# Improving the cellular immunogenicity of recombinant Modified Vaccinia virus Ankara using green fluorescent protein as model system

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Für

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# I. INTRODUCTION

Infectious diseases are still one of the leading causes of death worldwide. Even if the diagnostic and therapeutic opportunities have been improved year by year, there are still a lot of infectious diseases completely untreatable. These diseases represent one of the major challenges in public health. For this reason the development of preventive measures such as vaccines is supposed to have high priority in medical research.

Generally many different strategies for the generation of new vaccines are pursued. Today's research works on live-attenuated or inactivated vaccines, subunit or DNA vaccines as well as on viral vector vaccines. As far as viral vectors are concerned, poxviruses serve as a promising platform for vaccine development and production. In this context Modified Vaccinia virus Ankara (MVA), a member of the family Poxviridae, has successfully been tested as a vector for various infectious antigens in phase I to IIb clinical trials. MVA is a highly attenuated and safety tested Vaccinia virus strain which can stably express high amounts of heterologous protein. This replication-deficient viral vector has been generated through more than 500 growth passages on chicken embryo fibroblasts. Recently published data show that MVA is able to trigger both, antigen-specific humoral immune response and cellular immune response. Especially the potent induction of antigen-specific T cells gets more and more into the focus of vaccine development. Enhancing this part of the immune reaction seems to be a promising approach in the control of viruses with a high antigenic shift potential such as influenza.

The aim of this project was to improve MVA as a viral vector through enhancing its cellular immunogenicity. Therefore green fluorescent protein (GFP) was used as a model antigen in the MVA vector system. Several recombinant MVA-GFP candidate vaccines were constructed containing GFP in combination with specific localization signals. The effects of myristoylation and a nuclear localization signal were investigated *in vitro* and *in vivo* and compared to unmodified GFP. Myristoylated GFP induced a significantly higher level of antigen-specific CD8+ T cells compared to all

other GFP variants tested. Thus, myristoylation could serve as a promising tool to enhance the cellular immunogenicity of specific target antigens expressed within the MVA vector system.

# II. LITERATURE REVIEW

## 1. T cell mediated immunity against major infectious diseases

### 1.1. T lymphocytes

In general, the immune system is divided into two parts, the innate and the adaptive immune system (CHAPLIN, 2010). Although the innate immune system responds universally and very rapidly in case of exposure to infectious agents (BEUTLER, 2004), it is rather unspecific (TOPFER et al., 2015) and without the capacity to induce immunological memory (WARRINGTON et al., 2011). In contrast, the activation of the adaptive immune system takes more time, but it allows for selective recognition of specific antigens (CHAPLIN, 2010). Moreover, it is able to develop immunological memory (ZINKERNAGEL, 2000). This allows the immune system to respond more strongly and faster in case of re-encounter with the same infectious agent (ZINKERNAGEL, 2000; SALLUSTO et al., 2010). For this reason the development of immunological memory is the basis of all vaccination strategies. Generally, efficient vaccines are represented by a strong and stable activation of the adaptive immune system.

The adaptive immune system can be further subdivided in a cellular and a humoral fraction. The cellular part of the adaptive immune system consists of T lymphocytes, whereas the humoral immune response is predominantly based on antibodies produced by B cells (CHAPLIN, 2010; JANEWAY, 2013). All blood cells originate from pluripotent hematopoietic stem cells in the bone marrow (WARRINGTON et al., 2011). These cells can either develop further to a myeloid or to a lymphoid progenitor cell. The myeloid progenitor cell is the origin of all cells which belong to the myeloid lineage, such as red blood cells, thrombocytes and different kinds of granulocytes (MURPHY et al., 2008). The lymphoid progenitor cell, however, is the precursor of B lymphocytes, T lymphocytes and natural killer cells (CHAPLIN, 2010; LUCKHEERAM et al., 2012). B cells mature

in the bone marrow, whereas T cells are initially released to the blood and develop further in the thymus (GERMAIN, 2002; BEVAN, 2004). In this process all T cells carrying functional T cell receptors survive positive clonal selection, whereas cells with receptors binding self-antigens are eliminated during negative clonal selection to avoid autoimmune reactions (KLEIN et al., 2014). After reentering the bloodstream, T cells constantly circulate between the blood and lymphatic tissues (GOWANS, 1996). These circulating mature T cells which have not yet met their specific antigen, are called naive T cells (MURPHY et al., 2008).

The T cell population can be subdivided into two major groups, cells carrying CD8 co-receptors (CD8+ T cells) and CD4 co-receptor (CD4+ T cells) positive cells (GERMAIN, 2002). CD4+ T cells are mainly involved in the defense of extracellular bacteria and parasites (LUCKHEERAM et al., 2012). Furthermore they are able to secret immuno-modulatory cytokines which can activate and recruit other immune cells such as macrophages, B lymphocytes or CD8+ T cells (BEVAN, 2004). Regulatory T cells can partially express CD4 on their surface as well. These cells are involved in an important mechanism which helps to protect the organism from an uncontrolled T cell response or autoimmunity (LUCKHEERAM et al., 2012). CD8+ T cells, on the other hand, are responsible for the defense of intracellular pathogens such as viruses and some bacteria. Their main function consists of recognizing and killing virus-infected cells. In this context, the induction of apoptosis is the main way to eliminate infections (WRIGHT et al., 2004; MURPHY et al., 2008). Therefore CD8+ T cells synthesize and store cytotoxic proteins such as perforin or granzyme in granules (MURPHY et al., 2008). Perforin is able to form pores in the plasma membrane which are essential for the translocation of granzyme to the cytoplasm of infected cells (MURPHY et al., 2008). After entering the cell, granzyme activates proteolytic caspase cascades which finally result in apoptosis (MURPHY et al., 2008). For this reason CD8+ T cells are also called cytotoxic T cells (MURPHY et al., 2008).

## 1.2. Function of the αβ-T cell receptor

The major task of T cells consists of recognizing foreign antigens presented through the body's own cells. Therefore all T lymphocytes carry about 30,000 identical antigen recognition receptors with unique antigen-binding sites on their cell surface (MURPHY et al., 2008). These T cell receptors (figure 1) are heterodimeric molecules consisting of two glycoprotein chains (α and β) which are linked via a disulfide bond (VAN DER MERWE & DUSHEK, 2011). The two transmembrane chains are composed of a constant region ($C_\alpha$; $C_\beta$) which is anchored into the plasma membrane, and a variable region ($V_\alpha$; $V_\beta$), responsible for the specific antigen recognition (MURPHY et al., 2008). The remarkably high diversity of the T cell receptor which is essential for the recognition of numerous different pathogens, is accomplished through somatic recombination of several gene segment sets (GRAWUNDER et al., 1998). In addition, co-expression of CD3 is essential for the activation of T lymphocytes (MURPHY et al., 2008). Generally, the formation of the T cell receptor is similar to the structure of immunoglobulin, but T lymphocytes are not able to bind intact antigen directly which is possible for immunoglobulin (WARRINGTON et al., 2011; JOFFRE et al., 2012). Indeed, they can only recognize antigens which are processed into short peptide fragments and bound to major histocompatibility complexes (MHC) (GOLDBERG & ROCK, 1992; MURPHY et al., 2008).

MHC molecules are membrane glycoproteins, encoded in the major histocompatibility complex of the genome (MURPHY et al., 2008). They can be subdivided into two differing groups, class I and II. MHC class I molecules are located on the surface of nucleated cells (WRIGHT et al., 2004; JOFFRE et al., 2012), whereas MHC class II molecules are only situated on cells of the immune system (WARRINGTON et al., 2011). The heterodimeric MHC class I molecules (figure 1) are assembled in the endoplasmic reticulum and form four different domains (WRIGHT et al., 2004). They consist of $\beta_2$-microglobulin and a larger α chain which is subdivided into the domains $\alpha_1$, $\alpha_2$ and $\alpha_3$ (WRIGHT et al., 2004; CHAPLIN, 2010). In this context, subunit $\alpha_3$ is responsible for the membrane anchoring, whereas $\alpha_1$ and $\alpha_2$ in combination generate the

peptide binding site (MURPHY et al., 2008). MHC class II molecules (figure 1) also include two chains, α and β, but both are anchored to the cell membrane and the α-chain differs from the α-subunit in MHC class I molecules (CHAPLIN, 2010). The two chains are subdivided into $\alpha_1$, $\alpha_2$ and $\beta_1$, $\beta_2$ domains, in such a way that $\alpha_1$ and $\beta_1$ build the peptide-binding cleft (MURPHY et al., 2008). Antigen fragments bound on MHC I are mainly recognized by CD8+ T cells (BEVAN, 2004), whereas complexes consisting of peptide fragments and MHC II are preferentially identified by CD4+ T cells (MURPHY et al., 2008).

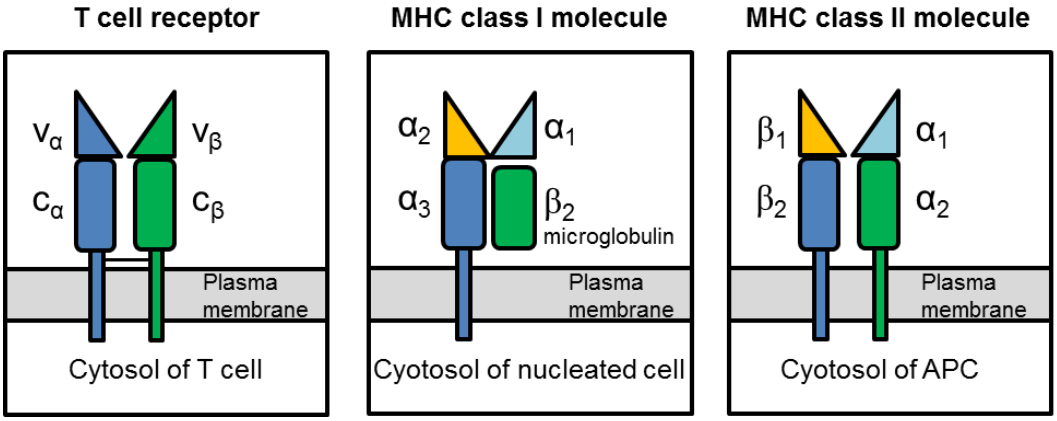


Figure 1: **Structure of T cell receptor, MHC class I molecule and MHC class II molecule** (MURPHY et al., 2008; modified)

### 1.3. Activation of naive T cells

Priming of naive T cells is triggered through their specific interaction with antigen-presenting cells (MURPHY et al., 2008; JOFFRE et al., 2012). In this context T cells bind to an antigenic peptide which is presented via MHC molecules and specifically fits to the T cell receptor. Apart from the interaction of the T cell receptor with the MHC complex additional signals are required to induce the clonal expansion of antigen-specific T cells, such as co-stimulatory molecules or cytokines produced by antigen-presenting cells (MURPHY et al., 2008).

The ligand presented on MHC molecules originates from extracellular pathogens or from intracellular ones. Extracellular microbes usually carry repetitive molecular structures consisting of lipopolysaccharides or

carbohydrates on their bacterial cell wall, which are known as pathogen-associated molecular patterns (PAMPs) (JANEWAY, 1989; IWASAKI & MEDZHITOV, 2015). Macrophages, dendritic cells or other cells of the innate immune system hold pattern recognition receptors (PRR) on their cell surface which are able to recognize the PAMPs (JANEWAY, 1989; IWASAKI & MEDZHITOV, 2015). The phagocytic cells furthermore express receptors for the interaction with complement proteins. Apart from direct binding, this additionally allows for recognition of opsonized pathogens. After recognition and phagocytosis, antigen-presenting cells process the pathogen intracellularly and present the newly formed antigenic peptides via MHC class II on their cell surface to CD4+ T cells (CHAPLIN, 2010). Antigen binding and processing furthermore also induce enhanced cytokine production through the phagocytic cell. This leads to the further activation of the innate immune response as well as the adaptive one. Thus, antigen presenting cells, especially dendritic cells, serve as a link between the innate and the adaptive immune system (WARRINGTON et al., 2011). Intracellular pathogens like viruses or some bacteria usually activate the immune system through another pathway. Here, intracellularly produced antigenic proteins are degraded in the proteasome and presented on MHC class I molecules. This leads to the activation of CD8+ T cells (MURPHY et al., 2008).

Apart from the two major pathways described above there are also other ways of antigen processing such as cross-presentation (CHAPLIN, 2010). This pathway plays a key role for the activation of dendritic cells by viral infections which do not directly affect the dendritic cells. Here, antigen-presenting cells receive antigen delivered by other infected cells via phagocytosis. This is mainly carried out by dendritic cells (CRESSWELL et al., 2005). Afterwards the exogenous antigen can also be processed via the proteasome and can finally be presented to cytotoxic T cells via MHC class I molecules (MURPHY et al., 2008; JOFFRE et al., 2012). Generally this strategy helps to control viruses which are able to avoid antigen processing through the endogenous pathway (CHAPLIN, 2010).

After initial priming T lymphocytes start to proliferate and differentiate into effector cells (AHMED & GRAY, 1996). In addition to the maturation of

effector T cells, priming leads to the development of a small fraction of memory T cells. These are long-lived T cells (AHMED & GRAY, 1996) which accelerate the immune response in case of a subsequent infection with the same pathogen responsible for priming. Furthermore memory cells also exist despite the absence of their specific pathogenic antigens (MURPHY et al., 2008). Generally, efficient vaccines should provide both, T cell effector cells as well as memory T cells. This allows efficient control of infectious pathogens including a higher magnitude of the secondary response compared to primary responses (MURPHY et al., 2008; SALLUSTO et al., 2010).

### 1.4. T cell response induced by vaccination

Up to date vaccines represent the most effective and cost-efficient preventive method in modern medicine (SALLUSTO et al., 2010; PULENDRAN & AHMED, 2011). The comprehensive use of vaccines is able to protect individuals from infectious diseases and can at best even result in the elimination of single pathogenic organisms (SALLUSTO et al., 2010). For decades the development of vaccines has predominantly been based on the induction of strong humoral immune responses. For this reason it is quite common that most of the vaccines currently available are based on the induction of antigen-specific antibodies (PLOTKIN, 2008). However, antibody inducing vaccines are not effective enough against numerous infectious pathogens which are more complex or even able to chance their antigenic structure, such as human immunodeficiency virus (HIV), influenza or Mycobacterium tuberculosis. The development of vaccines against HIV for example has so far not been successful (PULENDRAN & AHMED, 2011).

These findings clearly demonstrate the need for novel innovative strategies in vaccine development. Moreover, data recently published also highlight the importance of the T cell response and confirm that protection against many threatening infectious diseases could be improved through an increased induction of the cellular immune system (KREMER et al., 2012a; ALTENBURG et al., 2014). The importance of the T cell response is now generally accepted and therefore vaccine development is more and

more focusing on the induction of T cells. The requirement of a strong T cell response is for example clearly indicated in the control of tuberculosis (HOFT, 2008) or HIV (PANTALEO & KOUP, 2004). Additionally, influenza is also believed to require an efficient cellular immune response. Here, the influenza-specific T cell response even seems to correlate inversely with the risk of elderly people developing sickness following vaccination (McELHANEY et al., 2006). Furthermore, it is known that the protection against some infectious diseases such as smallpox depends on T cell memory. In fact, antibodies can prevent infection with smallpox and usually protect against severe disease, but the infection will only be asymptomatic through the help of the cellular immune response (PLOTKIN, 2008).

# 2. Modified Vaccinia virus Ankara (MVA) as vector vaccine

## 2.1. MVA: a member of the family Poxviridae

Modified Vaccinia virus Ankara (MVA) is assigned to the genus Orthopoxvirus which belongs to the family Poxviridae (MAYR et al., 1975). Poxviruses (figure 2) typically hold a single linear double-stranded DNA genome of about 130 to 300 kilo base pairs which encodes a multiplicity of viral proteins (MOSS, 1996). The virions of poxviruses are larger than most other animal viruses and can even be seen in light microscopy (200 nm - 400 nm). Basically, the structure of the enveloped virions is relatively complex. Additionally to the biconcave core, poxviruses form two lateral bodies. The core includes the nucleocapsid with the s-shaped viral genome and DNA associated proteins (MOSS, 2013).

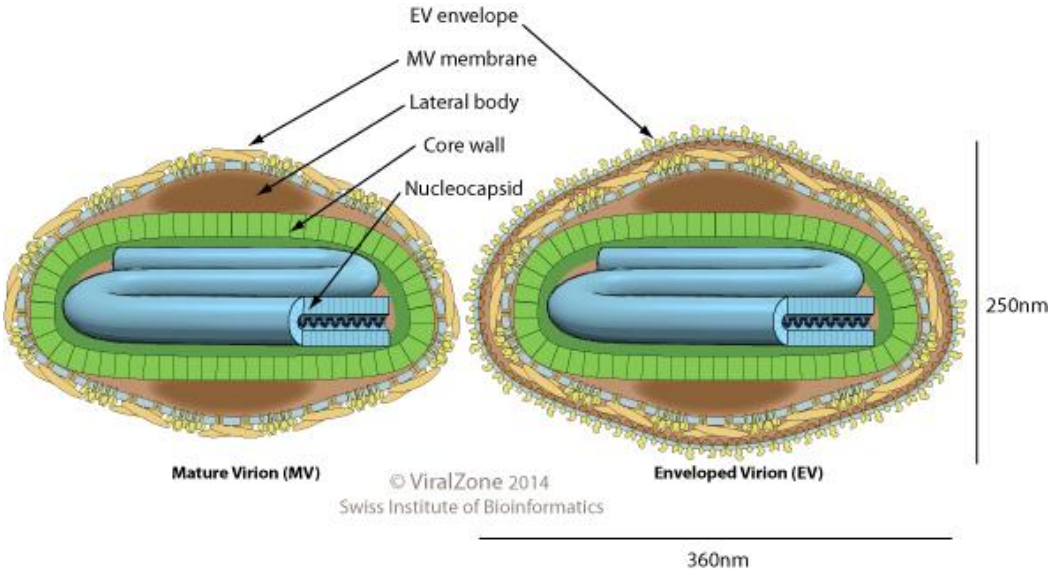


Figure 2: **Structure of poxvirus virions**
(Source: ViralZone; www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics; with permission)

The life cycle of poxviruses includes several steps which finally lead to the release of newly formed infectious virions (figure 3). After attachment and fusion with the plasma membrane, the core of the virions including the DNA is released to the cytoplasm of the host cell (MOSS, 2013). Early genes which are essential for DNA replication, transcription and translation of intermediate genes, are immediately transcribed (MOSS, 1996). In the next step uncoating takes place and DNA replication starts (McFADDEN, 2005). Poxviruses themselves encode all enzymes and factors which are essential for the replication. This is remarkable because it allows replication in the cytoplasm independently from the host cell (MOSS, 1996). Replication restricted to the cytoplasm furthermore prevents viral DNA from integrating into the host cell genome. In contrast, other DNA viruses usually need the cellular replication system and are partly able to integrate their genome into the DNA of the host cell (MOSS, 1996). In the further course of the life cycle, intermediate as well as late genes are successively transcribed (McFADDEN, 2005). Amongst other things the products of intermediate genes are essential for the initiation of the late gene transcription. In contrast, late genes encode mainly structural proteins which are necessary for the formation of infectious virions

(MOSS, 2013). Generally, this type of genetic programming is known as a cascade mechanism because the products of each stage regulate the next.

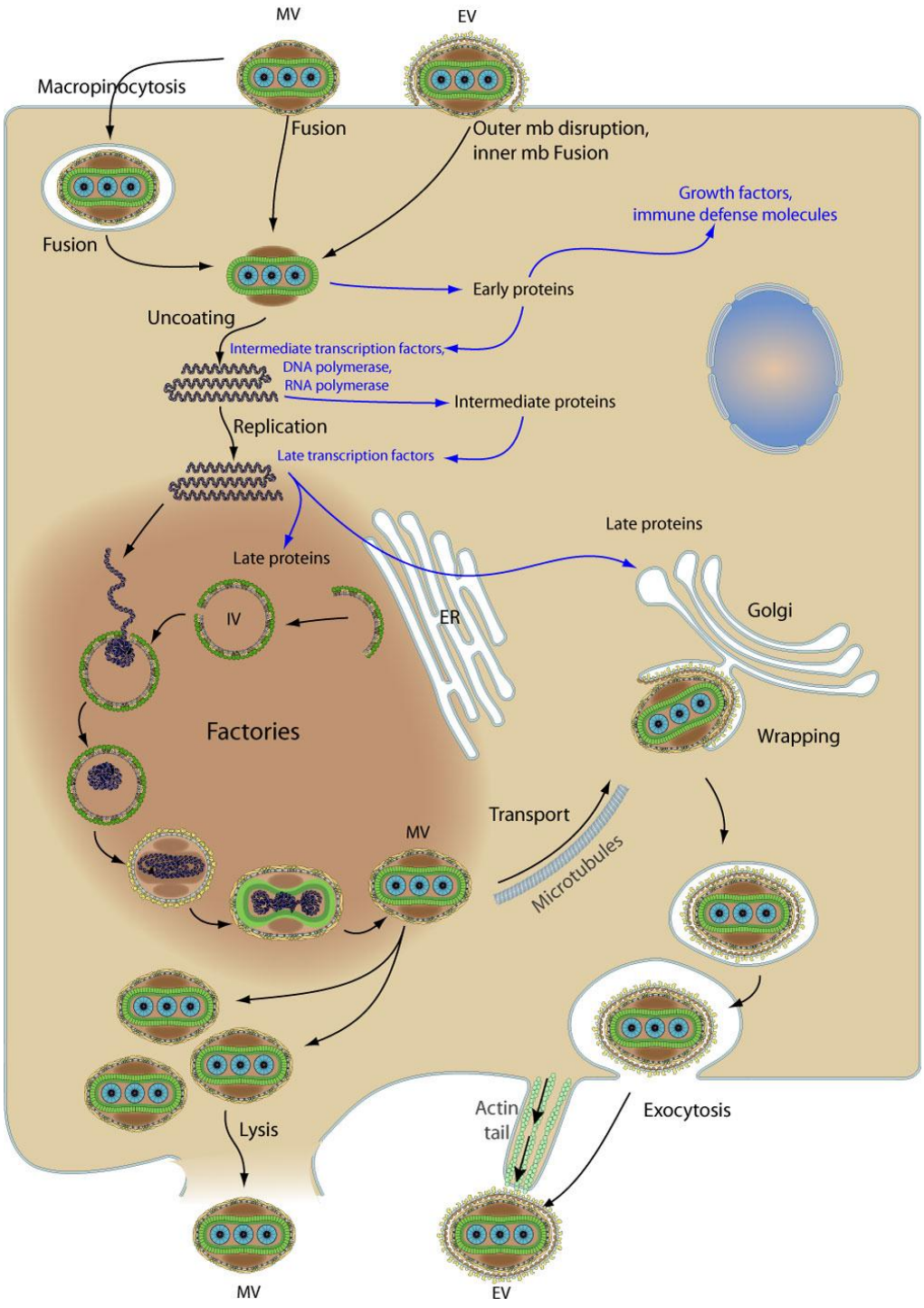


Figure 3: **Life cycle of poxviruses**
(Source: ViralZone; www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics; with permission)

After assembly and packaging of the newly replicated DNA, the viral particles mature to infectious intracellular virions (mature virion, MV) and get wrapped in the trans-Golgi (HILLER & WEBER, 1985; SCHMELZ et al., 1994). The wrapped virions are directly transported to the plasma membrane (STOKES, 1976). Finally, the virions are released to the extracellular space as enveloped virions (EV) through fusion with the cell membrane (MOSS, 2013).

## 2.2. Origin of MVA

The World Health Organization's (WHO) smallpox eradication program finally resulted in the elimination of smallpox in 1980. Vaccinia virus, the virus used as vaccine during the program, was immunogenic, but it partly induced severe side effects (KENNEDY et al., 2009; GILBERT, 2013). Thus, it naturally was not the best vaccine possible, above all for people with immunodeficiency. For this reason, the development of safe and efficient smallpox vaccines was urgently needed. In this context, MVA was developed at the LMU Institute for Microbiology and Infectious Diseases of Animals in Munich (MAYR & MUNZ, 1964). It was derived from Vaccinia virus strain Ankara after 516 serial passages on chicken embryo fibroblasts (MAYR et al., 1975). This resulted among other things in six major deletions of DNA, including several structural proteins, host range genes (MEYER et al., 1991) and immune evasion genes (ANTOINE et al., 1998). Apart from the six major deletions the genome was furthermore affected by a couple of point mutations, shorter deletions and insertions (ANTOINE et al., 1998; MEISINGER-HENSCHEL et al., 2007). Overall, the loss of genomic original material amounts to approximately 15% (about 30,000 base pairs) in comparison to the genome of the parenteral Chorioallantois Vaccinia virus Ankara (CVA) (MEYER et al., 1991). Thus, MVA lost the bigger part of its growth capacity and became replication deficient in mammalian cells including human cells (MAYR et al., 1975; CARROLL & MOSS, 1997; DREXLER et al., 1998). For this reason MVA can even be used as a safe vaccine for immunocompromised people, such as cancer patients receiving chemotherapy or people who have contracted immunodeficiency virus. This finding basically resulted in the

preferred use of MVA in vaccine development compared to the replication competent Vaccinia virus. But despite the inability of MVA to replicate early, intermediate as well as late gene expression still occurs in mammalian cells (SUTTER & MOSS, 1992). In general, the high-level attenuation of the virus allows handling of MVA in Germany under biosafety level 1 conditions (ZKBS, 1997) and safe application in animal experiments as well as in human clinical trials.

Originally, non-recombinant MVA was used for the vaccination of more than 120,000 people in Germany (STICKL et al., 1974; MAYR et al., 1978). During that period of time many field studies investigated and confirmed the excellent safety profile of MVA (STICKL et al., 1974; MAYR et al., 1975; MAYR et al., 1978). Today, MVA is approved in the European Union and Canada as a smallpox vaccine for active immunization, even for immunocompromised people (VOLLMAR et al., 2006; KENNEDY & GREENBERG, 2009).

## 2.3. MVA: a vector for foreign genes

After the eradication of human smallpox, MVA has been used as a safe viral vector for the generation of candidate vaccines for the prevention of infectious diseases and cancer. In general, MVA has several useful characteristics which make it suitable as a viral vector for vaccine development (DREXLER et al., 2004; COTTINGHAM & CARROLL, 2013; GILBERT, 2013; KREIJTZ et al., 2013; VOLZ & SUTTER, 2013; ALTENBURG et al., 2014): i) The genome of MVA shows a high plasticity and is relatively large which allows the insertion of foreign gene sequences. ii) MVA is replication deficient in mammalian cells which indicates the use even in immunocompromised people. iii) Several studies showed an excellent safety record of MVA after use as a vaccine in humans. iv) MVA is able to induce humoral as well as cellular immune responses. v) Commonly available laboratory protocols allow easy and efficient handling of MVA.

As a representative of poxviruses MVA shows a high genetic plasticity which enables the insertion of heterologous gene sequences. The

generation of recombinant MVA usually proceeds according to a well-established and standardized laboratory system using a vector plasmid (KREMER et al., 2012b). Thus, foreign genes can be inserted into the viral genome via homologous recombination between flanking sequences in the vector and the MVA genome (MOSS, 1996). Besides the gene of interest, the vector plasmid also needs to include a promoter sequence. The promoter has to be Vaccinia virus specific because poxviruses avoid using the transcriptional system of the host cell through holding their complete own transcription machinery (MOSS, 1996). Furthermore the vector plasmid should also contain a marker gene which facilitates specific selection of recombinant MVA. Screening for the expression of fluorescent marker genes allows efficient isolation of recombinant viruses through several plaque passages in the cell culture. Finally, recombinant MVAs are able to synthesize high amounts of viral as well as recombinant protein in the cytosol of infected cells. This provides the basis for the function of a well-working expression vector (SUTTER & MOSS, 1992).

## 2.4. MVA as a platform for vaccine development

Preclinical studies already showed the suitability of MVA as a viral vector more than 15 years ago (MOSS et al., 1996). So far, many different recombinant MVAs delivering antigens of various infectious diseases have been constructed and characterized. Previously published data for example indicate the use of recombinant MVA for the prophylaxis of malaria or human immunodeficiency virus (COSMA et al., 2003; MOORTHY et al., 2003b; MOORTHY et al., 2003a). Several recombinant MVAs have already been tested in phase I to phase IIb clinical trials including vaccines against viral infections (table 1a) such as influenza, hepatitis or human deficiency virus (HIV) and also against bacteria and parasites (table 1b) (KREIJTZ et al., 2013). Vaccination with these recombinant MVAs has generally been well-tolerated. Safety data from these clinical trials have reported no severe or serious side effects (MOORTHY et al., 2003b; GILBERT, 2013; GOMEZ et al., 2013). Moreover, the MVA candidate vaccines were immunogenic as predicted in previous preclinical studies, and they induced humoral as well as cellular

immune responses (GILBERT, 2013). For this reason MVA serves as a promising platform for future development of efficient and safe vaccines.

Table 1a: **Clinical trials of recombinant MVA vaccines targeting viruses** (KREIJTZ et al., 2013; modified)

| Target disease | Antigen | Phase | Immunogenic | Safe | References |
|---|---|---|---|---|---|
| AIDS | HIV-1 Nef | I/II | yes | yes | (COSMA et al., 2003) |
| | HIV-1 Env<br>HIV-A gag/pol | I/II | yes | yes | (BAKARI et al., 2011)<br>(MUNSERI et al., 2015) |
| | HIV-B<br>Env/gag/pol/nef | I/II | yes | yes | (GOMEZ et al., 2011)<br>(GARCIA et al., 2011)<br>(GOMEZ et al., 2015) |
| Hepatitis B | HBV S | I | yes | yes | (CAVENAUGH et al., 2011) |
| Influenza | NP and M1 | I/II | yes | yes | (BERTHOUD et al., 2011)<br>(LILLIE et al., 2012)<br>(ANTROBUS et al., 2012) |
| | HA | I/IIa | yes | yes | (KREIJTZ et al., 2014)<br>(DE VRIES et al., 2015) |

Table 1b: **Clinical trials of recombinant MVA vaccines targeting bacteria and parasites** (KREIJTZ et al., 2013; modified)

| Target disease | Antigen | Phase | Immunogenic | Safe | References |
|---|---|---|---|---|---|
| Malaria | P. falcip. AMA1 | I | yes | yes | (SHEEHY et al., 2012) |
| | P.falcip. ME-TRAP | IIb | yes | yes | (BEJON et al., 2006) (BEJON et al., 2007) |
| | P. falcip. ME-TRAP | IIb | yes | yes | (MOORTHY et al., 2004) |
| | P. falcip. ME-TRAP | I | yes | yes | (HODGSON et al., 2015) |
| Tuberculosis | 85A (MTB) | IIb | yes | yes | (TAMERIS et al., 2013) |
| | 85A (MTB) | I | yes | yes | (SHEEHAN et al., 2015) |

Besides the advanced investigation of promising antigens in clinical trials, preclinical studies consistently investigate new MVA vaccine candidates. In this context, recently published data for example highlight the suitability of MVA as a vector for more complex diseases such as Middle East respiratory syndrome coronavirus (MERS-CoV) (SONG et al., 2013).

## 2.5. MVA's antigen presentation pathway

Generally, thorough knowledge of MVA's life cycle and its antigen presentation pathway is essential for the development of new effective vaccine candidates. In this context, the main mechanism of processing intracellular viral antigens delivered by MVA, is the endogenous pathway

(figure 4) for MHC I presented antigens (TSCHARKE et al., 2015).

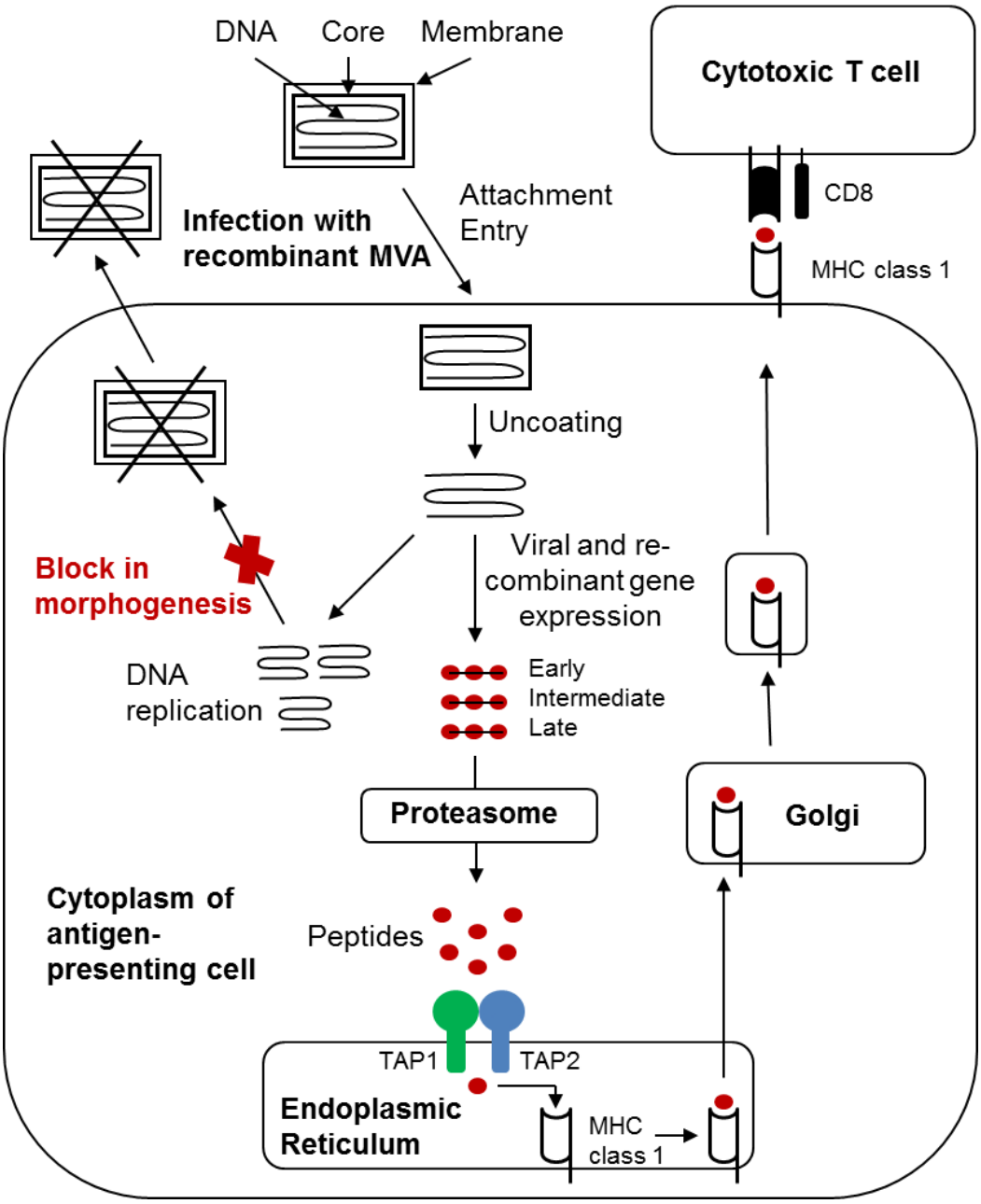


Figure 4: **Endogenous antigen presentation in MVA infected antigen-presenting cells** (MURPHY et al., 2008; MOSS, 2013; modified)

Proteases process viral proteins in the proteasome to small peptide fragments which are immediately transported into the endoplasmic reticulum (GOLDBERG & ROCK, 1992). The required length of the peptides for complexation with MHC I molecules is 8-10 amino acids (WRIGHT et al., 2004; CRESSWELL et al., 2005). The ATP-dependent

transport of the peptides across the endoplasmic membrane is mediated by two proteins called TAP 1, TAP 2 (transporters associated with antigen processing) which build a heterodimer (ANDROLEWICZ et al., 1993; CRESSWELL et al., 2005). This transport system belongs to the ATP-binding cassette family (ABC) and consists of four domains, two transmembrane and two ATP-binding domains (HIGGINS, 1992). The encoding sequences of TAP 1 and TAP 2 are located within the MHC. It is remarkable that the transcription and translation of these genes can be enhanced by interferons which are produced by various cells of the immune systems as a response to viral infections (MURPHY et al., 2008). In the endoplasmic reticulum the viral peptide fragments are loaded on the MHC class I molecules (CHAPLIN, 2010). This complex is subsequently transported to the cell surface and presented to cytotoxic T cells (MURPHY et al., 2008). Generally, this strategy allows the immune system to detect transformed cells as well as infected cells (JOFFRE et al., 2012).

Apart from the endogenous pathway, MVA is also able to activate cytotoxic T cells via cross-presentation by dendritic cells (TSCHARKE et al., 2015).

# 3. Target antigens for MVA-induced CD8+ T cells

## 3.1. Influenza antigens

Influenza viruses cause infections in the respiratory system of humans as well as of animals. They show a high genetic variability and an enormous capacity for antigenic drift (HAMPSON & MACKENZIE, 2006; GAMBLIN & SKEHEL, 2010) which can lead to the development of new viral subtypes. This strategy allows the virus to evade the humoral immunity of the host (WONG & PAMER, 2003). For this reason novel influenza subtypes especially possess pandemic potential because they often cannot be recognized and eliminated by antibodies induced by vaccination with the seasonal influenza vaccine or by a previous infection (HILLAIRE et al., 2011). Here, the virus-specific cellular immune response is considered to play a key role in the control of the virus and could also strongly support the induction of cross-protective immunity (HILLAIRE et al., 2011;

ALTENBURG et al., 2014). Different studies have already shown the relevance of CD8+ T cell responses in challenge experiments (FLYNN et al., 1998; WONG & PAMER, 2003). Furthermore, it is known that memory T cells are essential for rapid protection of mice in case of reinfection (FLYNN et al., 1999; WONG & PAMER, 2003). Therefore the strong activation of T cells is a vital target in today's development of more broad-protective vaccines. Successful development and licensing of such vaccines is a major aim regarding the high morbidity and mortality of epidemic influenza outbreaks which are especially threatening for elderly or immunocompromised people.

In 1994 a study first described that vaccination with recombinant MVA expressing hemagglutinin (HA) and nucleoprotein (NP) is able to protect mice from lethal challenge with influenza virus (SUTTER et al., 1994). Today several influenza antigens are known and investigated for their potential to induce T cell responses. Recently published data evaluated different MVA vaccines encoding influenza antigens for their potential to induce antigen-specific CD8+ T cell responses. In this context, hemagglutinin, nucleoprotein and matrixprotein 1 (M1) are promising antigens used in the MVA vector system. Hemagglutinin is a glycoprotein located on the surface of influenza virions (GAMBLIN & SKEHEL, 2010). It mediates binding of the virus particle to the host cell and is also involved into the fusion of the viral envelope with the endosome membrane (GARTEN & KLENK, 1999). In contrast, nucleoprotein and matrixprotein belong to the structural proteins of the virus. Nucleoprotein is known as an RNA-binding protein which is responsible for the encapsidation of viral RNA (PORTELA & DIGARD, 2002). Thus, it also interacts with matrixprotein 1 and other viral molecules. It furthermore includes a nuclear localization signal and mediates the translocation of viral RNA into the cell nucleus. M1 is also a RNA-binding protein (YE et al., 1999), but it is able to bind the plasma membrane as well. Simultaneous binding of RNA and membrane through M1 is important for the encapsidation of the RNA into the viral envelope. Table 2 shows a selection of recombinant MVA recently tested expressing influenza proteins. It summarizes their potential to induce cellular immune responses against homologous and heterologous

influenza subtypes.

Table 2: **Influenza antigens and their potential to induce antigen-specific CD8+ T cell responses**

| Influenza virus | Antigen | Induction of antigen-specific CD8+ T cells | Protective in influenza challenge | References |
|---|---|---|---|---|
| H1N1 | HA+NP | yes | yes | (SUTTER et al., 1994) |
| H5N1 | NP | yes | yes (H5N1, H7N1, H9N2) | (HESSEL et al., 2014) |
| H3N2 | NP+M1 | yes | yes | (BERTHOUD et al., 2011) |
| H1N1+H5N1 | HA (H1N1) + NP (H5N1) | yes | yes (H1N1, H5N1), partial protection (H3N2) | (BREWOO et al., 2013) |

### 3.2. Model antigens

Experimental studies are consistently testing antigens of various infectious diseases delivered by MVA for their potential to induce strong T cell responses. Here, it is also worthwhile to carefully study modification tools for the promising antigens experimentally pre-selected. Many different modification tools are considered because of their capacity to enhance the immunogenicity of antigens such as localizing target antigens to subcellular compartments, co-expression of different immunogenic molecules or the use of different promoters (GASTEIGER et al., 2007). In this context model antigens play a key role because they allow to test the potency of antigenic modification tools independently of specific antigens.

In the next step the combination of the most promising tools and pre-selected antigens can be investigated for their immunogenicity. This procedure clearly decreases the number of experiments and studies which would usually be necessary to test all combinations of modifications and antigens. Several model antigens with different characteristics are established for the MVA vector system. Consequently the most commonly used model systems are to be presented in the following chapter.

### 3.2.1. Ovalbumin

The chicken egg white protein ovalbumin (OVA) belongs to the serpin superfamily (HUNT & DAYHOFF, 1980). The name of this superfamily is based on the function of the main part of all associated proteins which typically are serin proteinase inhibitors (GETTINS, 2002). The exact function of the non-inhibitory OVA is still unknown, but it is supposed to be a storage protein (GETTINS, 2002; BENARAFA & REMOLD-O'DONNELL, 2005). OVA's X-ray structure was finally determined in 1990 (STEIN et al., 1990). This well-conserved protein has so far been used as a model antigen for recombinant MVA in many different experimental studies. Cells infected with recombinant MVA expressing OVA (MVA-OVA) usually secret ovalbumin constantly to the medium (BECKER et al., 2014). For this reason OVA serves as an appropriate model antigen for the comparison of modification tools which are supposed to have an influence on the strength of the antigen expression such as different promoters (BECKER et al., 2014). Furthermore, secretion to the medium allows analysis of protein expressed without affecting the cells. OVA is also well-established and frequently used as a model antigen for the analysis and comparison of T cell responses in immunization experiments (BAUR et al., 2010; BECKER et al., 2014). It is not only used as a model system, but also as a promising antigen in the development of vaccines for the prevention of allergic symptoms caused by food allergies. Thus, previously published data clearly suggest MVA-OVA as a potential vaccine against allergic symptoms induced by chicken egg white (ALBRECHT et al., 2008; BOHNEN et al., 2013).

### 3.2.2. β-Galactosidase

β-Galactosidase is a member of the enzyme family glycosyl hydrolases which are typically able to hydrolase O-glycosyl linkages (HENRISSAT, 1991). For this reason these enzymes play a key role in the carbohydrate metabolism of the human body. Enzyme deficiencies triggered through mutations in the β-Galactosidase gene can result in GM1-gangliosidosis or Morquio B syndrome. Both are rare autosomal recessive lysosomal storage diseases (CACIOTTI et al., 2011). GM1-gangliosidosis results in a pathological accumulation of acidic lipids in the lysosomes, especially in cells of the central nerve system. In contrast, Morquio B syndrome is a mucopolysaccharidosis which mainly affects the skeletal and the cardiovascular system (CACIOTTI et al., 2011). The β-Galactosidase used in molecular biology is originated from E. coli and is encoded by the lacZ gene. This gene belongs to the inducible lac operon system (OSBOURN & FIELD, 2009). It helps the bacteria to permanently adapt their enzyme expression to the availability of alimentary substrate. In the presence of lactose and in the simultaneous absence of glucose, the lac operon system is activated and encodes - apart from other molecules - β-Galactosidase which is essential for the lactose metabolism. β-Galactosidase catalyzes the hydrolysis of the disaccharide lactose in glucose and galactose (LEONARD et al., 2015). In molecular biology β-Galactosidase serves on the one hand as a reporter for gene expression because it is able to stain tissue cultures blue in the presence of enzyme specific substrate (ARMIT, 2015). On the other hand it is also used as a viral tumor associated antigen in cancer research. These antigens allow the generation of recombinant anti-cancer vaccines on the basis of MVA. Furthermore, β-Galactosidase delivered by MVA is able to specifically induce T lymphocytes (CARROLL et al., 1997). For this reason research especially makes use of recombinant MVA delivering β-Galactosidase in studies about novel treatment options against cancer, in which cytotoxic T cells play the key role (CARROLL et al., 1997). T cell leucemias induced by T cell lymphotropic virus (DE THE et al., 1994) or Burkitt's lymphoma caused by Epstein-Barr virus (MOSS et al., 1994) for example belong to this kind of cancer. More than 15 years ago MVA expressing β-

Galactosidase has also successfully been used to protect and treat pulmonary metastases which deliver β-Galactosidase (CARROLL et al., 1997).

### 3.2.3. Green fluorescent protein (GFP)

In general, fluorescent proteins serve as an innovate tool in molecular biology. They allow the visualization, observation and thus the better understanding of processes within cells, bacteria or viruses. Today many different fluorescent proteins are established for laboratory use such as mCherry (red fluorescent protein), mOrange (orange fluorescent protein) or Venus (yellow fluorescent protein) (KREMERS et al., 2011). But the discovery of green fluorescent protein and its use in cell culture is still considered as the milestone in the development of fluorescent proteins. GFP was derived from the green fluorescent jellyfish Aequorea victoria and firstly described in 1962 (SHIMOMURA et al., 1962). The crystal structure of GFP was finally published in 1996 (YANG et al., 1996). Generally, the field of fluorescent proteins has been studied very well over years. In 2008 the Nobel Prize in chemistry was finally awarded to Martin Chalfie, Osamu Shimomura and Roger Tsien for their outstanding scientific studies about GFP (CHALFIE, 2009; REMINGTON, 2011). As early as in 1994 Chalfie published the suitability of GFP as a marker for gene expression in prokaryotic as well as in eukaryotic cells. It is remarkable that the expression of GFP has no obvious negative influence on cell growth or function which allows monitoring of gene expression and protein localization in living cells (CHALFIE et al., 1994). Moreover, the fluorescence is not affected by treatment with formaldehyde which is useful for the analysis of fixed cell preparations (CHALFIE et al., 1994). By now GFP has been established in many experimental settings. It is very well characterized and probably the most commonly used fluorescent protein in molecular biology. GFP is a protein of approximately 240 amino acids which can spontaneously absorb blue light (maximum at 395 nm). Furthermore it is able to very stably emit green fluorescence (peak at 309 nm) (CHALFIE et al., 1994). GFP can efficiently be targeted to the major cell organelles as for example the cell nucleus, plasma membrane,

endoplasmic reticulum or mitochondria (TSIEN, 1998). So far GFP has also been used as a visualizable tool in many studies dealing with recombinant MVA (for example: COSMA et al., 2004; PASCUTTI et al., 2011; WONG et al., 2011) including the analysis of T cell responses (DI PILATO et al., 2015).

In this study we used GFP as a model antigen to analyze the influence of localization signals on the induction of antigen-specific cellular immunogenicity. We preferred working with GFP as a model antigen because it has several characteristics which are beneficial for the experimental setting: i) GFP is - in difference to OVA - not secreted to the medium which could influence and disturb the experimental testing systems. ii) GFP allows direct visualization of the different subcellular protein localization sites. iii) GFP as a model antigen allows to analyze the effect triggered by localization signals independently of specific infectious antigens. iv) GFP is very well characterized and established in many experimental settings. v) GFP has already successfully been used in the MVA vector system.

# III. OBJECTIVES

Due to the need of new efficient and safe vaccines for the control of complex infectious diseases and due to the suitability of MVA as an effective and safe viral vector this work describes:

(i) The generation of recombinant MVA expressing GFP in combination with different localization signals

(ii) *In vitro* characterization of the recombinant MVA

- ➔ Analysis on DNA level
- ➔ Analysis on protein level
- ➔ Fluorescence microscopy

(iii) *In vivo* characterization of the recombinant MVA

- ➔ Vaccination experiment in mice
- ➔ Analysis of the induced CD8+ T cell response

# IV. RESULTS

The manuscript is presented in form accepted for publication (MARR et al., 2016).

## Myristoylation increases the CD8+ T cell response to a green fluorescent protein prototype antigen delivered by Modified Vaccinia virus Ankara

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**Activation of CD8+ T cells is an essential part of immune responses elicited by recombinant Modified Vaccinia virus Ankara (MVA). Strategies to enhance T cell responses to antigens may be particularly necessary for broadly protective immunization against influenza A virus infections or for candidate vaccines targeting chronic infections and cancer. Here, we tested recombinant MVAs that target a model antigen, green fluorescent protein (GFP), to different localizations in infected cells. *In vitro* characterization demonstrated that GFP accumulated in the nucleus (MVA-nls-GFP), associated with cellular membranes (MVA-myr-GFP) or was equally distributed throughout the cell (MVA-GFP). On vaccination, we found significantly higher levels of GFP-specific CD8+ T cells in MVA-myr-GFP vaccinated BALB/c mice than in those immunized with MVA-GFP or MVA-nls-GFP. Thus, myristoyl modification may be a useful strategy to enhance CD8+ T cell responses to MVA-delivered target antigens.**

Modified Vaccinia virus Ankara (MVA) is a replication-deficient and safety-tested Vaccinia virus strain that can be engineered as a vector virus encoding foreign antigens (SUTTER & MOSS, 1992; SUTTER et al., 1994). Today, MVA vectors serve as an established platform technology for developing vaccines against infectious diseases and cancer (KREIJTZ et al., 2013; VOLZ & SUTTER, 2013; ALTENBURG et al., 2014; SEBASTIAN & GILBERT, 2016). Various recombinant MVA have been tested successfully in phase I to phase IIb clinical trials, and have been found to be safe and immunogenic, inducing both target antigen-specific antibodies and cellular immune responses (GILBERT, 2013; GOMEZ et al., 2013). In a recent phase I study, immunizations with recombinant MVA delivering the hemagglutinin (HA) antigen of Influenza A virus H5N1 (MVA-HA) induced high levels of H5-specific antibodies (KREIJTZ et al., 2014).

In addition, the ability to activate strong cellular immune responses is an important factor for the use of recombinant MVA in the search for influenza vaccines with improved efficacy. Enhancing antigen specific T cell responses might be a promising strategy for developing broadly protective

vaccines against influenza A virus. So far, two major parameters are reported to influence efficient T cell responses: (i) optimal use of early promoters for recombinant gene expression to support direct antigen presentation and priming of T cells (BRONTE et al., 1997; KASTENMULLER et al., 2006; KASTENMULLER et al., 2007) and (ii) the synthesis and delivery of stable mature protein antigens as preferred substrates for efficient priming of T cells by cross-presentation (GASTEIGER et al., 2007; PASCUTTI et al., 2011).

Localizing target antigens to subcellular compartments is also considered as an innovative approach to enhance the cellular immune response (GASTEIGER et al., 2007). To analyze the effect of different antigen localizations on immunogenicity, we chose green fluorescent protein (GFP) as a model antigen and assessed the induction of GFP epitope-specific CD8+ T cells mediated by recombinant MVA producing either unmodified GFP or GFPs containing nuclear localization (nls) or myristoylation (myr) signals. The gene sequences encoding the target antigens GFP, myr-GFP and nls-GFP were introduced at the site between the Vaccinia virus (VACV) G1L and I8R genes (all gene nomenclatures as established for VACV strain Copenhagen; GOEBEL et al., 1990) by homologous recombination and placed under transcriptional control of Pvgf promoter sequences (Fig. 1a). Pvgf is a natural Vaccinia virus promoter controlling the abundant expression of VACV ORF C11R mRNA (YANG et al., 2015).

Recombinant MVA expressing unmodified GFP served as a base-line vaccine. To achieve increased transport of GFP to cellular membranes, we added a myristoylation signal (MAURER-STROH et al., 2002; CHAN et al., 2011), and for nuclear localization we tagged the GFP with a specific nuclear localization signal (WANG et al., 1997) that translocates GFP to the cell nucleus (Fig. 1b). As a further control, recombinant virus MVA-P7.5-GFP was used, encoding GFP under the transcriptional control of the natural VACV early-late promoter P7.5 (MACKETT et al., 1982), the first and probably still most widely used promoter for constructing recombinant Vaccinia viruses.

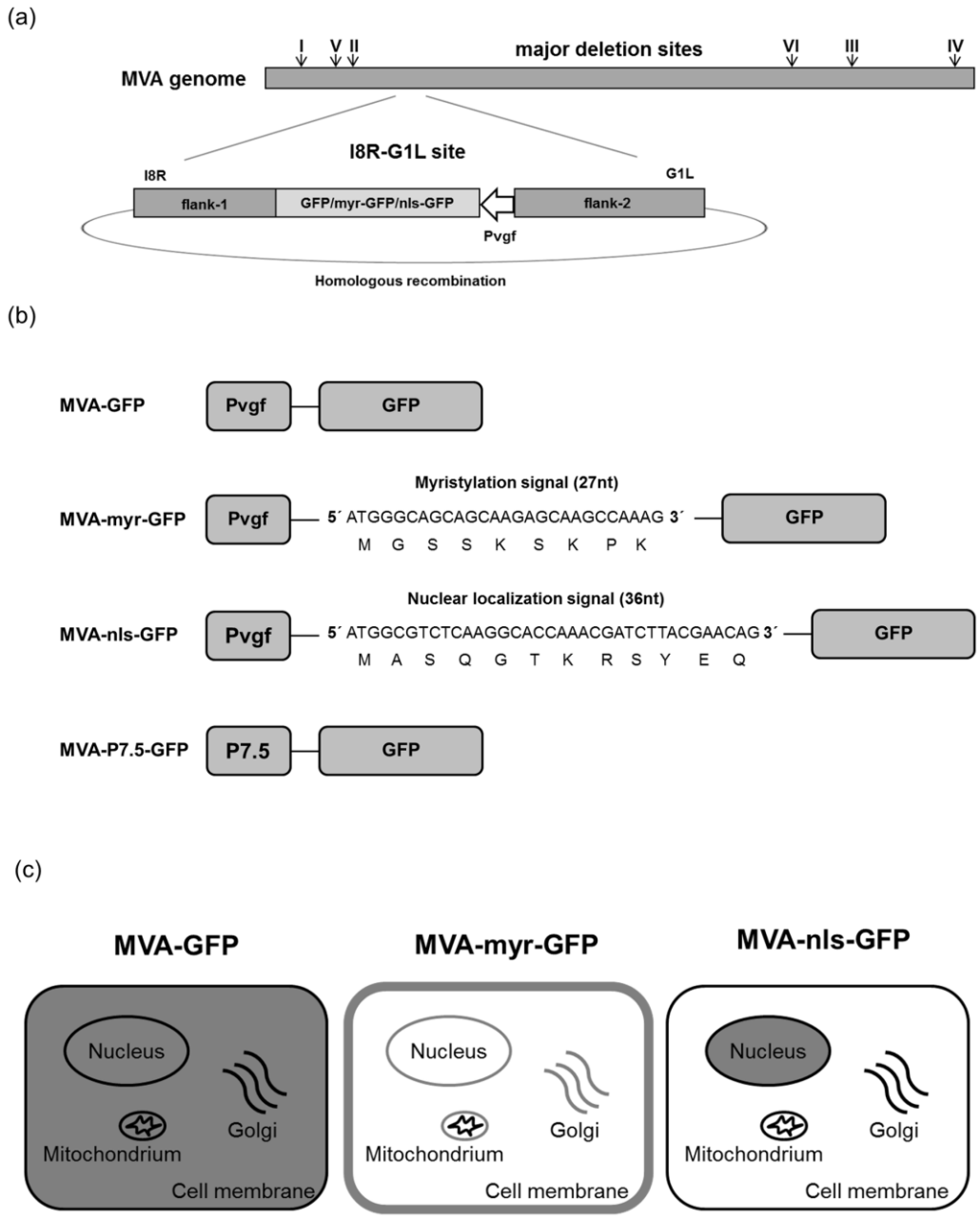


**Fig. 1. Design of the recombinant MVAs.**
(a) Schematic diagram of the MVA genome containing the six major deletion sites (I to VI). The I8R-G1L site was used to insert GFP, myr-GFP or nls-GFP, under transcriptional control of the early Vaccinia virus promoter Pvgf (GTTTATATTACTGAATTAATAATATAAAATTCCCAATCTTGTCATAAA, underlined A indicates transcriptional start site +1 as described by Broyles and coworkers BROYLES et al., 1991). (b) Schematic representation of the MVA constructs with the nucleotide and amino acid signal sequences used to construct the different GFP variants. (c) Scheme of GFP localization within infected cells, with GFP represented by grey shading.

Our resulting recombinant MVA viruses should equally distribute GFP throughout the cell or deliver GFP predominantly to the cell nucleus (MVA-nls-GFP) or cellular membranes (MVA-myr-GFP) (Fig. 1c). These viruses were purified and quality controlled according to standard procedures for generating recombinant MVA vaccines (KREMER et al., 2012b). During plaque purification, we analysed genetic integrity and stability by PCR using oligonucleotide primers to confirm MVA identity (PCR of the six major deletion sites of MVA in MVA-nls-GFP, MVA-myr-GFP and MVA-GFP, MVA-P7.5-GFP) and correct insertion of GFP gene sequences within the MVA genome: MVA-nls-GFP, MVA-myr-GFP and MVA-GFP between the G1L and I8R genes, and MVA-P7.5-GFP insertion in deletion site III (Fig S1).

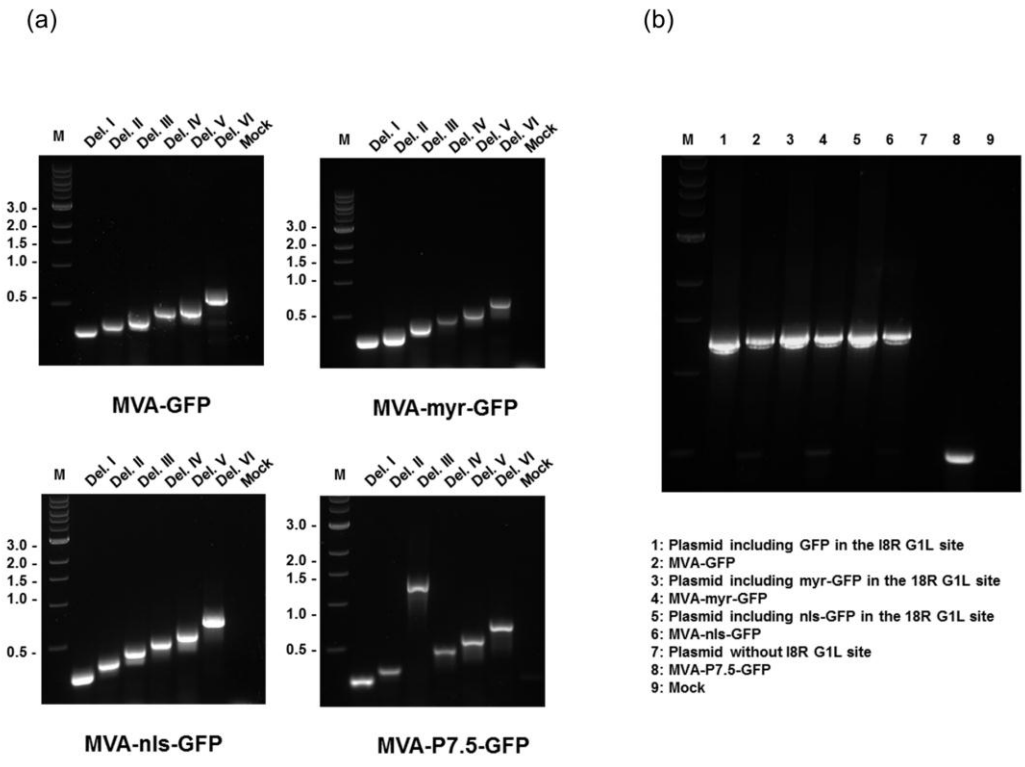


**Fig. S1. Detection of genetic integrity and stability of the recombinant MVAs.**
(a) PCR of the six major deletion sites of MVA. (b) PCR of the I8R-G1L insertion site.

To analyze growth behavior, we infected permissive DF-1 and non-permissive MEF and NIH3T3 cells with the recombinant viruses and

collected cells and supernatants at the indicated times post-infection (Fig. S2). Titration by immunostaining was performed as described previously (KREMER et al., 2012b). The recombinant MVAs replicated efficiently in the avian cell line (DF-1), but not in cells of mammalian origin (MEF, NIH3T3) (Fig. S2). These findings confirmed the expected MVA phenotype, allowing handling of the recombinant viruses under biosafety level 1 conditions.

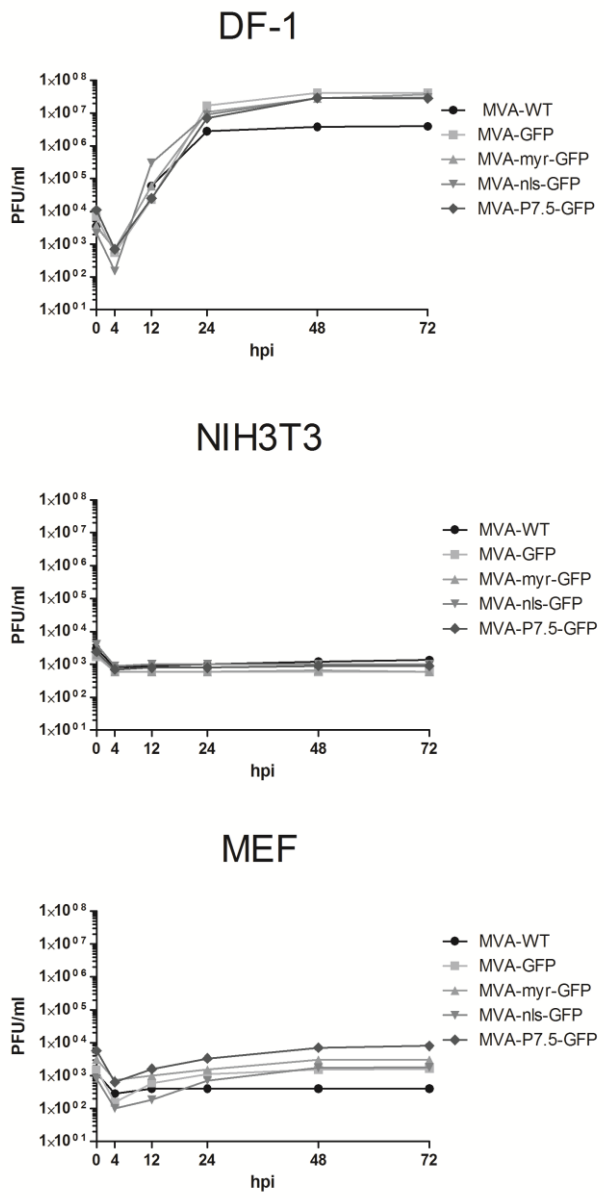


**Fig. S2. Recombinant MVA replication in cell lines.**
DF-1, MEF and NIH3T3 cells were infected at an m.o.i. of 0.01 for multistep growth analysis. Results are expressed as the average of three independent experiments.

Next we assessed the correct expression and predicted cellular localization of the GFPs by immunofluorescence microscopy of MVA-GFP-infected cells (Fig. 2a). Infected DF-1 cells were fixed 24 h post infection and immunostained as described previously (BOULANGER et al., 2002) using primary anti-GFP (Life Technologies) and secondary Alexa Fluor 488 antibody (Life Technologies). Cell nuclei were stained with DAPI (300 nM). As anticipated, we observed different patterns of green fluorescence, with varying cellular localizations depending on the MVA-GFP construct. Serial optical sections showed that MVA-nls-GFP expressed protein was predominantly located within the cell nucleus. In contrast, MVA-myr-GFP expressed GFP that accumulated primarily on membranous structures, e.g. nuclear and cytoplasmic membranes, but was notably absent within the nucleus. MVA-GFP and MVA-P7.5-GFP rarely showed specific stained areas of green fluorescence, indicating equal distribution of GFP throughout the infected cell.

To further analyze GFP synthesis, total cell lysates from infected CEF cells were analyzed by Western blotting using an anti-GFP rabbit antibody (Life Technologies; diluted 1:250) and a secondary anti-rabbit antibody (Cell Signaling Technology). We compared GFP expression at 24 hours post-infection in DF-1 cells (Fig. 2b). β-actin-antibody (Thermo Scientific; 1:500) was included to provide a loading control. Similar amounts of GFP were detected for all viruses tested. We also added cytosine arabinoside (AraC, 1 mg/ml) to the medium to inhibit viral DNA replication and synchronize all infections to strict early gene expression. As expected, the amounts of GFP produced by the control MVA-P7.5-GFP were clearly reduced compared with infection in the absence of AraC. In contrast, the addition of AraC increased the expression of GFP produced by MVA-GFP, MVA-myr-GFP and MVA-nls-GFP because of prolonged activity of viral early transcription.

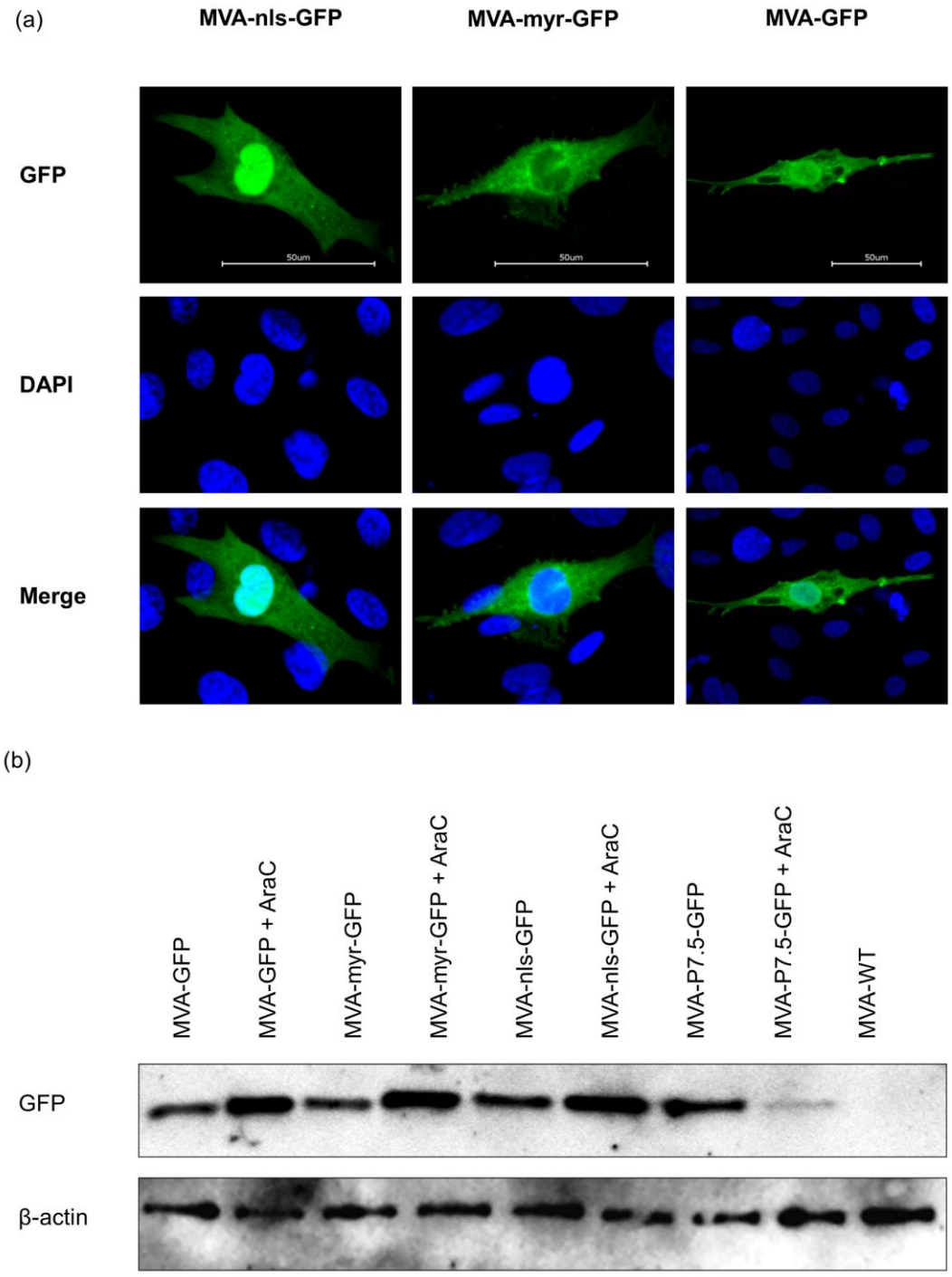


**Fig. 2. Characterization of recombinant GFP proteins**.
(a) Immunofluorescence staining of DF-1 cells infected with MVA viruses at an m.o.i. of 0.05 on cover slides and fixed 24 h post infection. Nuclei were stained with DAPI. Fluorescent images were captured with Keyence BZ-X710 at 100x magnification. (b) Western blot analysis of cell lysates prepared from DF-1 cells infected with MVA-GFP, MVA-nls-GFP, MVA-myr-GFP and MVA-P7.5-GFP at an m.o.i of 5, with or without the addition of cytosine arabinoside (AraC), 24 hours post infection.

To assess the immunogenicity of these recombinant MVA-GFP candidate vaccines *in vivo*, we investigated the activation of GFP-specific CD8+ T cells in BALB/c mice. Groups of BALB/c mice were inoculated once (at day 1) or twice (at day 1 and 21) intramuscularly with $10^8$ PFU of the MVA-GFP constructs, or with corresponding amounts of phosphate-buffered saline (PBS) as a control (Fig. 3). Mice were sacrificed at day 8 or 28 and spleens were processed for enzyme-linked immunospot assays (ELISPOT) to detect interferon gamma (IFN-γ)-secreting CD8+ T cells. MVA-immunized mice induced significantly higher numbers of cells producing IFN-γ following GFP-specific peptide stimulation than mock-inoculated control mice. Interestingly, after the second immunization at day 21, MVA-myr-GFP induced significantly higher levels of GFP-specific CD8+ T cells than either MVA-GFP or MVA-nls-GFP. Notably, the levels of GFP-specific CD8+ T cells induced by infection with MVA-myr-GFP were similar to those seen after MVA-P7.5-GFP immunization. In addition, we monitored for MVA-specific CD8+ T cells and confirmed very comparable $F2_{26\text{-}34}$ peptide-specific (TSCHARKE et al., 2006) responses for all MVA-based vaccines (Fig. 3c, d).

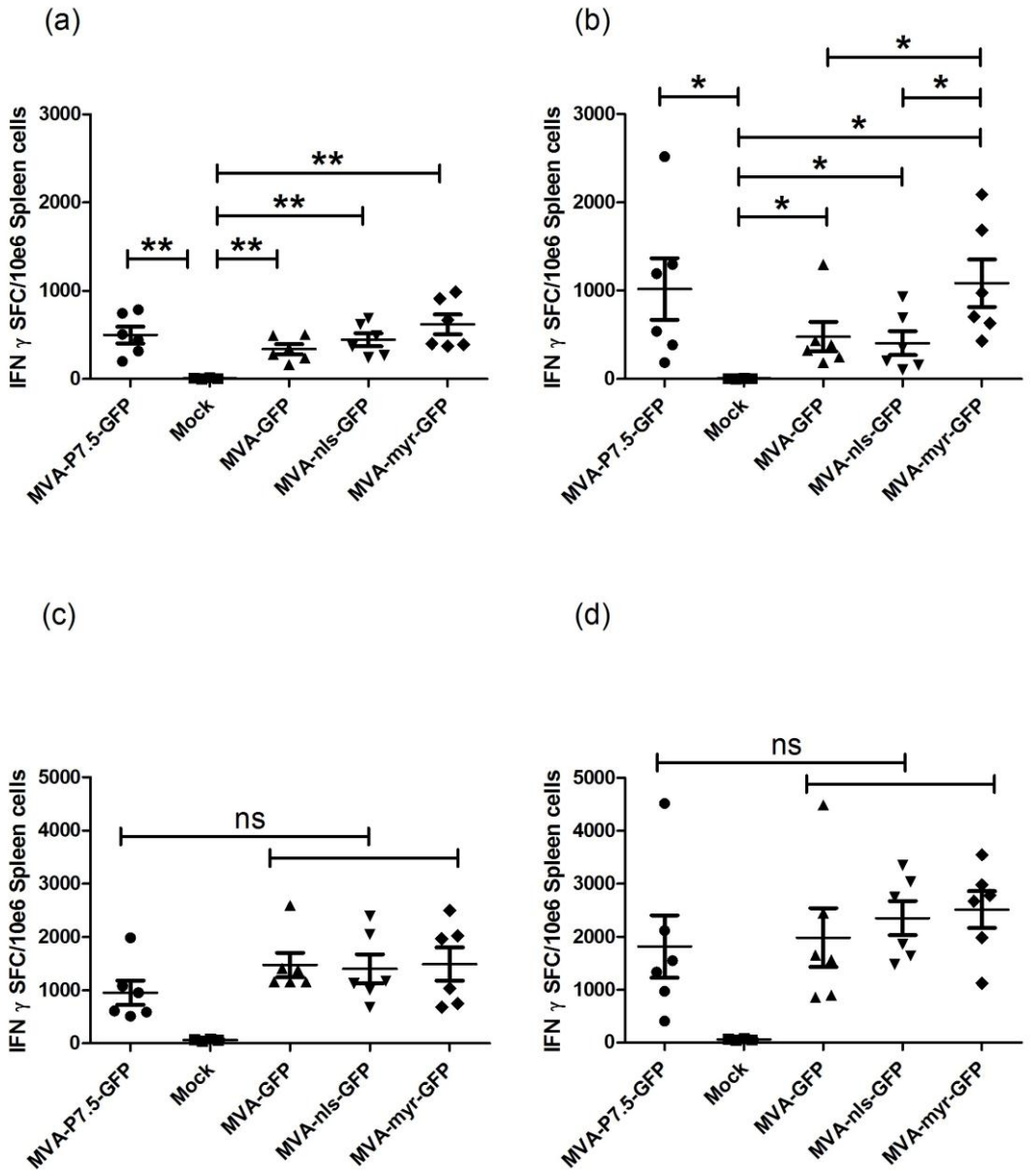


**Fig. 3. Immunogenicity of recombinant MVA-GFP candidate vaccines.** Groups of Balb/c mice (n=6) were immunized intramuscularly with $10^8$ PFU MVA or mock vaccinated (PBS, n=4). (a, c) At day 8 post vaccination, or (b, d) at day 28, following a boost at day 21, GFP-specific (a, b) or MVA $F2_{26-34}$-specific (c, d) IFNγ-producing CD8+T cells were measured using an ELISPOT kit (Mabtech) for mouse IFN-γ following the manufacturer's instructions. The cells were stimulated with $GFP_{200-208}$ peptide (HYLSTQSAL, 2 ng/ml, Thermo Fisher Scientific) or MVA $F2_{26-34}$ peptide (SPGAAGYDL, 2 ng/ml, Thermo Fisher Scientific) automated ELISPOT plate reader software (A.EL.VIS Eli.Scan software) was used to count and analyze the spots and differences between the groups were compared by t-tests using GraphPad Prism for Windows (GraphPad Prism Software, USA). Data are representative of two independent experiments. Statistically significant differences between the groups are shown as follows: *, $P < 0.05$; **, $P < 0.01$, ns, not significant.

Our aim was to generate an MVA vector vaccine that optimizes the induction of antigen-specific T cells. Recent data have increasingly highlighted the importance of antigen-specific T cells for generating protective immunity against infectious diseases, particularly in the context of more complex viruses such as influenza virus and human immunodeficiency virus, which characteristically mutate their antigenic structure very rapidly (BRANDLER et al., 2010; KREMER et al., 2012a). Moreover, long-lived memory CD8+ T cell immunity is considered important for cross-protection to ensure broader efficacy against different virus strains (BROWN & KELSO, 2009). Such vaccines are urgently needed to control pathogens with pandemic potential such as influenza (AHLERS & BELYAKOV, 2010).

Previous studies have proposed cross-priming as the most important mechanism for antigen presentation upon primary immunization to induce efficient T cell response (GASTEIGER et al., 2007). Here, antigen-presenting cells such as dendritic cells package the antigen expressed by donor cells using a major histocompatibility complex class I-molecule on their cell surface.

We analysed whether localizing the antigen to different cellular sites could affect the T cell immunogenicity of recombinant MVA. For this purpose we constructed recombinant MVA expressing GFP or GFP linked to either a nuclear localization signal or a cell membrane-locating myristoyl group. Unmodified GFP delivered by MVA distributes equally throughout an infected cell and is not secreted, providing a good base line to analyse the influence of selected cell compartments. Our immunostaining results confirmed the accumulation of GFP to different subcellular localizations. Indeed, for this purpose GFP may be superior to other commonly used model proteins, such as ovalbumin, which is glycosylated in the endoplasmic reticulum/Golgi compartments and secreted from the cell (NORDER et al., 2010; BECKER et al., 2014).

The choice of the early Pvgf promoter should not only drive early protein synthesis but also avoid the potential hiding of antigens in viral factories forming at late times of infection (KATSAFANAS & MOSS, 2007); both

parameters should facilitate efficient endogenous antigen presentation, which is needed to appropriately compare the different localization signals. Moreover, recent *in vitro* studies demonstrated the unique transcription strength of Pvgf (YANG et al., 2015), which additionally recommends the use of this promoter.

The localization signals of the recombinant MVA-GFP viruses did not influence either their growth kinetics compared with non-recombinant wildtype virus or the expression levels of the modified antigens. Direct comparison of strict early expression induced by AraC treatment clearly indicated the much more efficient early gene expression by the Pvgf promoter compared with the well-established P7.5 promoter, as shown by GFP protein amounts detected in Western blot analysis.

*In vivo,* we observed comparable activation levels of GFP epitope-specific CD8+ T cells for all vaccines after single immunization. However, after boost vaccination, expression of myristoylated GFP significantly enhanced the induction of GFP CD8+ T cells compared with the nuclear localization signal or unmodified GFP. These data are relevant because they confirm that GFP accumulation on cellular membranes has beneficial effects for activating antigen-specific CD8+ T cells. Moreover, these results support the hypothesis of Gasteiger suggesting that subcellular localization of target antigens could optimize the antigen characteristics to the requirements of the MVA vector system (GASTEIGER et al., 2007). Optimal interaction between the target antigen and the host cell system could then also result in enhanced cross-presentation and thereby induce an elevated immune response. Interestingly, early Pvgf in combination with myristoylation produced an immunization efficacy comparable to early-late P7.5-GFP. Pvgf is clearly the stronger early promoter, whereas the P7.5 promoter can be assumed to allow for higher levels of recombinant gene expression when combining early and late transcriptional activities (YANG et al., 2015). However, based on previous work (KASTENMULLER et al., 2007), we also expected a possible disadvantage of the early-late P7.5 promoter in boosting the CD8+ T cell response, which we did not observe. Thus, it could be that the late gene expression provided by P7.5 does contribute to the *in vivo* amplification of

GFP-specific CD8+ T cells. Subsequent studies will be necessary to investigate precisely the effect of myristoylation on antigen cross-presentation and the activation of cytotoxic T cells in the context of MVA early-late gene expression. In addition, while myristoylation can be expected to enhance the immunogenicity of MVA-produced antigens similar to GFP, it will be interesting to further test other target proteins including glycosylated and membrane-anchored antigens.

Taken together, our data support the idea that myristoylation could be a promising strategy for modifying antigens in the development of MVA-based vaccines against threatening infectious diseases. The recombinant MVA-GFP-myr prototype that we developed here merits further analysis in the context of real antigens, for example the nucleoprotein antigen of influenza A virus.

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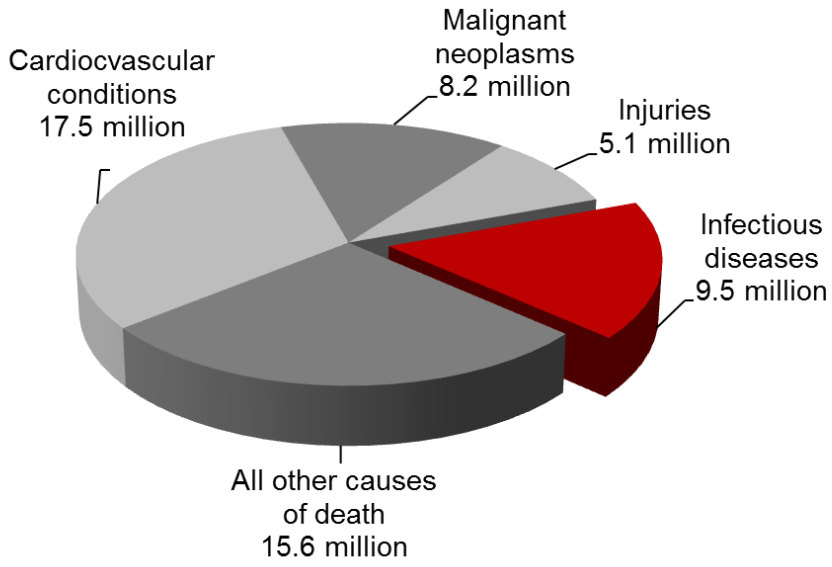
# V. DISCUSSION

In spite of ongoing progress in medicine, there are still several infectious diseases especially threatening for public health. These diseases are either hard to treat or completely untreatable (such as HIV), show high pandemic potential (for example influenza) or can't be prevented at all because of the lack of efficient vaccines. Therefore the development of novel vaccines including the investigation of innovative new vaccination strategies has high priority in medical research. Regarding the excellent safety profile and the results in preclinical as well as clinical trials, MVA serves as a promising platform in the development of vector vaccines. The objective in this study was to further enhance the cellular immunogenicity of recombinant MVA. For this purpose recombinant MVAs were constructed expressing green fluorescent protein as a model antigen under the transcriptional control of the strong poxvirus specific promoter Pvgf in combination with different subcellular localization signals. All constructed recombinant MVAs showed genetic stability and replicated efficiently in DF-1 cells. Moreover, the replication deficiency in mammalian cells confirmed safe handling of the recombinant MVA under biosafety level 1 conditions. The different MVA-GFP candidate vaccines efficiently produced green fluorescent protein and induced GFP-specific CD8+ T cells in vaccinated mice. In addition, combining GFP to a myristoylation signal statistically enhanced the cellular immunogenicity of GFP. Thus, myristoylation could serve as a promising tool to modify recombinant MVA expressing target proteins of various infectious diseases.

**Why does development of vaccines still have high priority in medical research?**

Today, infectious diseases yearly cause about 10 million deaths worldwide despite an obvious decrease through medical progress compared to data published about ten years ago (MORENS et al., 2004). Thus, infectious diseases still amount to about 17% of the annual 56 million deaths in the world in 2012 and represent one of the main problems in public health (figure 5). Children in developing countries are mainly affected

(GUERRANT & BLACKWOOD, 1999). Figure 5 shows the leading causes of death worldwide and highlights the major infectious diseases. The figure excludes deaths through complications or consequences of past or chronic infections to allow comparison with previous studies (MORENS et al., 2004).




| Infectious diseases | Annual deaths (millions) |
|---|---|
| Respiratory infections | 3.06 |
| HIV | 1.53 |
| Diarrhoeal diseases | 1.50 |
| Tuberculosis | 0.94 |
| Malaria | 0.62 |
| Childhood-cluster diseases | 0.27 |
| Hepatits B and C | 0.19 |

Figure 5: **Leading causes of death worldwide in 2012**
(Data published by World Health Organization 2014 (WHO methods and data sources for country-level causes of death 2000-2012); http://www.who.int/healthinfo/global_burden_disease/estimates/en/index1.html)

According to the data published in 2014 by the WHO respiratory infections including influenza, HIV, tuberculosis and malaria are besides the diarrhoeal diseases the most lethal infectious diseases. Unfortunately, the development of an efficient and safe vaccine against HIV has so far not been successful. Furthermore today no universal influenza vaccine is available which would be needed to efficiently protect against all influenza viruses including novel subtypes with pandemic potential. These more complex diseases are known to require cellular immune responses for protection (PANTALEO & KOUP, 2004; HOFT, 2008; ALTENBURG et al., 2014). For this reason it is important to focus not only on the induction of antibodies, but also on the strong activation of T cells in the development of new vaccines. Generally, the development of vaccines efficient against complex infectious diseases has high priority in medical research because vaccination is regarded to be the most effective and cost-efficient prevention method (SALLUSTO et al., 2010; PULENDRAN & AHMED, 2011).

**Factors with potential influence on T cell immunity triggered by MVA**

The activation of the cellular immune system does not only lead to the generation of effector and memory T cells. It furthermore also supports the B cell response (WEBER et al., 2009). Enhanced B cell response results in antibody production and supports the efficacy of the vaccine as well. Many different factors for the design of poxvirus vectors are considered to have positive effects on the activation of the humoral immune system. These factors can either modify the vector backbone or the target antigen.

The induction of T lymphocytes can for example be influenced through the amount of target protein delivered. It is known that the amount of heterologous protein clearly correlates with the immune response of vaccinated mice (WYATT et al., 2008; GARCIA-ARRIAZA & ESTEBAN, 2014). These data suggest that recombinant MVA should produce the highest amount of protein which is possible without affecting the stability and growth behavior of the virus (WYATT et al., 2008). High production levels of protein can mainly be achieved through the use of strong and efficient promoters. For this reason numerous experimental studies

consistently investigate and compare natural poxvirus-specific promoters as well as new synthetic promoters for their efficacy and strength (for example BAUR et al., 2010; BECKER et al., 2014; YANG et al., 2015). It is remarkable that in poxvirus vector systems additionally to the expression strength of the target antigen also the expression time during the life cycle of the virus can be specifically chosen. The expression time is even considered to be crucial for the type of the immune response (activation of CD4+ or CD8+ T cells). CD4+ T cells mainly recognize late antigens, whereas activation of cytotoxic T cells is predominantly associated with early expression (MOUTAFTSI et al., 2007; YANG et al., 2011; GARCIA-ARRIAZA & ESTEBAN, 2014). Today strong and well-characterized early, early-late as well as late promoters for the MVA system are available. These different promoters have specific advantages and disadvantages depending on the experimental approach and setting. Early-late expression allows antigen expression during the whole life cycle of MVA. In contrast, the use of late promoters only leads to antigen expression during the late cycle phase. Furthermore it can be associated with hiding of antigen in viral factories which are formed at later times of MVA infections (KATSAFANAS & MOSS, 2007). For this reason early promoters are considered to be better suitable for efficient induction of the endogenous pathway. Thus, early promoters are usually used in studies which aim at investigating T cell responses (BAUR et al., 2010). Apart from the promoter strength the spacer length also seems to play an important role. 2015 a study firstly confirmed the positive effect of an increased promoter spacer length on the activation of T lymphocytes (DI PILATO et al., 2015).

Another strategy for the improvement of the cellular immune response experienced over the last years consists in co-expression of different molecules. In this context, it is a good approach to combine different antigens of the same target virus. The combination of specific T cell antigens with antigens which induce strong antibody production should allow to efficiently activate both, the humoral immune system and the cellular immune system. Furthermore it is also possible to combine antigens of different viral subtypes for the induction of cross-protective

immunity. This could especially be supportive in the development of influenza vaccines. Besides the co-expression of different antigens, another promising approach is to combine target antigen with co-stimulatory molecules such as cytokines. Co-delivery of cytokines under the control of poxvirus specific promoters or exogenous inoculation can result in enhanced activation and recruitment of immune cells (GARCIA-ARRIAZA & ESTEBAN, 2014). IL-2 (BERTLEY et al., 2004), IL-12 (ABAITUA et al., 2006), IFNγ (ABAITUA et al., 2006) or GM-CSF (CHAVAN et al., 2006) have amongst other cytokines successfully been used in the MVA vector system. In general, for the construction of recombinant vectors co-expressing several proteins, the insertion of larger DNA sequences is required. In this context MVA serves as an appropriate vector because of its large genome and high genetic plasticity.

Next, the immune response could be improved through the combination of different vaccines in prime-boost vaccination protocols (for review see GARCIA-ARRIAZA & ESTEBAN, 2014). Today, poxvirus vectors combined with other vaccines are commonly used in preclinical and clinical trials. Here, the poxvirus vector can be used as the priming vaccine as well as the booster component. Recombinant MVAs have so far successfully been used in several experimental studies combined with DNA vectors (GOMEZ et al., 2007), recombinant influenza viruses (GONZALEZ-ASEGUINOLAZA et al., 2003), adenoviruses (REYES-SANDOVAL et al., 2010; RATTO-KIM et al., 2012) or other poxvirus vectors (SANTRA et al., 2007). Results from these data clearly recommend further investigation and use of heterologous prime-boost vaccination strategies.

Additionally to the improvement of the vector backbone described above another approach is to optimize the target antigen. In this context the choice and suitable design of the target antigen certainly plays the key role. Optimal adjustment of the antigen to the specific requirements of the delivering vector system is essential to efficiently activate the immune system (GASTEIGER et al., 2007). Data published recently also highlight the importance of long-lived antigen for the induction of the cellular immune response. It is generally supposed that long-lived antigen could

enhance the cross-presentation pathway and therefore induce a stronger activation of cytotoxic T cells (GASTEIGER et al., 2007). Furthermore the antigen chosen should preferably have full length instead of being shortened. Full length antigen can support immune reaction not only against a single, but potentially also against multiple T cell epitopes (GASTEIGER et al., 2007). In this context we should keep in mind that basically only 2% of all peptide fragments which are generated through processing of foreign proteins within the cell have an amino acid structure which can bind to MHC and is consequently able to activate T cells (WEBER et al., 2009).

Data published recently including this study also consider effects of the target protein's localization on the immunogenicity of recombinant MVA (GASTEIGER et al., 2007). Target protein can generally be expressed all over an infected cell or it can be located in a specific subcellular compartment. Distinct subcellular localizations can be achieved through the use of cellular localization signals. But on the other hand it is also possible to abrogate the natural subcellular distribution of a target antigen through removing its own localization signal. The purpose of such modifications is to improve the accessibility of target protein to the cellular apparatus processing protein. Generally, enhanced protein depletion and presentation of antigenic material obviously seem to support the activation of T lymphocytes.

Another parameter which is discussed for further enhancement of recombinant MVA's immunogenicity is the use of adjuvants. Adjuvants are commonly used in many vaccines currently available. The major task of adjuvants is to enhance the efficacy of vaccines by triggering rapid, strong and long-lasting immune responses (GUPTA & SIBER, 1995). Moreover adjuvants are also able to selectively modulate immune responses to MHC class I or MHC class II molecules. This could be helpful in the control of diseases caused by intracellular pathogens (GUPTA & SIBER, 1995). In addition, adjuvants containing synthetic T cell epitopes have already been developed which can specifically enhance the cellular immunogenicity (HADDEN, 1994). However, the use of adjuvants in the MVA vector system is controversially discussed for good reasons. On the one hand

adjuvants indeed enhance the immunogenicity of many vaccines available and could therefore also be useful in the MVA vector system. But on the other hand previous work clearly considers adjuvants as dispensable for most MVA based vaccines because of the intrinsic immunogenicity of the vector (ALTENBURG et al., 2014). Furthermore the use of adjuvants in vaccines is generally discussed. Many adjuvants are known to have great toxic potential and can induce adverse and severe side effects following vaccination (GUPTA & SIBER, 1995). For this reason the dispensability of adjuvants is considered to be one important factor which supports the generation of safer vaccines. Therefore MVA renouncing the addition of adjuvants serves as a promising vaccine platform.

**Importance of T cell response for efficacy of MVA based vaccines**

Today it is consensus that protective vaccines against more complex diseases such as HIV, tuberculosis or malaria require both, induction of strong T cell immunity and efficient activation of the humoral immune system. Especially vaccines against HIV lack a coordinated B and T cell response (GOMEZ et al., 2011; GOMEZ et al., 2012) because T lymphocytes are crucial for the control of viremia during acute infections (McELRATH & HAYNES, 2010). Additionally a large population-based study generally provides evidence that Gag-specific T cell responses can efficiently control chronic infections and are affiliated with lower viral loads (KIEPIELA et al., 2007; McELRATH & HAYNES, 2010). Several recombinant MVA vaccines expressing Gag have already been tested for their potential to induce cellular immune responses (for review see GOMEZ et al., 2012). The results of these studies clearly indicate MVA as a promising Gag vector for combined induction of the cellular immune system and the humoral immune system.

In case of malaria, T cell activation is also required for the protective efficacy of vaccines because inducing protective immunity against sporozoites only through humoral immune responses is obviously challenging (HILL et al., 2010). Several animal studies and human clinical trials have confirmed the key role of cytotoxic T cells in the elimination of the parasites (DOOLAN & HOFFMAN, 2000; DE BARRA et al., 2014). In

general, protection against malaria is clearly related to high levels of CD8+ T cells (SCHNEIDER et al., 1998; DE BARRA et al., 2014). Moreover, cytotoxic T cell immunity even seems to be the vital factor for prevention of parasitemia and clearance of all parasites (EWER et al., 2015). For this reason malaria vaccine development is focused on the generation of vaccines which can activate both, B cells and T lymphocytes.

The currently available tuberculosis vaccine, Bacille Calmette-Guérin (BCG), is commonly used especially in developing countries (BROOKES et al., 2008). However, a meta-analysis published in 1994 reports that BCG generally only reduces the risk of tuberculosis by 50% (COLDITZ et al., 1994). It efficiently protects children from tuberculous meningitis, but the efficiency against pulmonary tuberculosis shows an inappropriately high variability (COLDITZ et al., 1994; TRUNZ et al., 2006; BROOKES et al., 2008). Therefore the development of new tuberculosis vaccines is still ongoing. Experimental studies indicate the important role of cytotoxic T cells for the induction of protective immunity in mice (FLYNN et al., 1992). In this context, a MVA vaccine expressing 85A antigen has been developed. This vaccine indeed induced 85A-specific T cells, when tested on volunteers in Africa (BROOKES et al., 2008) and proved to be well tolerated when tested in a large phase 2b trial in about 1,400 infants (TAMERIS et al., 2013). These data showed that the 85A antigen alone may not be sufficient to provide protective immunity, but the safety data clearly recommend further development of tuberculosis vaccines on the basis of MVA.

Even if it is often underlined that T cell based immunity through vaccination with recombinant MVA is not able to completely prevent an infection alone, it indeed can trigger rapid protection. In this context the general rapid activation of T cells which can of course also be achieved in the MVA vector system is especially vital. T cell responses usually only need about four days to develop. In contrast antibody production takes obviously longer with approximately eight to ten days (MURPHY et al., 2008). So even if the T cell immunity is not able to prevent the infection and the disease following, the rapid response can play the key role in the prophylaxis of infectious diseases such as influenza. This point has been

confirmed in recent studies which investigated MVA's potential to induce short term protection using ectromelia virus (ECTV) challenge infections in mice (KREMER et al., 2012a; VOLZ et al., 2014). ECTV, the causative agent of mousepox, is probably the best animal model available for human smallpox infections. The results clearly show that the early protective capacity of MVA depends on the induction of CD4+ and CD8+ T cells. Here, MVA-specific T cells could already be detected at day two, whereas the number of MVA-specific antibodies didn't start to increase until day eight. These studies furthermore show the adequacy of low-dose immunization and consider the dispensability of the humoral immune response for rapid protection.

Activation of CD8+ T cells has recently also been confirmed in experimental studies with a recombinant MVA vaccine delivering spike glycoprotein of Middle East respiratory syndrome coronavirus (MERS-CoV) (SONG et al., 2013; VOLZ et al., 2015). MERS is a zoonotic infectious disease which has its natural reservoir in camels. It has caused severe human respiratory diseases in Middle Eastern countries since 2012. The MERS triggered respiratory syndrome is reported to result in death of about 20% to 40% of those infected (MACKAY & ARDEN, 2015). In 2015 the MVA-MERS-CoV vaccine was also successfully tested in a challenge experiment using dromedary camels (HAAGMANS et al., 2016). The success of this vaccination is hypothesized to be based on the induction of MERS-CoV neutralizing antibodies. However, the potential contribution of vaccine induced CD8+ and/or CD4+ T cells to protective immunity is not known and warrants further investigation. Generally, the development of camel-specific MERS-vaccines is very vital for the protection of humans who work in close contact with the animals.

Furthermore the protective efficacy of recombinant MVA expressing nucleoprotein of influenza also indicates the importance of T cell activation in the MVA vector system. As previously mentioned, nucleoprotein is able to efficiently induce T cells. Previous immunization experiments using recombinant MVA delivering nucleoprotein highlight that protective immunity against lethal challenge with influenza virus is affiliated with high CD4+ and CD8+ T cell responses (HESSEL et al., 2014). These data also

confirm that pre-existing CD4+ T cell responses obviously correlate with protection even without influenza specific antibodies (WILKINSON et al., 2012; HESSEL et al., 2014). The efficacy of a T cell-based MVA influenza vaccine expressing nucleoprotein and matrixprotein 1 has also been demonstrated in a phase 2a influenza challenge study published recently (LILLIE et al., 2012).

The exact mechanism of short term protection triggered by T cells is still subject of present research. However, it is supposed that rapid T cell immunity could probably bypass the time until the infection leads to the additional and long-lasting protective antibody response. This approach is basically also regarded as progressively important for the prevention of new threatening infections such as Ebola or MERS corona virus.

**Role of T cells in MVA-based cancer immunotherapies**

However, T cells are not only important for the protective capacity of vaccines against infectious diseases. They are also considered to play a key role in the development of efficient new cancer therapies. Here, T cells could be determining factors for the efficacy of vaccines on the basis of tumor-associated antigens. Such vaccines are especially promising in the control of immunogenic tumors like melanomas. Spontaneous regression of melanomas is obviously associated with infiltration of T cells (LOWES et al., 1997). Therefore research investigates more and more tumor-associated target antigens for their potential to induce tumor-specific T cell responses.

In this context, tyrosinase serves as a promising tumor-associated target antigen for cancer immunotherapies because it is regularly expressed in melanomas (CHEN et al., 1995). Its natural function is being the key enzyme of the melanin biosynthesis. Expressed by MVA, this enzyme is able to efficiently trigger tyrosinase- and melanoma-specific cytotoxic T cell responses *in vitro* and *in vivo* (DREXLER et al., 1999). In a phase I clinical study melanoma patients were treated with dendritic cells which had been infected with recombinant MVA delivering tyrosinase (DI NICOLA et al., 2003). In general, this treatment was well tolerated and

triggered tyrosinase-specific T cells *in vivo* (DI NICOLA et al., 2004). Apart from tyrosinase, tyrosinase-related-protein-2 (TRP-2) also serves as an appropriate target antigen for T cell based cancer therapy. It is remarkable that the expression of TRP-2 even seems to correlate with the resistance of melanoma cells against radio- and chemotherapy (PAK et al., 2004). TRP-2 delivered by recombinant MVA can efficiently be recognized by dendritic cells and therefore also activate T lymphocytes (PASCHEN et al., 2005).

However, melanoma is not the only type of cancer which responds positively on T cell based immunotherapies. In approximately 30% human breast cancer is associated with the overexpression of human epidermal growth factor receptor-2 (HER-2/neu) (SLAMON et al., 1989). This overexpression has also been demonstrated in ovarian and renal cancer (SLAMON et al., 1989). HER-2/neu is a proto-oncogenic glycoprotein which belongs to the tyrosinase-kinase receptors family (COUSSENS et al., 1985). Overexpression of HER-2/neu is related to a down-regulation of MHC class I molecules and disturbance of CD8+ T cell mediated cell lysis (HERRMANN et al., 2004). The down-regulation of MHC-class I molecules can result in further tumor progression (HERRMANN et al., 2004). For this reason it seems to be a promising approach to use HER-2/neu as a tumor-associated antigen and recombinant MVA has been used for efficient delivery of HER-2/neu antigen to human dendritic cells (KASTENMULLER et al., 2006).

**Future perspectives**

Today safe and highly effective viral vectors on the basis of the well-characterized MVA are commonly generated. Further preclinical as well as clinical studies are essential to define the recombinant MVAs which are able to fully protect against specific diseases. Thus, further enhancement of the cellular immune response certainly plays an important role, especially in the development of vaccines against complex infectious diseases. In general, recombinant MVAs are also suitable candidates for the development of cancer immunotherapies. In this context, the combination of MVA with further innovative treatment tools such as the

simultaneous use of checkpoint inhibitors seems to be a very promising approach for future research.

# VI. SUMMARY

**Improving the cellular immunogenicity of recombinant Modified Vaccinia virus Ankara using green fluorescent protein as model system**

Infectious diseases are still threatening for public health and cause about 17% of the annual deaths worldwide despite great medical progress over the last decades. In this context more complex diseases such as influenza, HIV, tuberculosis or malaria are especially problematic. These infectious diseases are characterized either by being untreatable or unpreventable because of the lack of efficient vaccines.

The development of vaccines has for a long period of time predominantly been based on the induction of humoral immune responses. But today the cellular immune system is also considered to play an important role in the prevention of more complex diseases. So the focus of vaccine development is changing more and more. In this context, Modified Vaccinia virus Ankara (MVA) serves as a promising platform for vaccine development because it is able to induce both, humoral immune responses and cellular immune responses. MVA is a safety tested, replication deficient poxvirus vector which can stably express heterologous proteins. Apart from being used as recombinant vector vaccine, MVA also serves as a promising tool in the development of cancer immunotherapies which are based on the induction of T cells.

In this study we investigated an innovative antigen modification tool for its potential to further enhance the cellular immunogenicity of recombinant MVA. For this purpose we introduced green fluorescent protein (GFP) in combination with different localization signals to the MVA genome. GFP was tacked either to myristoyl acid or to a nuclear localization signal and compared with unmodified GFP. The genetic integrity and stability of the recombinant MVAs was confirmed via polymerase chain reaction (PCR). Moreover, growth kinetics of the candidate viruses in DF-1, MEF and NIH3T3 cells showed the replication deficiency of the generated MVAs in mammalian cells. The stable expression of the GFP variants was verified

using immunoblot analysis. Furthermore, microscopical analysis confirmed the expected specific subcellular localization sites of GFP triggered through the different localization signals. All MVA-GFP candidate vaccines induced GFP-specific CD8+ T cells after intramuscular inoculation of BALB/c mice. However, after boost vaccination modification by myristoylation signal resulted in an increased level of antigen-specific CD8+ T cells statistically significant compared to unmodified GFP or to nuclear localized GFP. Therefore the addition of myristoylation successfully enhanced the cellular immunogenicity of the recombinant MVA. Generally, this result supports the further use of myristoylation as promising modification tool for the generation of MVA based vaccines delivering specific antigens of infectious diseases.

# VII. ZUSAMMENFASSUNG

## Verbesserung der zellulären Immunogenität von rekombinantem Modifizierten Vaccinia Virus Ankara unter Verwendung von Grün fluoreszierendem Protein als Modellsystem

Trotz des großen medizinischen Fortschritts während der letzten Jahrzehnte stellen Infektionskrankheiten eine nicht zu unterschätzende Bedrohung für das öffentliche Gesundheitswesen dar. Infektionskrankheiten zählen mit einem Anteil von 17% an den jährlich weltweit registrierten Todesfällen immer noch zu den Haupttodesursachen. Dabei gelten vor allem komplexere Infektionen wie Influenza, HIV, Tuberkulose oder Malaria als besonders problematisch. Diese Infektionskrankheiten weisen häufig einen schweren Verlauf auf oder sind nicht heilbar. Aufgrund des Fehlens wirksamer und sicherer Impfstoffe ist zudem die Prävention dieser Krankheiten nur eingeschränkt oder überhaupt nicht möglich.

Die Entwicklung neuer Impfstoffe basierte lange Zeit hauptsächlich auf der Induktion des humoralen Immunsystems. Inzwischen rückt aber auch die Aktivierung des zellulären Immunsystems immer mehr in den Fokus, da viele neuere Forschungsarbeiten in effizienten T-Zell-Antworten den Schlüssel für die Prävention komplexerer Infektionskrankheiten sehen. In diesem Zusammenhang dient Modifiziertes Vacciniavirus Ankara (MVA) als eine vielversprechende Plattform für die Impfstoffentwicklung, denn MVA kann sowohl die Aktivierung des humoralen als auch des zellulären Immunsystems induzieren. Das Pockenvirus MVA kann stabil Fremdgene exprimieren und dient somit als viraler Vektor in der Impfstoffentwicklung. Da MVA in menschlichen Zellen und Säugetierzellen nicht replizieren kann, gilt die Verwendung dieses Vektors als besonders sicher. Außer in der Impfstoffentwicklung wird MVA auch in der Entwicklung von neuen Krebstherapien, die auf der Induktion von T-Zellen basieren, eingesetzt.

Im Rahmen dieser Arbeit wurde eine neue Methode zur Modifizierung von Proteinen auf ihre Immunogenität im MVA Vektorsystem getestet. Dazu wurden Gensequenzen für Grün fluoreszierendes Protein (GFP) in

Kombination mit unterschiedlichen Lokalisierungssignalen in das MVA Genom integriert. GFP wurde dabei entweder mit einer Myristoylierungs- oder einer nukleären Lokalisierungssequenz versehen und mit unmodifiziertem GFP verglichen. Die genetische Stabilität der rekombinanten MVAs wurde mittels Polymerase-Ketten-Reaktion nachgewiesen. Wachstumskurven auf DF-1, MEF und NIH3T3 Zellen bestätigten sowohl die funktionierende Replikation in Zellen aviären Ursprungs als auch die fehlende Vermehrungsfähigkeit der rekombinanten Viren in Säugetierzellen. Zudem wurde die stabile Expression der verschiedenen GFP-Varianten mithilfe von Immunfluoreszenz-Analysen gezeigt. Die erhoffte subzelluläre Lokalisation von GFP in Abhängigkeit von den eingesetzten Lokalisierungssequenzen wurde mittels Fluoreszenzmikroskopie bestätigt. Im Tierversuch konnten alle rekombinanten MVA nach intramuskulärer Injektion die Aktivität GFP-spezifischer zytotoxischer T-Zellen induzieren. Nach der zweiten Immunisierung war die Aktivität der CD8+ T-Zellen, induziert durch myristoyliertes GFP, signifikant erhöht im Vergleich zu den weiteren GFP Varianten. Die hier untersuchte Myristoylierung konnte somit erfolgreich die zelluläre Immunogenität des rekombinanten MVA steigern. Myristoylierung empfiehlt sich deshalb auch als zukunftsträchtige Methode zur Modifizierung weiterer Antigene im MVA Vektorsystem.

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# IX. ABBREVIATIONS

| | |
|---|---|
| ABC | ATB-binding cassette family |
| AIDS | Acquired immunodeficiency syndrome |
| AMA1 | Apical membrane antigen 1 |
| APC | Antigen-presenting cell |
| ATP | Adenosine triphosphate |
| BALB/c | Albino, laboratory bred strain of house mouse |
| BCG | Bacille Calmette-Guérin |
| CD4 | Cluster of differentiation 4 |
| CD8 | Cluster of differentiation 8 |
| CEF | Chicken embryo fibroblasts |
| CVA | Chorioallantois Vaccinia virus Ankara |
| DAPI | 4’,6-Diamidin-2-phenylindol |
| DF-1 | Chicken embryo fibroblast cell line prepared by Doug Foster (University of Minnesota) |
| DNA | Desoxyribonucleic acid |
| ECTV | Ectromelia virus |
| ELISPOT | Enzyme-linked immuno spot assay |
| ENF | HIV gene encoding for envelope proteins |
| EV | Enveloped virion |
| GAG | HIV gene encoding for proteins of matrix and capside |
| GFP | Green fluorescent protein |
| GM1 | Monosialotetrahexosylganglioside 1 |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HA | Hemagglutinin |
| HBV-S | Hepatits B virus surface protein |
| HER-2/neu | Human epidermal growth factor receptor 2 |
| HIV | Human immunodeficiency virus |

| | |
|---|---|
| hpi | Hours post infection |
| IAV | Influenza A virus |
| IL | Interleukin |
| INF | Interferon |
| Kb | Kilobase |
| kDa | Kilodalton |
| LMU | Ludwig-Maximilians-Universität München |
| M1 | Matrixprotein1 |
| MEF | Mouse embryonic fibroblasts |
| MERS-CoV | Middle East respiratory syndrome coronavirus |
| ME-TRAP | Thrombospondin related adhesive protein fused to a multi-epitope |
| mg | Milligram |
| MHC | Major histocompatibility complex |
| ml | Milliliter |
| MOI | Multiplicity of infection |
| mRNA | Messenger ribonucleic acid |
| MTB | Mycobacterium tuberculosis bacteria |
| MV | Mature virion |
| MVA | Modified Vaccinia virus Ankara |
| NA | Neuraminidase |
| NEF | Negative factor |
| ng | Nanogram |
| NIH3T3 | Mouse embryonic fibroblast cell line derived from albino mice from the National Institutes of Health, Bethesda |
| nM | Nanomolar |
| NP | Nucleoprotein |
| ORF | Origin of replication |
| OVA | Ovalbumin |
| PAMP | Pathogen associated molecular pattern |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| P. falcip | Plasmodium falciparum |
| POL | HIV gene encoding viral enzymes |
| PRR | Pattern recognition receptor |
| RNA | Ribonucleic acid |
| TAP | Transporters associated with antigen processing |
| TRP | Tyrosinase-related-protein |
| WHO | World Health Organization |
| WT | Wildtype |
| VACV | Vaccinia virus |

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