mock) served as negative control; Cells treated with Staurosporine were used as positive control; GAPDH served as loading control, C7L served as control for viral load.

3.3.2. Lack of F1 protein causes karyorrhexis in infected cells

One distinct event of apoptotic cell death is the fragmentation of the cell's nucleus (ZAMZAMI & KROEMER, 1999). To visualize the effect of the missing F1 protein on nuclear morphology, we examined the nuclei of MVA infected cells and screened for signs of fragmentation. To do so, we infected HeLa cells with MVA-OVA, MVAΔΔ-OVA and MVAΔΔΔ-OVA at an MOI of 5. 15h post infection, cells were fixed and nuclei were stained to visualize karyorrhexis. Uninfected cells were used as a control (Fig. 17).

DAPI staining of cell nuclei of uninfected cells showed no signs of cell nucleus fragmentation. At the same time, nuclei of cells infected with MVA-OVA and MVAΔΔ-OVA also showed no signs of karyorrhexis. However, a large proportion of cells infected with MVAΔΔΔ-OVA showed nuclear fragmentation (Fig. 17). These results confirm that lack of F1 protein not

only allows for activation of Casp-3, but also that cells infected with viruses lacking the F1 protein are able to undergo apoptosis.

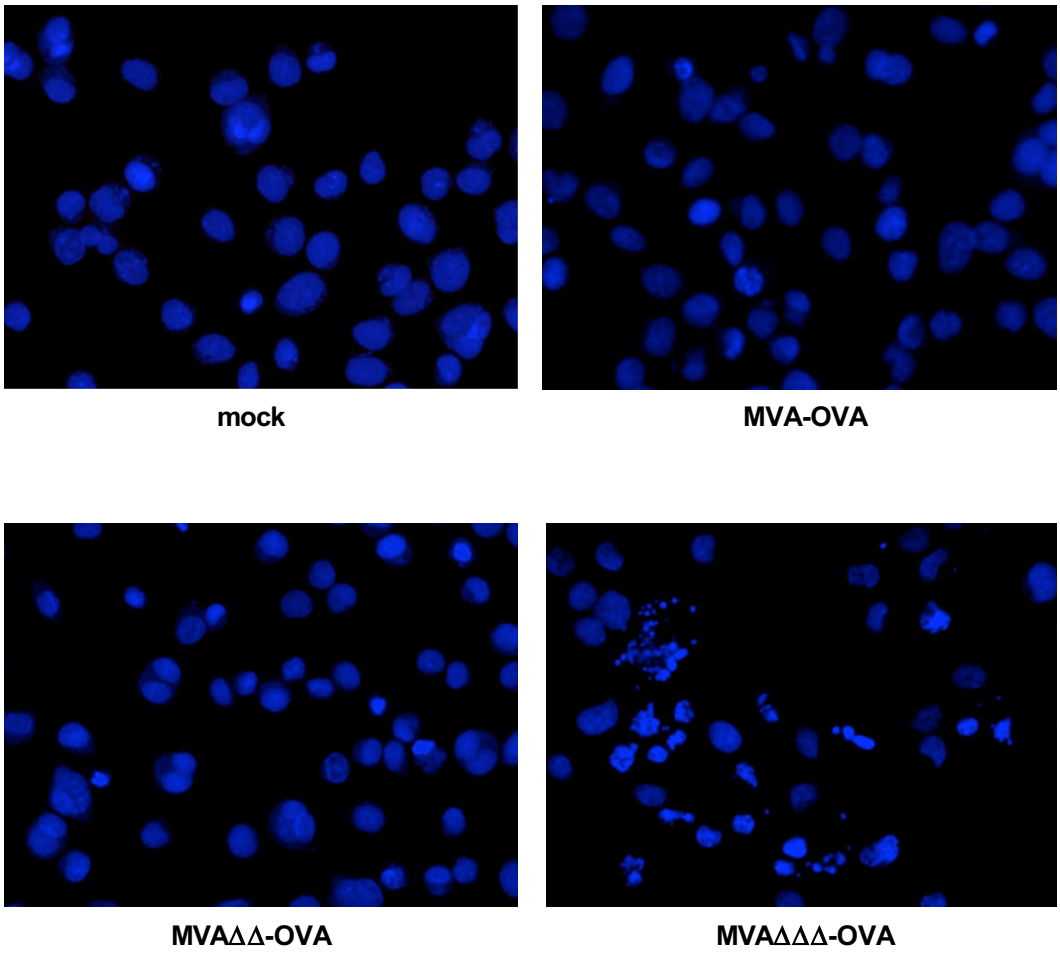

Fig. 17: Induction of apoptosis by MVAΔΔΔ-OVA. DAPI staining of cells infected with the indicated viruses at an MOI of 5. Nuclei were stained 15h p.i.. Uninfected cells (mock) were used as a negative control.

4. *In vivo* evaluation of immunogenicity and efficacy

Following the genetic and functional characterization of the constructed MVA viruses, this last part of the study focuses on the comparison of our constructed vaccine candidates *in vivo*. For that purpose, we evaluated the immunogenicity and efficacy of our constructs in two separate mouse models.

4.1. Evaluation of immunogenicity

To determine immunogenicity, we quantitatively compared immune responses induced by our vaccine candidates. For that purpose, C57BL/6 mice were grouped into groups of five. All mice were vaccinated intramuscularly at day 0 (prime) with 10^7 PFU of either MVA-OVA, MVAΔΔ-OVA, MVA-ΔΔΔ-OVA or PBS as control. At day 14 p.i., all mice received a second dose of the same virus/PBS as boost. At day 21 p.i., mice were sacrificed, sera and spleens were collected and samples were analyzed for the presence of OVA-specific T-cells.

The experiments were performed externally in cooperation with our collaborators from Boehringer Ingelheim at the research facility in Biberach under the guidance of Dr. Aleksandra Kowalczyk.

4.1.1. Induction of OVA-specific T-cells

To determine if a prime/boost immunization induces OVA-specific T-cells, a FACS analysis of splenocytes was performed. SIINFEKL was used as an OVA-specific peptide to stimulate CD8+ T-cells and determine the amount of CD8+ T-cells capable of being activated.

As expected, in mice immunized with PBS as control no OVA-specific CD8+ T-cells were detectable among the T-cell population. In contrast, all mice mounted OVA-specific T-cell responses when immunized with any of the recombinant MVA vaccines. This response is highly significant for all MVA vaccinated groups in comparison to the control group that received PBS as mock vaccine (Fig. 18). Overall, the three MVA-OVA vaccines induced comparable levels of OVA-specific T-cells. Yet, we observed a slightly higher amount of OVA-specific T-cells in mice immunized with MVAΔΔ-OVA

compared to the other groups, albeit no significant increase could be detected (Fig. 18).

These results show that our mutant MVA viruses are indeed able to induce CD8+ T-cells specific for OVA after prime/boost vaccination at levels that are at least comparable to those elicited by non-modified recombinant MVA.

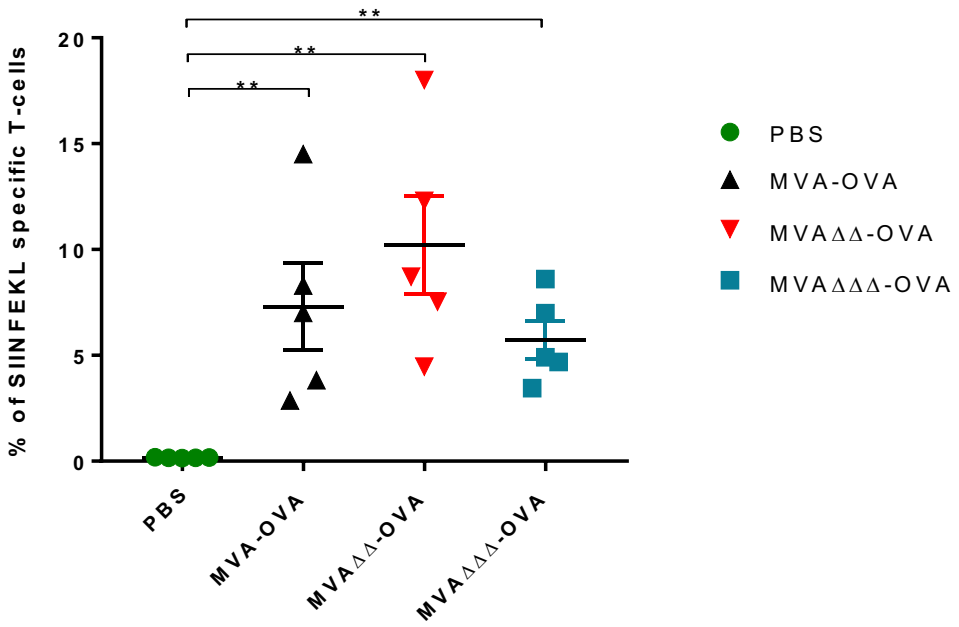

Fig. 18: Analysis of SIINFEKL specific T-cells. Mice were immunized i.m. in a prime/boost setting with 10^7 PFU of the indicated viruses (n=5). Mice inoculated with PBS were used as control. 21 days p.i., the amount of SIINFEKL-specific CD8+ T-cells was determined using flow-cytometry (FACS); values are given as mean with SEM. Statistically relevant differences between results are indicated with **, P<0.01.

4.2. Evaluation of efficacy

For comparing efficacy, all three candidate vaccines were tested in a therapeutic setting using an E.G7 OVA-expressing murine tumor model. E.G7 is a murine T-cell lymphoma cell line that, when injected subcutaneously, leads to the formation of a solid tumor with tumor cells that stably express OVA-epitopes as a unique antigen (ZHOU et al., 1992). Therefore, OVA-specific immune responses can affect tumor growth. With that said we decided to compare efficacy of our candidate vaccines by

evaluating and comparing the outcome of therapeutic immunizations after tumor challenge.

For that purpose, female C57Bl/6 mice were grouped into seven experimental groups with 12 animals per group. All mice received $0.5x10^6$ E.G7–OVA tumor cells subcutaneously into the flank at day 0. When tumors were palpable at day six, mice were intramuscularly vaccinated once with MVA-OVA, MVAΔΔ-OVA, MVA-ΔΔΔ-OVA or PBS as control. For dosage evaluation, two groups always received the same vaccine candidate with either 10^6 PFU or 10^7 PFU (Fig. 19A). After vaccination, tumor growth and survival rate of each group were monitored and evaluated at least twice a week until the end of the experiment at day 30 (Fig. 19B). Tumor growth was evaluated by determining tumor volume using a caliper. Mice were sacrificed once they reached a previously set end point respectively at the end of the experiment.

A

Group 1	PBS	
Group 2	MVA-OVA	$1x10^6$ PFU
Group 3	MVAΔΔ-OVA	$1x10^6$ PFU
Group 4	MVAΔΔΔ-OVA	$1x10^6$ PFU
Group 5	MVA-OVA	$1x10^7$ PFU
Group 6	MVAΔΔ-OVA	$1x10^7$ PFU
Group 7	MVAΔΔΔ-OVA	$1x10^7$ PFU

B

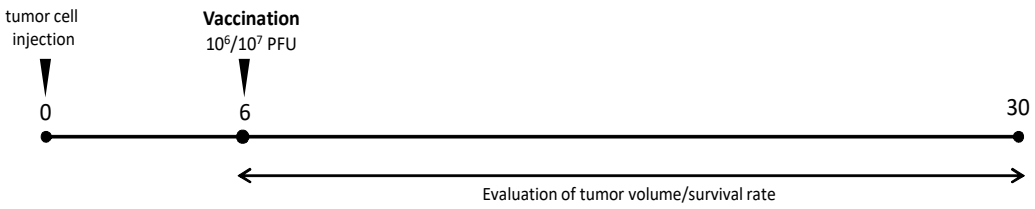

Fig. 19: E.G7–OVA Tumor model. Grouping of mice (A) and timetable of experimental setup (B).

The experiment was performed externally in cooperation with our collaborators from Boehringer Ingelheim at the research facility in Biberach under the guidance of Prof. Dr. Klaus Erb.

4.2.1. Immunization slows tumor growth and prolongs survival

Median survival for PBS vaccinated mice was 21 days.

Of the groups vaccinated with 10^6 PFU, mice vaccinated with MVA-OVA showed median survival of 25.9 days whereas mice vaccinated with MVAΔΔ-OVA showed median survival of 25.95 days. The longest median survival was observed for animals vaccinated with MVAΔΔΔ-OVA with 27 days. Of the groups that were vaccinated with a one log higher dose of MVAs (10^7 PFU) median survival was 25.075 days for MVA-OVA, 25.125 days for MVAΔΔ-OVA and 25.175 days for mice vaccinated with MVAΔΔΔ-OVA.

In terms of survival rate, a significantly prolonged survival was observed for groups that received any of the recombinant MVAs compared to the PBS receiving control group ($p<0.001$). This could be observed for both dosages tested (Fig. 20 A and B). However, out of the groups that received 10^6 PFU, no statistical significances were observed between the group that received MVA-OVA and the groups that received either MVAΔΔ-OVA or MVAΔΔΔ-OVA. Nevertheless, survival rate was significantly higher for animals that received 10^6 PFU of MVAΔΔΔ-OVA compared to animals that received 10^6 PFU of MVAΔΔ-OVA ($p<0.05$). At the same time, out of the groups that received 10^7 PFU, no statistical significances were observed between any of the groups that received recombinant MVAs.

Concerning tumor growth, all groups that received a recombinant MVA-vaccine show significantly smaller tumor volumes starting at day 15 ($p<0.001$) when compared to the control group. However, no statistical differences could be detected between groups that received different viruses, except at day 26, where tumor volume was significantly lower for animals that received 10^6 PFU MVAΔΔΔ-OVA compared to animals that received 10^6 PFU MVAΔΔ-OVA ($p<0.05$) (Fig. 20 A and B).

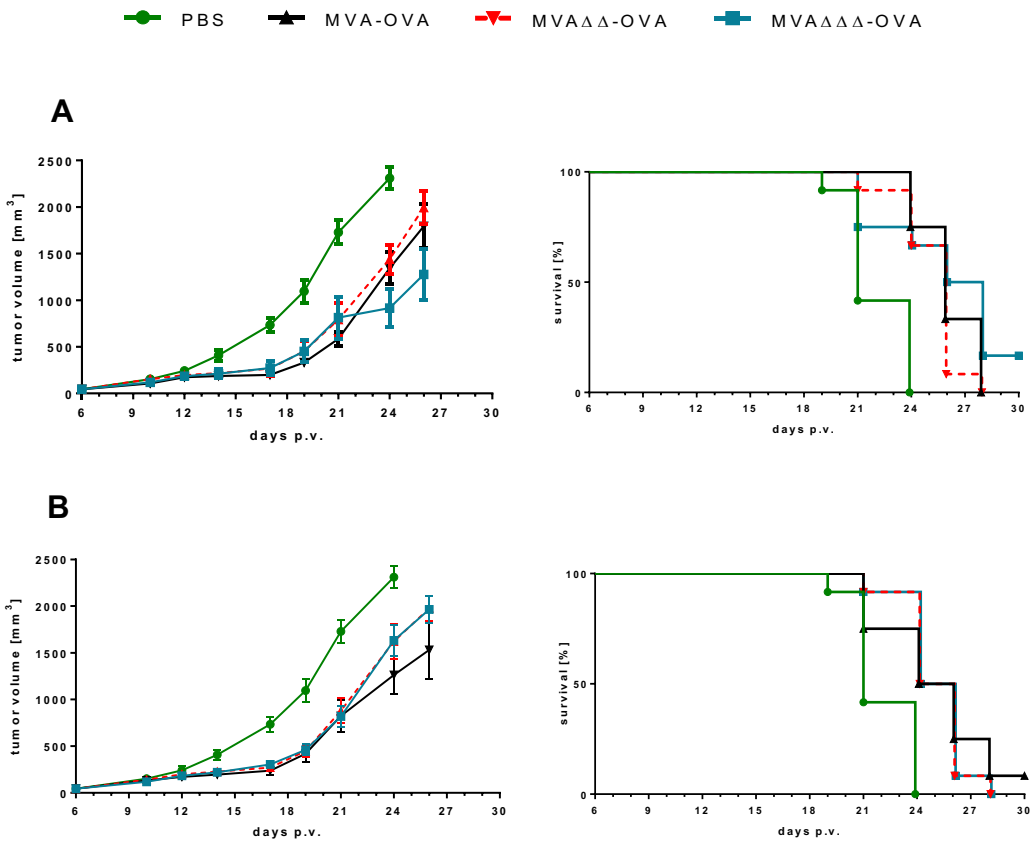

Fig. 20: Efficacy of modified recombinant MVA-OVA vaccine candidates. C57Bl/6 mice (*n*=12) were inoculated with 0.5x10^6 E.G7–OVA cells s.c.. When tumors were palpable at day 6 post inoculation, mice were vaccinated with either 10^6 PFU (A) or 10^7 PFU (B) of the indicated viruses. One group received PBS as control. Tumor growth and survival rate were analyzed over time. p.v. = post vaccination. Values of tumor volume are shown as mean +/- SEM+.

Taken together, these results indicate that a therapeutic vaccination of mice with recombinant MVA vector vaccines is successful and is capable of significantly increasing survival rate while significantly slowing tumor growth. Outcomes of both dosages were similar, indicating that 10^6 PFU was already sufficient to induce a potent immune response that was able to decelerate tumor growth and prolong survival. However, no significant differences could be detected between efficacies of wildtype MVA-OVA compared to our constructs. Nevertheless, MVAΔΔΔ-OVA performed significantly better compared to MVAΔΔ-OVA when given at a dosage of 10^6 PFU.

V. DISCUSSION

Prophylactic vaccines against various pathogens are generally very effective and protect against all types of infectious diseases. Therapeutic cancer vaccines on the other hand are much less successful. One reason for that is the occurrence of antigens. Pathogen-targeting vaccines can be based on pathogen-specific non-self antigens. Such antigens are naturally immunogenic and very potent in eliciting a strong immune response. With cancer vaccines, antigen identification is much more complicated. Target antigens need to be tumor-specific and unique to or at least overexpressed on tumor cells as compared to healthy cells (https://www.ncbi.nlm.nih.gov/pubmed/25483639). Over the last years, the identification of possible tumor antigens has been heavily studied and to date, several promising antigens for different types of cancer have been detected. However, successful therapeutic cancer vaccination doesn't only require the right antigen. Tumors embed themselves in their own microenvironment, in which the immune system is heavily down regulated. Therefore, the immune responses elicited need to be strong enough to overcome immune evasion of the tumor. In that context, the development of viral vector based therapeutic cancer vaccines has gained considerable interest over the years.

This study focused on the improvement of MVA as a viral vector, especially for immunotherapeutic approaches. For that reason, we constructed two genetically modified recombinant MVA viruses expressing OVA as a model antigen. Both constructs (MVAΔΔ-OVA and MVAΔΔΔ-OVA) were deleted in two (MVAΔΔ-OVA) respectively three (MVAΔΔΔ-OVA) immunoregulatory genes in order to potentially boost the vector's efficacy. MVA-OVA served as a recombinant non-modified control virus. At first, a detailed genetic and functional *in vitro* characterization was performed. Replication analysis of constructs in comparison to MVA-OVA as well as wildtype MVA F6 showed no lack of replication capacity in susceptible primary CEF cells. In contrast, both constructs were unable to productively replicate in human HaCat cells as expected, confirming no change in the biosafety level 1 profile of MVA. Furthermore, it was shown that both constructs were able to stably express

OVA at levels equal to MVA-OVA in both chicken and murine cell lines. Successful deletion of the desired genes (184R, 029L, 008L) in both constructs was genetically confirmed with no change in backbone integrity. The results confirmed that simultaneous deletion of two respectively three immunomodulatory genes from the MVA genome still allows for the construction of a stable recombinant MVA. Furthermore, deletions were also ratified on a functional level *in vitro*. Lack of the F1L protein (029L) was shown to increase apoptosis in infected cells. Absence of the vIL-18bp (008L) and IL-1βR (184R) was confirmed through missing binding activity of the respective Interleukin. Additionally, immunogenicity and efficacy of the constructs was tested *in vivo* using a prime/boost vaccination study and a murine tumor model. The immunogenicity study revealed that both constructs are able to elicit antigen-specific CD8+ T-cells at levels comparable to non-mutated MVA-OVA. Results from the efficacy study reinforced MVA's therapeutic potential with delayed tumor growth and longer survival of vaccinated compared to non-vaccinated mice. However, in this particular setting, no significant differences were detectable between immunogenicity and efficacy of non-mutated MVA and our two mutated constructs.

Why is the development of new alternative cancer therapies important?

Today, cancer is among the leading causes of death worldwide (Fig. 21). According to the WHO, 8.8 million people have died from cancer in 2015 alone. That means, in 2015 almost 1 in 6 deaths globally was caused by cancer. At the same time, cancer is also an economical burden. In 2010 the annual economic cost of cancer was estimated to be 1.16 trillion US$ (WHO data: http://www.who.int/cancer/en/). But despite the obvious need and intensive research over the last years, cancer therapy is developing slowly. Classical treatments of cancer, including radiotherapy, chemotherapy and surgical intervention, are still considered gold-standard therapies for many cancers. They present however many limitations for usage including severe side effects due to lack of specificity and toxicity (LA-BECK et al., 2015). Therefore, the development of new effective treatment options has become a high priority in medical research, with one focus being on the enhancement of treatment specificity to limit negative effects on healthy

tissue.

In that context, viral immunotherapy has gained considerable interest over the years and viral vector-based therapeutic cancer vaccines are gaining ground. However, despite first promising results, so far immunogenicity and efficacy of these vectors is still not strong enough to fully overcome the strong immune tolerance exhibited by tumors. Thus, research efforts to improve vector platforms need to be intensified to develop next generation vectors with increased immunogenicity and efficacy.

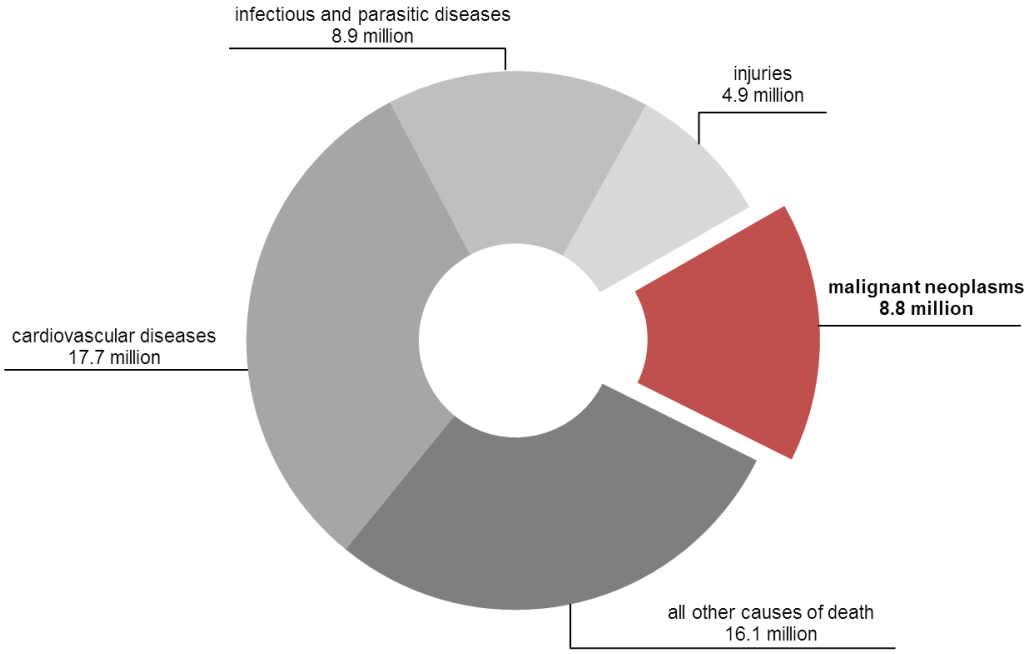

Fig. 21: Leading causes of death worldwide in 2015. According to data published by the WHO in 2016: WHO methods and data sources for global causes of death 2000-2015. Global Health Estimates Technical Paper WHO/HIS/HSI/GHE/2016.3. Geneva: World Health Organization; 2016 (http://www.who.int/healthinfo/global_burden_disease/GlobalCOD_method_2000_2015.pdf).

Reasons for choosing MVA as a vector platform

Several different viral vectors are currently being investigated as platforms for the development of therapeutic cancer vaccines (LAROCCA & SCHLOM, 2011). One distinct feature of viral vectors is their replicative capacity in humans with different replicating and non-replicating vectors that are being developed. Undeniable, one advantage of replicating vectors such

as VACV is their oncolytic potential. Such oncolytic viruses are either naturally occurring or engineered to be tumor selective when replicating and therefore they are able to directly lyse infected tumor cells (KELLY & RUSSELL, 2007; FUKUHARA et al., 2016). But although this concept holds great potential in theory, concerns about the safety of replicating vectors have been raised. Especially immunocompromised people such as cancer patients are at risk of experiencing severe side effects after vaccination due to the incompetence of the immune system to control viral replication of the vector. Additionally, the vector's toxicity, environmental shedding and the rates of possible mutations including the reversion to the potentially harmful wild type virus are being discussed (RUSSELL et al., 2012; BUIJS et al., 2015).

MVA is a non-replicating viral vector with a remarkable safety profile, also under immunocompromised conditions (STITTELAAR et al., 2001). Because of this, it can be safely administered to cancer patients, also as add-on or follow-up treatment after radio- or chemotherapy, when patients are known to be heavily immunocompromised. Several clinical trials to date have confirmed this (HARROP et al., 2013; QUOIX et al., 2016). Moreover, MVA is known to make stable recombinants and to be able to successfully deliver foreign antigens to the host cell (SUTTER & MOSS, 1992), making way for an efficient and strong antigen-specific immune response, an important feature for overcoming immune tolerance in tumors (PERKUS et al., 1985). Also, with more and more potential TAAs being discovered, the potential of delivering several antigens at the same time can be of great advantage (PERKUS et al., 1985). It is known that for efficacy of cancer vaccines, a strong T-cell response plays a crucial role. Early on it was shown that TAAs are recognized by both CD4+ and CD8+ T cells and that activated cytotoxic CD8+ T cells are able to directly lyse TAA presenting tumor cells (BOON et al., 1994). Moreover, spontaneous regression was observed for some-melanomas and was clearly associated with T cell activation and infiltration (LOWES et al., 1997), underlining the importance of an adequate T-cell response. In that context, many studies have already shown that recombinant MVAs are able to activate high levels of antigen-specific CD8+T cells (VOLZ & SUTTER, 2013; KREIJTZ et al., 2014; VOLZ et al.,

2016) upon immunization.

Deletion of immunomodulatory genes

Despite the fact that the majority of immunomodulators known in VACV have been deleted in MVA during the attenuation process, some immunomodulatory genes are still conserved in MVA (MEYER et al., 1991; ANTOINE et al., 1998). These gene functions are responsible for counteracting cellular antiviral responses triggered after MVA infection. A number of them have been identified, studied and were shown to influence both, the innate as well as the acquired immune response (GARCIA-ARRIAZA & ESTEBAN, 2014). Deleting one or more of those still intact genes thus provides an opportunity, to enhance MVA's efficacy as a vector platform.

The cellular induction of the apoptotic pathway is one antiviral mechanism MVA has to prevent after infection in order to keep the infected cell vital and be able to complete its life cycle. Thus, it is of no surprise that MVA encodes proteins with anti-apoptotic function, including the F1 protein (FISCHER et al., 2006). Deletion of the F1 protein in both VACV and MVA was previously shown to result in the induction of apoptosis in human cell lines (WASILENKO et al., 2005; FISCHER et al., 2006). This phenotype could be confirmed in this study for HeLa cells infected with MVAΔΔΔ-OVA. Interestingly, western blot analysis of apoptosis associated caspase-3 activation in infected cells also showed a signal for activated caspase-3 for MVA-OVA and MVAΔΔ-OVA infected cells. However, this signal was also present in uninfected cells and much weaker than the signal detected in cells infected with MVAΔΔΔ-OVA. This signal seems to be cell culture dependent and not specific for viral infection. At the same time, levels of activated caspase-3 in cells infected with MVAΔΔΔ-OVA were much higher and comparable to levels in cells treated with Staurosporine, a known highly potent inducer of apoptosis and activator of Caspase-3. This is in line with the observation that in cells infected with MVAΔΔΔ-OVA or treated with Staurosporine, the signal for uncleaved inactive caspase-3 vanished nearly completely in contrast to the strong signal detectable in cells infected with MVA-OVA, MVAΔΔ-OVA and mock infected cells.

Because apoptotic cell death results in a shut-down of the cell and thus of

the cellular machinery exploited by the virus, it could potentially be harmful for the viral life cycle and thus antigen production over time. However, deletion of the F1L ORF in VACV Copenhagen showed no signs of disruption of the virus life cycle (WASILENKO et al., 2005). In this study, multistep growth analysis also revealed no differences in replication capacity in primary CEF cells between MVAΔΔΔ-OVA and wildtype MVA F6 over the course of 72h despite apoptosis. At the same time, western blot samples obtained 15hpi from MVAΔΔΔ-infected cells (MOI 5) show strong activation of caspase-3 and overall signs of apoptosis. However, western blot samples of MVAΔΔΔ-OVA infected cells (MOI 5) obtained for protein analyses at 24hpi showed no differences in OVA production compared to MVA-OVA and MVAΔΔ-OVA with intact F1L. Thus, the onset of apoptosis didn't negatively influence antigen production in this study.

MVA also encodes several proteins that are able to bind to and interact with cytokines in order to down-regulate the immune response (GARCIA-ARRIAZA & ESTEBAN, 2014). Two of such cytokine binding proteins of MVA are the vIL-18bp and the vIL1βR (ALCAMI & SMITH, 1992; FALIVENE et al., 2012). Previous studies have confirmed that single deletion of each protein from the MVA genome has no negative impact on viral replication or antigen production (FALIVENE et al., 2012; ZIMMERLING et al., 2013). This was confirmed to be also true for the simultaneous deletion of both ORFs during this study. Neither MVAΔΔ-OVA nor MVAΔΔΔ-OVA showed any disturbance in replication capacity in primary CEF cells or antigen production during the course of this study.

VACV's vIL1βR was previously identified to be expressed late during the virus life cycle (ALCAMI & SMITH, 1992). This was confirmed in this study by ELISA analysis of supernatants from infected cells that were incubated with IL-1β. In supernatants collected 4hpi, comparable levels of IL1β were detectable in MVA-OVA and mock infected cells. However, in supernatants collected 8hpi and 24hpi, levels of IL-1β were significantly lower in supernatant from MVA-OVA infected cells compared to mock infected cells. This confirms expression of vIL1βR as a late protein in MVA. At the same time this also confirmed binding affinity of the receptor to mature IL-1β as described in the literature (ALCAMI & SMITH, 1992; BLANCHARD et al.,

1998). Visually lower levels of IL-1β in supernatants from MVA-OVA infected cells compared to supernatants from MVAΔΔ-OVA, MVAΔΔΔ-OVA and mock infected cells were most likely due to residues of vIL1βR in the sucrose stock preparation of MVA-OVA. In supernatants from MVAΔΔ-OVA and MVAΔΔΔ-OVA levels of IL-1β remained equally high over time, confirming absence of vIL1βR.

MVA's vIL18bp is described as a binding protein with high affinity to mature IL-18 (SYMONS et al., 2002). This study confirmed the existence of a soluble IL-18 binding protein in the supernatant of MVA-OVA infected cells with the help of an ELISA. At the same, this binding protein was confirmed to bind mature IL-18 at high levels. IL-18 is known to stimulate IFN-γ production in natural killer cells (NKs) and T-cells. (BORN et al., 2000; READING & SMITH, 2003). In this study, we incubated supernatants of MVA infected cells with IL-18 in the presence of Con A and subsequently used them to stimulate freshly prepared murine splenocytes. Splenocytes stimulated with supernatant from mock infected cells that contained high levels of IL-18 also showed high levels of IFN- γ production, confirming IL-18's function as an inducer of IFN-γ in immune cells. At the same time, splenocytes stimulated with supernatant from MVA-OVA infected cells showed significantly lower levels of IFN-γ. This not only confirmed the existence of a functional vIL-18bp in MVA but also, that IL-18 bound to a binding protein is no longer biologically active.

In vivo evaluation of immunogenicity and efficacy

Single deletion mutants that have previously been constructed and described in the literature already showed promising results in terms of enhanced immunogenicity compared to wildtype MVA (FALIVENE et al., 2012; PERDIGUERO et al., 2012b; ZIMMERLING et al., 2013). In this study, we decided to evaluate both immunogenicity and efficacy of our recombinant mutant MVA viruses compared to non-mutant recombinant MVA *in vivo.* Thus, we constructed all viruses to express OVA as a model antigen, which is a well-established concept. It is non-toxic and inert in mice, thus has no negative effect on the animals that could potentially influence results (CARROLL & FITZGERALD, 2004). To evaluate immunogenicity, the amount of OVA-specific CD8+ T-cells was analyzed by flow-cytometry

using SIINFEKL as the OVA-specific peptide to stimulate T-cells. To evaluate efficacy we conducted a second experiment using the EG.7 tumor model which is an established model system and, compared to other OVA-expressing tumor models such as the B16, it is considered to be less aggressive and thus in order opinion a good choice for a first efficacy study (DE TITTA et al., 2013).

The well-established dosage most commonly used for immunization experiments with MVA is $1x10^8$ PFU, with strong immune responses detectable (VOLZ & SUTTER, 2013). However, to have a higher chance of detecting differences between our constructs and MVA-OVA, we first decided to compare viruses with a reduced dosage of 10^7 PFU in our immunogenicity experiment. Upon prime/boost immunization of mice we were able to detect OVA-specific CD8+ T-cells at similar amounts in all our recombinant MVA viruses while mice immunized with PBS showed no OVA-specific T-cells as expected. However, we were unable to detect significant differences in the amounts of OVA-specific T-cells elicited by our three viruses. One possible reason for that might be the dosage of 10^7 PFU. Although this is already a one log reduction compared to the most commonly used dosage for immunization experiments, it might still be too high to detect differences in immunogenicity between viruses. At the same time, OVA is known to be a very strong and immunodominant antigen. 10^7 PFU might already be enough to saturate the system, making it unable to reveal differences. Against this background, we decided in order to increase our chances to detect differences in efficacy, to compare viruses again with a dosage of 10^7 PFU and additionally also with a lower dosage of 10^6 PFU. Nevertheless, in both dosage experiments results were very similar. We were able to confirm the general efficacy of MVA-based therapeutic vaccines in a tumor model. Regardless of the dosage, all tested viruses were able to significantly prolong survival while slowing down tumor growth. This data shows that MVA can be effective in rather low doses, an option that is of great advantage in the clinic, where dose reduction is of great importance. However, we were not able to observe any significant differences in efficacy between MVA-OVA and our two constructs. This could once again be due to the chosen dosages. Although we already

decided to reduce vaccine dosages compared to simple immunization experiments, the chosen dosages of 10^7 and 10^6 PFU might still be too high in order to detect differences in efficacy. This is supported by the fact, that both dosages more or less delivered the same results, showing that the amount of virus delivered with a smaller dose was still able to elicit comparable immune responses as the higher dose. Taken together with the fact that OVA was shown to be strongly expressed by MVA, the system's set-up might simply not be sensitive enough to detect differences. It would be an option in future experiments to possibly further reduce dosages or look for a more sensitive model system as read-out. The studies conducted for the single knock-out viruses compared immunogenicity rather than efficacy to compare viruses and show differences. Measuring antibody and T-cell responses in more detail might be a more sensitive system to detect significant variations and could be used in further studies. Nevertheless, this data confirms that MVA can be effective in rather low doses, an option that is of great advantage in the clinic, where dose reduction is important.

Future perspectives

Today, safe and effective vaccine candidates on the basis of MVA are constantly being generated and tested for various infectious diseases. At the same time, more and more MVA based therapeutic cancer vaccines are being investigated, some of which have already entered clinical trials (AMATO et al., 2010; HARROP et al., 2013; QUOIX et al., 2016). However, until now no candidate vectors have been able to elicit strong enough immune responses to be highly effective, let alone curative against tumors. One possible approach that seems very promising is the combination of several therapies to maximize effect (DRAKE, 2012). Many studies have already combined MVA based immunotherapy with conventional first-line therapies (i.e. radiotherapy, chemotherapy) with promising results (HARROP et al., 2007; HARROP et al., 2008; AMATO et al., 2010; QUOIX et al., 2016). The recent successful development of other immunotherapies, above all the advancements made in the field of checkpoint inhibitors (MARTIN-LIBERAL et al., 2017), will likely make way for diverse combinations of different immunotherapies. For example, a recent study revealed that the efficacy of the checkpoint inhibitor CTLA-4 synergized with

an MVA-based vaccine resulting in a significantly improved median survival in a mouse model (FOY et al., 2016). Nonetheless, further enhancements of the humoral and especially the cellular immune response is certainly of great importance. Hence, improvements on the vector itself should be pursued further in order to maximize the vectors capabilities of inducing the strongest immune responses possible. Additionally, research on identifying and improving cancer specific target antigens is equally important.

VI. SUMMARY

Characterization of vaccinia virus MVA candidate vaccines mutated in viral genes modulating inflammasome activation

Despite ongoing intensive research efforts, cancer is still among the leading causes of death worldwide. In 2015 alone, an estimated 8.8 million people died from malignant tumors. Classical treatments (i.e. chemotherapy, radiotherapy and surgical treatment) are still considered to be standard of care for most types of cancer, although they clearly lack specificity and cause a variety of negative side effect in treated patients.

Among the many approaches for novel cancer treatments, the field of viral immunotherapy is on the rise which uses therapeutic application of viral vectors to induce anti-tumoral immune responses. In that context, the live attenuated Modified Vaccinia virus Ankara (MVA) is being investigated as a potential viral vector for the construction of therapeutic cancer vaccines. It has already been successfully tested as a vector platform for the construction of prophylactic vaccines against various infectious diseases in several clinical studies. MVA has an exceptionally good safety profile due to its replication deficiency in human cells and can thus be administered to risk groups with no adjuvants needed. Nevertheless, it is able to efficiently deliver one or more recombinant antigens and induces strong antigen-specific humoral and cellular immune responses. However, previous studies have shown that MVA along with all other potential vectors is not yet able to elicit immune responses strong enough to fully overcome the tumor's immune tolerance.

The aim of this study was to improve MVAs potential as a viral vector by enhancing its immunogenicity and efficacy. Therefore, two genetically modified recombinant MVA viruses deficient in two (MVAΔΔ-OVA) respectively three (MVAΔΔΔ-OVA) immunomodulatory genes were constructed and characterized *in vitro* as well as *in vivo* using Ovalbumin (OVA) as a model antigen. Genetic and functional analysis showed successful deletion of the desired genes from the MVA genome. OVA expression was confirmed for all constructs at high levels. Multi-step

analysis of virus growth proved replication deficiency in human cells (HaCat), whereas replication competence was retained in primary chicken embryo fibroblasts (CEF) as expected. *In vivo* analysis of viruses was performed in mice with 10^7 PFU to compare immunogenicity and two different dosages (10^6 and 10^7 PFU) to compare efficacy in an E.G7 tumor model. Data revealed that immunization with all recombinant MVA viruses led to the induction of comparable amounts of OVA-specific CD8+ T-cells with no significant differences between viruses. Animals in the tumor model that received therapeutic vaccination with any recombinant MVA survived significantly longer and had significantly slower tumor growth than PBS vaccinated mice. However, no significant differences could be observed between any of the two constructs and MVA-OVA. Instead, significant differences could be observed between both construct: Upon immunization with 10^6 PFU, mice vaccinated with MVAΔΔΔ-OVA showed significantly longer survival and slowed tumor growth at the end of the experiment. All in all, these data suggest that MVA has great potential as a therapeutic cancer vaccine and more sensitive studies should be pursued in the future to reinvestigate differences in efficacy and immunogenicity for such deletion mutants.

VII. ZUSAMMENFASSUNG

Charakterisierung von MVA-Impfstoffkandidaten mutiert in verschiedenen viralen Genen zur Modulation der Aktivierung des Inflammasoms

Trotz intensiver Forschung im Bereich der Krebstherapie gehört Krebs immer noch zu den häufigsten Todesursachen weltweit. Rund 8.8 Millionen Menschen starben allein im Jahr 2015 an bösartigen Tumorerkrankungen. Die drei klassischen Therapiemethoden (Chemotherapie, Strahlentherapie sowie die operative Tumorentfernung) gelten auch heute noch als Standardtherapie bei den meisten Krebsarten, obwohl sie unspezifisch und somit durch Schädigung von gesundem Gewebe mit vielen Nebenwirkungen verbunden sind.

Nach neuen Therapieansätzen wird deshalb seit Jahren intensiv geforscht. Eine Strategie, die sich in den letzten Jahren als vielversprechend erwiesen hat, ist die virale Immuntherapie Dabei werden virale Vektorimpfstoffe entwickelt und eingesetzt, um spezifische anti-tumorale Immunantworten im Patienten hervor zu rufen. In diesem Zusammenhang wird das Modifizierte Vaccinia Virus Ankara (MVA) als potentieller therapeutischer Vektor erforscht. MVA wird bereits zur Entwicklung prophylaktischer Impfstoffen gegen diverse Infektionskrankheiten erfolgreich in klinischen Studien getestet. Es kommt ohne die Zugabe von Adjuvantien aus und eignet sich aufgrund seines Replikationsdefizites im Menschen auch zur Therapie immunsupprimierter Risikopatienten. Trotz Replikationsdefizit exprimiert MVA problemlos rekombinante Proteine und induziert eine antigen-spezifische humorale und zelluläre Immunantwort. Allerdings haben Studien zur Nutzung viraler Vektorimpfstoffe als Krebstherapeutika gezeigt, dass die induzierten Immunantworten bei allen Vektoren noch nicht stark genug ist, um die vom Tumor induzierte Immuntoleranz vollständig zu durchbrechen.

Das Ziel der vorliegenden Studie war es deshalb, MVA als Vektorimpfstoff hinsichtlich seiner Effizienz zu verbessern. Dafür wurden im viralen Genom von MVA zwei (MVA$\Delta\Delta$-OVA) bzw. drei (MVA$\Delta\Delta\Delta$-OVA) Gene mit

bekannter immunmodulatorischer Funktion deletiert und anschließend *in vitro* sowie *in vivo* charakterisiert. Dabei wurde Ovalbumin (OVA) als Modellantigen verwendet. Die zunächst durchgeführten genetischen und funktionellen Analysen bestätigten die erfolgreiche Deletion der gewünschten Gene. Die Expression von OVA wurde ebenfalls erfolgreich nachgewiesen. Wachstumsanalysen haben gezeigt, dass die rekombinanten MVA Viren weiterhin nicht fähig waren, in humanen Zellen zu replizieren. Die Replikationsfähigkeit in primären Hühnerzellen (CEF) blieb hingegen wie erwartet unverändert. Eine *in vivo* Evaluation erfolgte hinsichtlich Immunogenität in einer Impfstudie mit einer Impfdosis von 10^7 PFU sowie bezüglich Effizienz im murinen EG.7 OVA Tumormodell, wobei zwei Impfdosen (10^6 und 10^7 PFU) vergleichsweise getestet wurden. Es zeigte sich, dass die Impfung mit allen rekombinanten MVA Viren zu einer vergleichbaren Induktion OVA-spezifischer T-Zellen führt, wobei keine signifikanten Unterschiede zwischen den drei Viren nachweisebar waren. In Mäusen, die mit einem rekombinanten MVA therapeutisch geimpft wurden, konnte ein signifikant verlangsamtes Tumorwachstum sowie eine signifikant verlängerte Überlebenszeit beobachtet werden im Vergleich zu mit PBS geimpften Mäusen. Jedoch konnten keine signifikanten Unterschiede zwischen den beiden Konstrukten und rekombinantem nicht mutiertem MVA nachgewiesen werden. Unterschiede hingegen konnten gezeigt werden zwischen den beiden Konstrukten bei einer Verabreichung von 10^6 PFU, wobei das Trippelkonstrukt zu einer signifikant längeren Überlebenszeit führte. Insgesamt haben die Ergebnisse gezeigt, dass MVA großes Potential als therapeutischer Impfstoff aufweist. Weitere sensitivere Methoden sollten in Zukunft angewendet werden, um die Deletionsmutanten erneut zu testen und eventuell vorhandene Unterschiede besser dar zu stellen.

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IX. APPENDIX

1. Chemicals, reagents and consumables/plasticware

Description	Supplier
6-/24-/96-well flat bottom plates	Sarstedt, Nümbrecht, Germany
96-well Nunc MaxiSorp™ microwell plates	BioLegend, San Diego, USA
Acetone (C_3H_6O)	Carl-Roth GmbH, Karslruhe, Germany
Acrylamide 30% (C_3H_5NO)	AppliChem, Darmstadt, Germany
Ammoniumpersulfate ($H_8N_2O_8S_2$)	AppliChem, Darmstadt, Germany
Bovine serum albumine (BSA)	Sigma-Aldrich, St. Louis, USA
Bromophenol blue	Merck, Darmstadt, Germany
Cell culture flasks (25/75/175 cm^2)	Sarstedt, Nümbrecht, Germany
Color plus protein ladder	New England BioLabs ® GmbH, Frankfurt am Main, Germany
Color Protein Standard, broad range	New England BioLabs ® GmbH, Frankfurt am Main, Germany
DAPI Nucleic acid stain	invitrogen™, Carlsbad, USA
Distilled water	In-house production, LMU, München, Germany
DMSO (C_2H_6OS)	Carl-Roth GmbH, Karslruhe, Germany
DTT ($C_4H_{10}O_2S_2$)	Sigma-Aldrich, St. Louis, USA
EDTA ($C_{10}H_{16}N_2O_8$)	ICN Biochemicals, Ohio, USA
GelRed™	Biotrend, Köln, Germany
Glycerol ($C_3H_8O_3$)	Carl-Roth GmbH, Karslruhe, Germany
Glycine ($C_2H_5NO_2$)	Carl-Roth GmbH, Karslruhe, Germany
Hydrochloric acid (HCl), 6N	Carl-Roth GmbH, Karslruhe, Germany
Hypoxanthine	Sigma-Aldrich, St. Louis, USA
LE Agarose	Biozym, Hessisch Oldendorf, Germany
Methanol (CH_4O)	Carl-Roth GmbH, Karslruhe, Germany
Monopotassium phosphate (KH_2PO_4)	Carl-Roth GmbH, Karslruhe, Germany
Mycophenolic acid (MPA)	Sigma-Aldrich, St. Louis, USA
Nitrocellulose blotting membrane, 0.2 µm	GE Healthcare, München, Germany
Nonfat dried milk powder	AppliChem, Darmstadt, Germany
Paraformaldehyde	Carl-Roth GmbH, Karslruhe, Germany
Potassium chloride (KCl)	Merck, Darmstadt, Germany

Quick-Load® 2-log DNA ladder	New England BioLabs ® GmbH, Frankfurt am Main, Germany
Red blood cell lysing buffer	Sigma-Aldrich, St. Louis, USA
SDS ($C_{12}H_25NaO_4S$)	Carl-Roth GmbH, Karslruhe, Germany
Sodium chloride (NaCl)	Carl-Roth GmbH, Karslruhe, Germany
Staurosporine solution from Streptomyces sp., 1mM	Sigma-Aldrich, St. Louis, USA
TEMED ($C_6H_{16}N_2$)	SigmaAldrich, St. Louis, USA
Tris ultrapure ($C_4H_{11}NO_3$)	AppliChem, Darmstadt, Germany
Tris-gycine buffer, 10x	Bio-Rad, München, Germany
Triton® X-100	AppliChem, Darmstadt, Germany
TrueBlue™ Peroxidase Substrate	seracare, Milford, USA
Trypan blue	Sigma-Aldrich, St. Louis, USA
Tween20	Sigma-Aldrich, St. Louis, USA
Xanthine	Sigma-Aldrich, St. Louis, USA
λ DNA - Hind III and φX174 DNA - Hae III Mix Ready to Use Marke	Finnzymes/Thermo Fisher Scientific, Walham, USA

2. Laboratory equipment and software

Equipment	**Supplier**
Avanti® J-26 XP Centrifuge	Beckmann Coulter, Krefeld, Germany
ChemiDoc™ MP, Imaging System	Bio-Rad, München, Germany
Sunrise™ microplate absobance reader	Tecan Trading AG, Männedorf, Switzerland
Neubauer chamber improved	Paul Marienfeld GmbH&Co.KG, Lauda-Königshofen, Germany
PeqSTAR 2X Thermocycler	PEQLAB Biotechnology GmbH, Erlangen, Germany
Optima™ LE-80K Ultracentrifuge	Beckmann Coulter, Krefeld, Germany
Sonoplus	Bandelin electronics, Berlin, Germany
Olympus CKX41	Olympus Life Sciences, Hamburg, Germany
Trans Blot Turbo system	Bio-Rad, München, Germany
KEYENCE BZ-X710 All-in-one Fluorescence Microscope	KEYENCE Deutschland GmbH, Neu-Isenburg, Germany

3. Commercial kits

Kit	**Supplier**
Mouse IFN-γ ELISA MAX™ Deluxe	BioLegend, San Diego, USA
Mouse IL-1β ELISA MAX™ Deluxe	BioLegend, San Diego, USA
Clarity™ ECL Western Blotting Substrate	Bio-Rad, München, Germany
QIAamp DNA Mini Kit	Qiagen, Venlo, Netherlands
NucleoSpin® Plasmid	MACHERY-NAGEL GmbH&Co. KG, Düren, Germany
NucleoSpin® Gel and PCR Clean-up	MACHERY-NAGEL GmbH&Co. KG, Düren, Germany

4. Buffers, solutions, SDS-gels

Buffers and solutions	**Conditions**
Loading buffer (LB), 6x	10 mM Tris-HCl pH 7.6 60% glycerol 60 mM EDTA 0.03% bromophenol blue add. distilled water
Lysis buffer, 1x	62.5 mM Tris-HCl pH 6.8 2% SDS 10% glycerol 0.01% bromophenol blue 50 mM DTT add. distilled water
PBS (pH 7.4)	140 mM NaCl $Na_2HPO_4 + 7H_2O$ KCl KH_2PO_4 add. distilled water
TAE (pH 7.8), 50x	2 M Tris-acetate 0.5 M NaCl 50 mM EDTA add. distilled water
TBS (pH 7.6), 10x	200 mM Tris base 1.4 M NaCl add. distilled water
Transfer buffer, 1x	25 mM Tris base 200 mM glycine 20% ethanol add. distilled water

Tris/Glycine/SDS (pH 8.3), 10x	25 mM Tris 192 mM glycine 0.1% SDS
Mycophenolic acid soultion	0.25% mycophenolic acid (10mg/ml in 0.1 N NaOH) 2.5% xanthine (10mg/ml in 0.1 N NaOH) 0.15% hypoxanthine (10mg/ml in 0.1 N NaOH) add. distilled water

SDS-gel	**Conditions**
Resolving gel (10%)	3.3 ml 30% acrylamide 2.5 ml 1.5 M Tris-HCl, pH 8.8 0.3 ml 10% ammonium persulfate 0.1 ml 20% SDS 8 µl TEMED add. 10 ml distilled water
Resolving gel (15%)	5.0 ml 30% acrylamide 2.5 ml 1.5 M Tris-HCl, pH 8.8 0.3 µl 10% ammonium persulfate 0.1 ml 20% SDS 8 µl TEMED add. 10 ml distilled water
Stacking gel (5%)	0.5 ml 30% acrylamide 0.38 ml 1.0 M Tris-HCl, pH 6.8 0.1 ml 10% ammonium persulfate 60 µl 20% SDS 6 µl TEMED add. 3 ml distilled water

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