# Characterization of fetal protection by vaccination with recombinant Modified Vaccinia virus Ankara in a Zika virus mouse model

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# I. ABBREVIATIONS

| | |
|---|---|
| **Aa** | amino acid |
| **ADE** | antibody-dependent enhancement |
| **ATCC** | American Type Culture Collection |
| **BSA** | bovine serum albumin |
| **BSL** | biosafety level |
| **°C** | Degree Celsius |
| **C (protein)** | capsid protein |
| **CAM** | chorioallantois membranes |
| **CDC** | Center for Disease Control and Prevention |
| **CEF** | chicken embryo fibroblasts |
| **CMC** | carboxymethyl cellulose |
| **CNS** | central nervous system |
| **$CO_2$** | carbon dioxide |
| **CVA** | chorioallantois vaccinia virus Ankara |
| **CZS** | congenital Zika syndrome |
| **DAPI** | 4′,6-Diamidin-2-phenylindol |
| **DC-SIGN** | Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin |
| **$ddH_2O$** | double-distilled water |
| **DENV** | Dengue virus |
| **DMEM** | Dulbecco`s modified Eagle`s medium |
| **DMSO** | dimethyl sulfoxide |
| **DNA** | deoxyribonucleic acid |
| **dNTP** | deoxynucleosidtriphosphate |
| **DPBS** | Dulbecco`s phosphate buffered saline |
| **dpc** | days post challenge |
| **dsRNA** | double-stranded RNA |
| **ECDC** | European Centre for Disease Control |
| **E (protein)** | Envelope protein |
| **ELISA** | enzyme-linked immunosorbent assay |
| **ELISpot** | enzyme-linked immunosorbent spot assay |

| | |
|---|---|
| **ER** | endoplasmic reticulum |
| **FBS** | fetal bovine serum |
| **FcR** | Fc receptor |
| **FFU** | foci forming units |
| **FDA** | Food and Drug Agency |
| **f.p.** | footpad |
| **GAGs** | glycosaminoglycane |
| **hpi** | hours post infection |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| **HE** | hematoxylin / eosin |
| **HIV** | Human Immunodeficiency Virus |
| **HRP** | horseradish peroxidase |
| **i.m.** | intra muscular |
| **IF** | immunofluorescence |
| **IFN** | interferon |
| **IFNAR** | type I interferon receptor knockout mice |
| **Ig** | immunoglobulin |
| **IL** | interleukin |
| **IRF** | interferon regulatory factor |
| **JEV** | Japanese encephalitis virus |
| **kDa** | kilodalton |
| **kb** | kilo base pairs |
| **l** | liter |
| **M** | Mol |
| **M-protein** | Membrane protein |
| **MEM** | minimal essential medium |
| **MHC** | major histocompatibility complex |
| **mg** | milligram |
| **min** | minute |
| **ml** | milliliter |
| **mM** | millimol |
| **MOI** | multiplicity of infection |
| **MVA** | Modified Vaccinia virus Ankara |
| **MVA-prME** | Modified Vaccinia virus Ankara expressing Zika virus structural proteins prM and E |

| | |
|---|---|
| **NO** | nitric oxide radicals |
| **NS** | non-structural protein |
| **NS1** | non-structural protein 1 |
| **NS2A** | non-structural protein 2A |
| **NS2B** | non-structural protein 2B |
| **NS3** | non-structural protein 3 |
| **NS4A** | non-structural protein 4A |
| **NS4B** | non-structural protein 4B |
| **NS5** | non-structural protein 5 |
| **OD** | optical density |
| **o/n** | over night |
| **ORF** | open reading frame |
| **PAMPS** | pathogen-associated molecular patterns |
| **PBS** | phosphate buffered saline |
| **PCR** | polymerase chain reaction |
| **PFU** | plaque-forming units |
| **PMA** | phorbol 12-myristate 13-acetate |
| **prM** | precursor membrane protein |
| **PRNT** | plaque reduction neutralization test |
| **rMVA** | recombinant Modified Vaccinia virus Ankara |
| **RNA** | ribonucleic acid |
| **RPMI** | Roswell Park Memorial Institute |
| **rpm** | rounds per minute |
| **RT** | room temperature |
| **RT-PCR** | reverse transcription polymerase chain reaction |
| **SARS-CoV-2** | severe acute respiratory syndrome coronavirus 2 |
| **SDS-PAGE** | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| **sec** | second |
| **SFC** | spot-forming cells |
| **SPF** | specific-pathogen-free |
| **ssRNA** | single stranded RNA |
| **TAE** | tris-acetate-EDTA |
| **Th** | T helper |
| **TMB** | 3,3′,5,5′-Tetramethylbenzidine |

| | |
|---|---|
| **TNF** | tumor necrosis factor |
| **V** | volt |
| **VACV** | Vaccinia virus |
| **VNT** | virus neutralization test |
| **VP-SFM** | virus production serum-free medium |
| **VSV** | vesicular stomatitis virus |
| **WB** | Western blot |
| **WHO** | World Health Organization |
| **WNV** | West Nile virus |
| **YFV** | Yellow fever virus |
| **ZIKV** | Zika virus |

# II. INTRODUCTION

Zika Virus (ZIKV) was discovered in 1947 and remained mostly ignored for the next decades. The first large outbreak occurred in 2007 in the Federated States of Micronesia and enabled the virus to spread to Oceania and the Americas. The following epidemic in 2015 and 2016 was associated with numerous congenital birth defects and cases of Guillain-Barré-Syndrome (GBS). This new severity led the World Health Organization (WHO) to declare the ZIKV outbreak as a Public Health Emergency of International Concern. Although cases of ZIKV infections have declined in recent years, the virus still poses a threat to public health. Increasing global connection and vector expansion because of climate change could facilitate new outbreaks and epidemics.

Following the devastating outbreaks in 2015 and 2016, intensive research in the fields of diagnostics, therapeutics and vaccine design to combat ZIKV have been undertaken. Despite multiple vaccine candidates showing promising results and some even reaching clinical trials, there is still no licensed vaccine against ZIKV available today. Many of the different approaches in vaccine design focus on the structural proteins of ZIKV as antigens. The combination of the precursor membrane (prM) and the envelope (E) protein of ZIKV have been proven to induce protection in different models and settings. Additionally to safety and efficacy, a key requirement of a ZIKV vaccine is fetal protection.

Modified vaccinia virus Ankara (MVA) is a well-established vaccine platform and is used for development of various vaccines against infectious diseases. Its replication deficiency and decade long use has demonstrated an exceptional safety profile. This study aims to investigate a recombinant MVA vaccine in a pregnancy mouse model. The goal was to gain more insight into prME as an antigen and to investigate its capability to induce fetal protection when delivered by MVA.

# III. LITERATURE REVIEW

## 1. Epidemiology of ZIKV

The Zika virus was first discovered in 1947 in Uganda. Researchers of the East African Virus Research Center held rhesus monkeys in the Zika Forest as sentinel animals to study Yellow Fever Virus (YFV) (Dick, Kitchen, and Haddow 1952). One monkey developed symptoms such as mild fever and the following investigation finally lead to the isolation and identification of the virus. In the following years ZIKV was only sporadically detected in humans (Dick, Kitchen, and Haddow 1952; Haddow et al. 2012). The first human ZIKV isolate was obtained from a 10 year old Nigerian in 1954 (Macnamara 1954) and the first isolate outside Africa was obtained 1969 from an *Aedes aegypti* mosquito in Malaysia (Marchette, Garcia, and Rudnick 1969).

Until the 21st century all incidences remained restricted to Africa and Southeast Asia and were characterized by mild symptoms (Hayes 2009; Brasil, Pereira Jr, et al. 2016).

The outbreaks in 2007 in the Yap Islands and in the Pacific Ocean mark the turning point when ZIKV gained international attention. In the Yap Islands the outbreak was initial falsely attributed to Dengue virus (DENV) although cases displayed uncharacteristic DENV symptoms such as mild fever and conjunctivitis (Musso and Gubler 2016).

The spectrum of ZIKV associated symptoms further expanded with the second outbreak 2013 and 2014 in French Polynesia, where about half of the entire population was infected (Musso et al. 2018). Cases with neurological disorders and fetal abnormalities occurred increasingly (Musso, Nilles, and Cao-Lormeau 2014; Musso et al. 2018). Additionally, incidences of inflammatory polyneuropathy Guillain-Barre syndrome were reported (Mier et al. 2018). Even though the virus shows close relation to the strains in the Yap Islands the exact origin remains unknown (Cao-Lormeau et al. 2014).

The detection of ZIKV in the north of Brazil in 2015 indicated the next step in the spread of the virus (Faria et al. 2016). Molecular clock analyses and

phylogenetic studies show that a single introduction of ZIKV already happened more than year earlier in 2013 (Faria et al. 2016).

The following epidemic was associated with numerous fetal impairments such as microcephaly and even abortion (Miranda-Filho Dde et al. 2016; Costa et al. 2016; Krow-Lucal et al. 2018). These impairments were later known under the term of congenital ZIKV syndrome (Moore et al. 2017).

By 2018 nearly 4000 such cases were reported in South America (Musso, Ko, and Baud 2019). The progression of the epidemic was favored by the wide spread of the ZIKV vectors *Aedes aegypti* and *Aedes albopictus* in Brazil (Marcondes and Ximenes Mde 2016). Additionally, the population of Latin America had never been exposed to ZIKV before and therefore was an easy target for the virus (Rossi et al. 2018)(Rodriguez-Barraquer et al. 2019).

**Figure 1: Spread of ZIKV.** ZIKV was first discovered 1947 in Africa and from there spread to Asia. The Asian ZIKV lineage later spread to the Pacific, the Americas and back to Africa. Created with BioRender.com

The severity and increasing frequency of the congenital ZIKV syndrome, together with the confirmation of its ability to be transmitted via sexual contacts and blood, led to the WHO declaring ZIKV in February 2016 as a “Public Health

Emergency of International Concern” (PHEIC) (Saiz et al. 2016).

Even though the effects of the ZIKV epidemics are clearly devastating, it is still debated whether the diseases are solely attributed to ZIKV or the result of multiple factors (Petzold et al. 2021). Different viruses, such as DENV, could act as a cofactor and even the prevalence of these diseases before the outbreak is not fully known (Petzold et al. 2021).

Lack of information about the course of individual cases further complicate the studies. A clear connection between a ZIKV infection and the occurrence of symptoms is often not given (Victora et al. 2016). The available data is also often not taken according to an international standard or not scientifically validated (Victora et al. 2016; Krow-Lucal et al. 2018). These factors make comparison difficult and led to an overestimation of cases of microcephaly (Krow-Lucal et al. 2018). There is also a great temporal and regional variety between the detection of the virus and the developing of neurological symptoms as seen in different cases in French-Polynesia and Brazil (Miranda-Filho Dde et al. 2016; Hay et al. 2018; Mier et al. 2018).

With the beginning of 2017 a remarkable shift in the ZIKV epidemic occurred: cases began to regress and by 2018 less than 30.000 cases were identified, which contrasted to the 500.000 registered cases in 2016. (Musso and Gubler 2016). The reasons behind this drastic decline in cases raised many questions that are still debated today. Since there were no preventive therapeutics or vaccines available at the time, herd immunity is considered to be the deciding factor. This course of events make sense for areas with a high incidence like French Polynesia or Brazil, but cannot explain the slowdown in other countries (Rodriguez-Barraquer et al. 2019; Aubry et al. 2017).

Genetic studies have shown, that the different outbreaks and epidemics of ZIKV were caused by different genetic lineages. Genetic studies enabled the distinction between an African and an Asian lineage. The American lineage was identified later and originated from the Asian lineage (Metsky et al. 2017; Liu, Shi, and Qin 2019). This lineage was responsible for the outbreaks in Micronesia and the Americas and is therefore responsible for the largest and most severe outbreaks. The Asian strain nevertheless still poses a threat to public health which was shown by the 2017 outbreak in India with 150 cases

(Watts et al. 2018). Although most outbreaks can be related to a specific lineage, the detailed impact on pathogenesis and spread is hard to retrace. When considering the time frame between 2013 and 2017, the Asian and the closely related American lineage were responsible for congenital birth defects and neurological afflictions (Wongsurawat et al. 2018). The African lineage however has in its decade long occurrence in Africa not been connected with these symptoms (Simonin et al. 2017). This remarkable difference could be explained by differences in the pathogenesis. For the African lineage, in vitro models for pregnancy have demonstrated its capability to damage placental cells heavily and therefore lead to an early pregnancy loss instead of further fetal development with brain damage (Sheridan et al. 2018). The fact that the outbreak in Angola 2016 with a cluster of birth defects was caused by the Asian lineage supports this hypothesis (Hill et al. 2019). When comparing cases of microcephaly overall, only a fraction occurred in Africa and the majority was connected to the outbreaks outside Africa in Brazil (WHO 2019).

ZIKV has spread over Africa, America, South-East Asia and the Western Pacific with autochthonous infections occurring in 87 countries (WHO 2019). Although ZIKV had reached Europe via more than 2000 imported infections in 2017 (Wilder-Smith, Chang, and Leong 2018), no autochthonous infection had been reported until 2019. In early 2019 the European Centre for Disease Control (ECDC) reported the first mosquito borne autochthonous ZIKV infection in southern France. While the main vector of ZIKV *Aedes aegypti* is not native to France, the closely related *Aedes albopictus* is established in some regions and also able to transmit the virus (Kraemer et al. 2019). This highlights the potential future danger of ZIKV, because all regions with the *Aedes* species are at risk and factors like climate change, increasing global connection and vector adaptation to urbanization could facilitate the vector expansion in new regions (Gould et al. 2017). The possibility of an outbreak or even an epidemic in Europe is therefore given (Giron et al. 2019). Fortunately for this possibility, *Aedes agypti* is far better suited because it carries high loads of ZIKV and thus is able to cause larger ZIKV outbreaks than *Aedes albopictus* (Hugo et al. 2019; Chouin-Carneiro et al. 2020).

## 2. Taxonomy, structure and viral life cycle

The ZIKV is a 40-50 nm small virus from the genus of Flavivirus (T. Pierson and Diamond 2013). Together with the Yellow fever virus (YFV), the Dengue virus (DENV), the West Nile virus (WNV) and the Japanese encephalitis virus (JEV) they form the Flaviviridae family. The common feature of Flaviviruses is their infection pathway: they utilize arthropods as vectors and are therefore called arboviruses (Musso and Gubler 2016). When infecting a host, arboviruses cause viremia after 2 to 6 days and enable a spread back to a vector. The latter are mostly mosquitoes and ticks, in which viral replication takes place first in intestinal epithelia and then in the salivary glands thereby making the vector infectious again (Modrow and Falke 1997). Although arthropods are the main route of infection, different ways of spreading are possible. Infection from mother to unborn child (Basurko et al. 2009), sexual intercourse and blood transfusions can lead to transmission (Tambyah et al. 2008; Oster et al. 2016). Interestingly Flaviviruses were initially not bound to vectors and developed this adaptation later (Gould et al. 2003).

ZIKV is enveloped and contains a single stranded RNA (ssRNA) genome with positive polarity that is 111kb in size (Musso and Gubler 2016). Like in other flaviviruses, the membrane of mature viruses derive from the host. The genome holds the information for one polyprotein in an open reading frame (ORF) with 100 – 400 nucleotide long untranslated regions (UTRs) both on the 5´ and 3´ end (Wolford and Schaefer 2020). The polyprotein is later cleaved by viral and cellular proteases into three structural proteins (capsid (C), precursor membrane (prM), envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Baronti et al. 2014; Musso and Gubler 2016). The structural proteins make up the viral composition and the non-structural proteins are essential for replication and assembly (Mukhopadhyay, Kuhn, and Rossmann 2005).

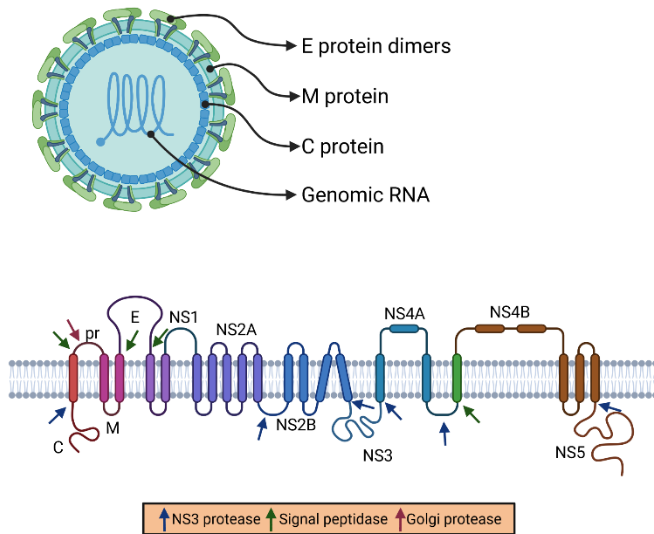


**Figure 2: Structure of a ZIKV particle and genome.** The genome is translated into a single polyprotein which is then processed by viral and host proteases. The viral particle consists of three structural proteins: envelope (E) protein, membrane (M) protein and capsid (C) protein. Created with BioRender.com

The infection of human cells by ZIKV follows a principle that is often found in the flavivirus family. Interaction between viral glycoproteins and cellular surface receptors that are often associated with sulfated glycosaminoglycans (GAGs) lead to receptor mediated endocytosis (Gollins and Porterfield 1985; Kim et al. 2017). In the case of ZIKV two types of receptors primarily interact with the E protein: the liver-specific ICAM3-grabbing-nonintegrin (L-SIGN) and the dendritic cell-specific intercellular adhesion molecule-3-grabbing-nonintegrin (DC-SIGN) (Jameson et al. 2002; Perera-Lecoin et al. 2013). This type of lectins are predominantly expressed on endothelial and dendritic cells which are targeted by ZIKV.

Once the viruses are taken up in endosomes, acidification leads to a change in the conformation of E proteins and subsequently to fusion of cellular endosome membrane and viral membranes (Allison et al. 1995; Smit et al. 2011). Now the

replication cycle of ZIKV starts with the release of RNA from the capsid into the cytoplasm (Lindenbach and Rice 2003). The viral RNA interacts with cellular ribosomes through its 5´-cap structure and translation of the polyprotein starts (Abrams, Solis, and Nath 2017). When the process reaches the amino-terminal site of the capsid, a sequence of this protein located in the carboxy-terminal region acts as a signal peptide and interrupts translation. This causes a transportation of the entire translation complex to the endoplasmatic reticulum (ER). Next, particles consisting of the structural proteins (E, prM and C), the nucleocapsid and a lipid membrane are formed (Guirakhoo et al. 1991). The transmembrane domains of prM and E function as an anchor in the lipid membrane of the ER (Saumya et al. 2021). The first cleavage is mediated by the host cell signal peptidase and produces the C-, prM-, E- and NS1 proteins (Abrams, Solis, and Nath 2017; Murray, Jones, and Rice 2008). After this post-translational processing, the replication of the original RNA genome at the ER membrane starts (Gillespie et al. 2010). A complex formed by the nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) supports further replication (Abrams, Solis, and Nath 2017). Specific steps that are mediated by NS5 and NS3 lead to the generation of multiple ssRNA strands. The NS5 contains a RNA polymerase which transcribes the ssRNA into double stranded RNA (dsRNA) multiple times while the helicase of NS3 unwinds and separates these dsRNA in between (Wu et al. 2015; Luo et al. 2008). The newly formed positive ssRNA strands are then methylated in a last preparation step before virus assembly by the NS5 (Wu et al. 2015). Immature virions assembled out of the C, prM, E proteins and the ssRNA are formed in the ER with heterodimers of prM and E on the surface (Heinz and Stiasny 2017). Most importantly a premature fusion of the E protein with cell membranes is prevented by the premature domain of prM through coverage of E (Abrams, Solis, and Nath 2017). The final step of maturing is achieved in the Golgi apparatus when furin cleaves prM into the mature and smaller M (Ma et al. 2004; Stadler et al. 1997). The now infectious virus particles leave the Golgi network through exocytosis (Mukhopadhyay, Kuhn, and Rossmann 2005).

## 3. Transmission

ZIKV can be transmitted through different pathways, but most infections by far are caused by mosquitoes (Gutiérrez-Bugallo et al. 2019). *Aedes* mosquitos are the main vector, more specifically *Aedes aegypti* is responsible for most transmissions (Gutiérrez-Bugallo et al. 2019). Even though *Aedes albopictus* has a much bigger distribution range and can transmit ZIKV, it plays a secondary role in transmissions (Gutiérrez-Bugallo et al. 2019). In addition to horizontal transmission from mosquitos to humans, flaviviruses like ZIKV can propagate vertically in mosquitos once the virus reaches the ovaries of infected mosquitos (Modrow and Falke 1997).

The ability for sexual and mother to fetus transmission is what makes ZIKV stand out among arboviruses (Musso, Ko, and Baud 2019). The sexual pathway of transmission however only makes up around 1 % of all recorded cases in Europe and the United States of America and while there is no reliable data for other countries, a similar distribution is suspected (Wilder-Smith, Chang, and Leong 2018). Most sexual transmitted ZIKV infections happen from male to female with no difference whether the male harbors a symptomatic or asymptomatic infection (Polen et al. 2018). Remarkably infectious virus particles can be found up to 30 days after the initial infection in testes and semen (Mead et al. 2018).

A transmission from mother to fetus occurs in 20 – 30 % of maternal ZIKV infections (Marbán-Castro et al. 2021; Pomar et al. 2018) and the symptomatic status of mothers does not influence the probability of transmission either (Pomar et al. 2018). There are different ways for ZIKV to infect fetuses, but the specifics are still debated. Human placentas are hemochorial, which causes a close contact between maternal blood and fetal chorionic villi (Schmidt and Kurjak 2001). Any kind of damaged to the placental barrier can make the fetus vulnerable to infections during the pregnancy (Coyne and Lazear 2016). After 12 weeks the barrier between mother and fetus is fully developed (Rossant and Cross 2001; Foidart et al. 1992). Despite this, a ZIKV infection of the fetus can occurre. As a matter of fact, ZIKV infections are possible at any time during pregnancy (Brasil, Pereira, et al. 2016; Reynolds et al. 2017). This suggests different pathways of ZIKV infections (Coyne and Lazear 2016). A likely possibility is that ZIKV can directly damage the placental barrier. In vitro studies

have shown that ZIKV can disrupt cellular tight junctions and therefore enable transmission through a paracellular pathway (Chiu et al. 2020). The maternal immune response is also suspected to damage the placental integrity (Coyne and Lazear 2016). Human placental trophoblasts on the other hand produce antiviral agents and are not susceptible to a ZIKV infection (Bayer et al. 2016). Despite this, an infection of progenitor cells is possible and therefore could be another pathway of transmission (Tabata et al. 2016).

Arboviruses including ZIKV are also known to be transmissible through blood transfusions (Musso, Stramer, and Busch 2016). The fact that ZIKV has been detected in whole blood up to two months after infection is a cause for concern (Lustig et al. 2016). Meta-analyses however have set the prevalence of ZIKV RNA at 1 – 2 % (Giménez-Richarte et al. 2021; Liu et al. 2019) and only isolated cases of transmission by blood transfusion have been reported (Barjas-Castro et al. 2016).

## 4. Clinical manifestation

The majority of ZIKV infections in adults result in an asymptomatic course of disease. Only 20 to 40 % experience often unspecific or flue like symptoms (Costa et al. 2016; Musso and Gubler 2016; Musso et al. 2018) such as fever, headaches, arthralgia, exanthema, myalgia and conjunctivitis (Petersen et al. 2016; Halani et al. 2021). This clinical manifestation often resembles symptoms from other flaviviruses such as DENV and WNV and result in an 11 % hospitalization rate and a 0, 1 % fatality rate overall (Halani et al. 2021).

Neurological disorders are possible sequelae from ZIKV infections with 75 % being Guillain-Barré Syndrome (GBS) (Halani et al. 2021). The outbreak in French Polynesia 2013 was the first time GBS was associated with ZIKV (Cao-Lormeau et al. 2016). Overall GBS occurs with an incidence of 2 to 3 cases per 10.000 ZIKV infections (Mier et al. 2018). GBS is an acute peripheral neuropathy with at least four different subtypes (Hughes and Cornblath 2005). Symptoms include ascending paresis, acute motor and sensory axonal neuropathy, inflammatory polyneuropathy and ophthalmoplegia (Hughes and Cornblath 2005; do Rosario et al. 2016; Dos Santos et al. 2016).

The worst effects of ZIKV often occur in fetuses as a result of maternal infections

during pregnancy. The fetal defects associated with maternal ZIKV infection are referred to as congenital Zika syndrome (CZS) (Costa et al. 2016). A variety of fetal and birth defects are associated with this syndrome, with microcephaly and abortion being the most well-known (Costa et al. 2016; Miranda-Filho Dde et al. 2016). Similar to other congenital infections, the pathology includes cortex calcifications, macular scarring, lesions in the medulla oblongata, retinal atrophy and congenital contractions (Moore et al. 2017; Pomar et al. 2018). In newborns the syndrome presents itself with many symptoms, such as cerebral palsy, epilepsy and severe intellectual disability (King and Irigoyen 2021). Contrary to striking impairments at birth, such as microcephaly, there is increasing evidence of neurological damage that becomes noticeable later in the children's life (López-Medina et al. 2020). These include developmental, visual and auditory impairment, cerebral palsy, dysphagia or epilepsy (van der Linden et al. 2022) As a result, long term studies and surveillance of children born from ZIKV infected mothers are essential.

## 5. Immune response to ZIKV

To combat infections the immune system must detect pathogens and afterwards mount an effective response. Two parts fill this role: the innate and the adaptive immune response.

A first detection of pathogens is mediated by pattern recognition receptors (PRRs) of host cells which initiates the immune response. (Boehme et al. 2004). Different PRRs can detect specific foreign pathogens through pathogen-associated molecular patterns (PAMPs) such as single-stranded RNA in the case of flaviviruses (Boehme et al. 2004).

Upon recognition of a flaviviral infection, genes that stimulate interferons (IFN) are activated and cytokines are produced, specifically type I (α, β), type II (γ), and type III (λ) IFN (Hamel et al. 2015; Bayer et al. 2016; Quicke et al. 2016). Type I interferons can be produced by all nucleated cells and likewise are recognized by most cells. Viral infections therefore primarily trigger the interferon I system (Ngono and Shresta 2018). ZIKV in particular is known to interfere with and attenuate the IFN response and therefore facilitate infection. In this context, NS5 directly antagonizes type I interferon and NS3 antagonizes the Interferon I pathway through proteolytic dependent and independent

activities (Ngono and Shresta 2018). By hampering the phosphorylation of signal molecules STAT1 and STAT2 and additionally reducing the interaction of STA2, viral replication is promoted and IFN response is reduced (Grant et al. 2016; Bowen et al. 2017). The importance of IFN in combating flaviviruses is demonstrated by mice lacking parts of the IFN signalling pathway. These mice are vulnerable to ZIKV infections (Shresta et al. 2004; Lazear et al. 2016) and produce other cytokines (Karaghiosoff et al. 2000; Shimoda et al. 2000).

When combating a ZIKV infection, the humoral immune response plays an important role in clearing viral particles through antibodies. These neutralizing antibodies are mainly targeted against the E protein, specifically against the domain III (Ngono and Shresta 2018; Dai et al. 2018; Throsby et al. 2006). Experiments have shown protection by these E specific antibodies, both through vaccines and passive transfer (Bayer et al. 2016; Muthumani et al. 2016). Antibodies directed against different structures don’t offer neutralizing capacity but nevertheless are induced by infections (Vázquez et al. 2002; Heinz and Stiasny 2012).

T cells are another important part of the immune response to ZIKV infections (Ricciardi et al. 2017; Wen et al. 2017). CD8+ T cells secrete cytokines like IFN-γ and tumor necrosis factor (TNF) that modulate the immune reaction to flaviviruses (Shrestha et al. 2006; Brien, Uhrlaub, and Nikolich-Zugich 2007). Furthermore CD8+ T cells support the elimination of infected cells through secretion of cytotoxic proteins (Shrestha, Samuel, and Diamond 2006; Ramos et al. 2012). The activation of CD8+ T cells and their capability to reduce viral burden have been demonstrated in mouse models (Huang et al. 2017; Pardy et al. 2017). In addition, protective capability against ZIKV infection has been proven for both ZIKV and DENV specific CD8+ T cells (Elong Ngono et al. 2017; Wen et al. 2017). While this demonstrates the importance of CD8 T cells, they can also do harm through mediation of ZIKV neuropathogenesis (Jurado et al. 2018; Manangeeswaran, Ireland, and Verthelyi 2016). CD4+ T cells also play an important role and support combating viral infections. They produce cytokines and help clear virus from the central nervous system (CNS) (Sitati and Diamond 2006). By supporting B cells and antibody maturation they also assist the humoral immune response (Sen et al. 1992; Pierson and Graham 2016).

Antibody-dependent enhancement (ADE) is a phenomenon mostly known in DENV infections but is also a risk for ZIKV and its corresponding vaccines (Martinez-Vega et al. 2017). When a DENV infection meets insufficient levels of neutralizing antibodies, the disease can be enhanced dramatically. This can happen after a second infection when previous antibody levels have declined or after an infection of a different serotype. ADE is caused by antibodies that bind to viral particles and are not able to fully neutralize them. Instead, monocytic cells take in the virus after recognition through immunoglobulin receptors (FCR) and facilitate viral replication (Halstead 2014). Early viremia and immune modulations then lead to a worse course of disease. Both CD4+ and CD8+ T cells recognize viral antigens through HLA class I and II molecules and produce different immunomodulatory cytokines like IFN-γ, TNFα, and IL2 which triggers capillary leakage (Roy and Bhattacharjee 2021). IFN-γ also enhances the expression of HLA II class receptors which in turn fosters viral antigen presentation and viral intake through antibody recognition (Rothman and Ennis 1999; Roy and Bhattacharjee 2021). Studies have also shown that infection of monocytic cells promote viral particle production and reduce antiviral immune responses through enhancement of IL-6 and IL-10 and downregulation of IL-2, IFN and nitric oxide radicals (NO) (Chareonsirisuthigul, Kalayanarooj, and Ubol 2007).

The increased risk of developing severe dengue fever due to pre-existing DENV antibodies has been demonstrated by multiple studies (Guzman, Alvarez, and Halstead 2013; Katzelnick et al. 2017). When considering ADE and ZIKV, three factors must be accounted for: Both ZIKV and DENV belong to the flaviviruses and are closely related, they are endemic to the same areas and anti-DENV antibodies are already proven to enhance ZIKV infections in vitro and in mice (Shukla, Ramasamy, et al. 2020). To date there is only limited data available on the impact of ZIKV infections in humans who have been previously infected with DENV. There has been no case reports of enhanced severity of ZIKV infections with prior DENV immunity (Katzelnick et al. 2020). A live attenuated DENV vaccine on the other hand is proven to enhance DENV infections in naive patients and therefore is only suited for people with previous DENV infections (Martinez-Vega et al. 2017; Thomas and Yoon 2019). This DENV vaccine has furthermore been proven to also enhance ZIKV infections (Shukla, Beesetti, et

al. 2020) and therefore highlights the importance of ADE consideration in vaccine design for flaviviruses.

## 6. Prevention and treatment

Since the epidemic of 2013/2014, tremendous efforts have been undertaken to study the entire complex around ZIKV. Great progress can be seen in the research of diagnostics, treatments, and vaccines, but nevertheless no licensed vaccine is available today (Wilder-Smith et al. 2018; Zhou et al. 2021). There are however multiple promising candidates of which ten have reached clinical phase I (Masmejan et al. 2018). The rapid decline in cases starting 2017 was a relief for global health but unexpectedly has complicated further vaccine development: fewer cases make safety and efficacy testing of vaccines in a clinical phase II setting difficult (Wilder-Smith et al. 2018; Vannice et al. 2019; Pattnaik, Sahoo, and Pattnaik 2020). Considering the importance of these assessments, alternative ways, including human infection models, are being investigated (Vannice et al. 2019). Two DNA-based vaccine candidates are currently under investigation in phase II clinical trial. They utilize ZIKV structural proteins prM and E like most vaccine approaches (Dowd et al. 2016; Gaudinski et al. 2018). In combination with data from different candidate vaccines, which utilize other platforms like mRNA technologies or recombinant viruses, protective capacity is sufficiently proven (Griffin et al. 2017; Bullard et al. 2018; Zhong et al. 2019; Richner et al. 2017).There are however some experimental vaccines which utilize different antigens such as the NS1 protein and report protective capacity in animal models (Brault et al. 2017). Apart from larger clinical trials, there is need for more detailed investigation in the field of fetal protection. First data suggest a protective extension from mother to fetus (Jagger et al. 2019; Hazlewood et al. 2020; Choi et al. 2021; Gambino et al. 2021; Hazlewood et al. 2022) but insight in correlates of protection and clinical data is badly needed.

The global search for a therapeutic is in a similar situation like the vaccine. There is no licensed preparation available (Musso and Gubler 2016; Masmejan et al. 2018), but promising candidates have been tested in various models (Masmejan et al. 2018; Bernatchez et al. 2020). Approaches utilize mechanisms like polymerase and protease inhibition to directly target the virus (Bernatchez

et al. 2020; Barrows et al. 2016) or to antagonize purine and pyrimidine synthesis (Baz and Boivin 2019). Nevertheless, these treatments only target symptoms of the infection and resemble treatment strategies used for other infectious diseases. In cases of GBS this means supportive intravenous immunoglobulins and plasma administration (WorldHealthOrganization 2016; Musso, Ko, and Baud 2019).

Children affected by CZS have a very low chance of survival even with intensive care (Adebanjo et al. 2017). The best approach is therefore frequent and extensive monitoring of pregnant women with suspected ZIKV infections. Regular monitoring by ultrasound (US) and close supervision from experts together with strong family support are the methods of choice during pregnancy (Musso and Gubler 2016; Adebanjo et al. 2017).

When combating ZIKV, the prevention of infections altogether is the most effective strategy. Since mosquito borne infections are by far the most common cause of infection, interventions like supplying insect repellents and mosquito nets to at-risk populations are recommended (Teixeira et al. 2016; Organization 2016). Screening of individuals at risk should be done with RT-PCR as the gold standard and serology as a supportive method (Magalhães et al. 2022). To prevent sexual transmission of ZIKV, protective intercourse is recommended for three months for men and two months for women after infection (WorldHealthOrganization 2016).

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

## 7.1 Taxonomy and History

MVA belongs to the vertebrate specific subfamily *Chordopoxviridae* of the *Poxviridae* family of viruses. The Genus *Orthopoxvirus* not only includes the ancestor of MVA, the Vaccinia Virus (VACV), but also the variola virus as the cause of human smallpox. This devastating infectious disease was globally eradicated through vaccination with different VACV strains (Bhattacharya 2008).

The VACV strain Ankara was initially used as a smallpox vaccine in Turkey, but several side effects including localized reactions, general vaccinia and potential deadly post vaccinal encephalitis compromised its safety (Mayr 2003). A different isolate of VACV was then obtained through passaging on chorioallantoic membranes of embryonated chicken eggs and thus named chorioallantois vaccinia virus Ankara (CVA) (Volz and Sutter 2017). This isolate was used as second generation smallpox vaccine in Germany from 1953 on, but showed secondary lesions in some cases after injection and therefore was also suspended in the use as a vaccine (Herrlich and Mayr 1957; Staib and Sutter 2003). Yet, the University of Munich continued to work with CVA in tissue cultures to study the host range biology of vaccinia virus (Mayr and Munz 1964). Through serial passaging in chicken embryo fibroblasts (CEF) the phenotype of CVA changed and, after over 516 passages, the new strain was named Modified Vaccinia virus Ankara (MVA) (Mayr and Stickl 1975).

Unlike CVA, MVA displays attenuated virulence and a replication deficiency in mammalian cells, while preserving infectivity and genetic stability (Mayr 1978; Carroll and Moss 1997; Drexler et al. 1998). After proving its potential as a smallpox vaccine candidate and no association of severe side effects (Stickl 1974), MVA was used as a smallpox vaccine in South Germany from 1968 to 1976 (Mayr and Danner 1978; Mahnel and Mayr 1994). Over 120.000 people, including immunocompromised and elderly people, were immunized by the end of 1976 and MVA received the approval as a pre-vaccine for smallpox in Germany in 1977 (Mayr and Danner 1978; Stickl 1974). After the worldwide

eradication of smallpox in 1977 the need for vaccines declined. Today MVA is licensed as a stand-alone third-generation vaccine against smallpox and monkeypox (EuropeanMedicinesAgency 2019; FDA 2021).

### 7.2 Structure and viral life cycle

Like other poxviruses MVA is a large enveloped DNA virus. Its structure consists of two lipid membranes, an outer membrane associated with tubules and an inner membrane. The core structure contains structural proteins, organized in an outer and a thinner inner layer with two flanking lateral bodies. The 178kb double stranded DNA genome is associated with nucleoproteins and located together with viral enzymes inside the core structure (Westwood et al. 1964). The genome itself ca be differentiated into the highly conserved central region containing genes for viral replication and the terminal regions with ORFs for host interaction (Moss 1996; Werden, Rahman, and McFadden 2008).

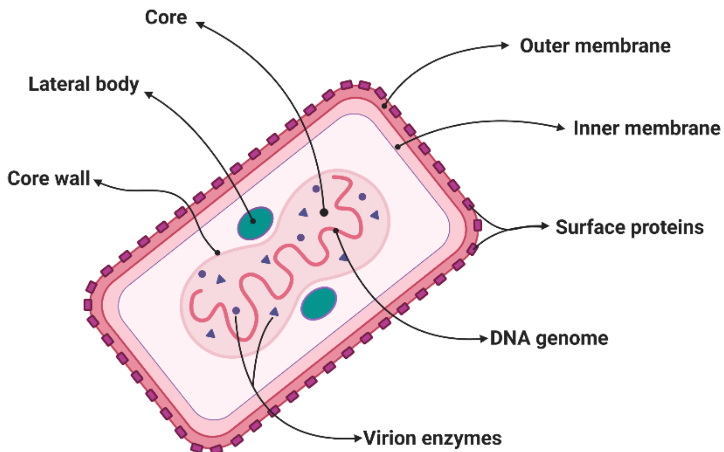


**Figure 3: Structure of MVA.** The envelope of MVA consist of an inner lipid membrane and an outer lipid membrane displaying surface proteins. The core structure is flanked by two lateral bodies and contains the DNA genome and viral enzymes inside a double protein layer. Created with BioRender.com

The viral life cycle of MVA starts with attachment to the cell followed by cell entry through endocytosis or fusion with the plasma membrane (Law et al. 2006; Moss 2006). The following transcription, replication and assembly takes place in the cytoplasm and is guided by a cascade like expression of genes (Moss 1996; Broyles 2003). The transcription of early genes is assisted by proteins from the viral core and results in the production of proteins vital for the transcription of intermediate and late genes and the release of viral DNA into the cytoplasm (Moss and Earl 2002). Subsequently DNA replication and the translation of intermediate and late proteins start. Structural proteins and early transcription factors are formed and assembly begins (Broyles 2003). The viral particles are then transported to the Golgi apparatus and wrapped. Finally the mature virions are released from the cell by fusion with the plasma membrane (Payne 1980; Ward and Moss 2001).

### 7.3 Properties as a vaccine vector

MVA exhibits a multitude of properties that makes it suitable as a platform for vector vaccines. As a poxvirus it allows the insertion of large foreign DNA sequences which results in a stable expression of diverse target proteins (Antoine et al. 1998). The expression is not only limited to the cytoplasm, but also independent from the host cells and strictly under the control of the viral transcription system (Sutter and Moss 1992). Although no infectious particles are being produced by MVA, all viral genes are expressed and allow an unrestricted production of viral and recombinant proteins. The ability to infect mammalian cells paired with replication deficiency can be explained by the passaging on CEF resulting in many mutations and the loss of approximately 15 % genomic information when compared to CAV (Meyer, Sutter, and Mayr 1991). The genetic stability of the MVA genome remains unaffected by these genetic changes and allows for large scale production of recombinant viruses as proven by the use as investigational drug product in different clinical studies (Cosma et al. 2003; Meyer et al. 2005; Kreijtz et al. 2014; Koch et al. 2020). Lastly MVA is easy and efficient to work with. Through decade long use sufficient methods and protocols that rely on standard technics are widely available (Kremer et al. 2012).

### 7.4 Immunogenic properties as a Vaccine

The characteristics of MVA to induce cellular and humoral immune responses while being replication-deficient in mammalian cells is a valuable feature for a vaccine platform. Poxviruses in general have a variety of features which modulate host immune responses and ultimately help them evade immune responses and facilitate their replication. Different genes of VACV target the innate immune system through immunomodulatory proteins expressed early in the viral infection (Smith et al. 2013). MVA lacks multiple of these genes, which enables it to activate the innate immune response early on (Altenburg et al. 2014). Poxviruses inhibit the apoptosis of infected cells and block different cytokine productions like interferon regulatory factor 3, nuclear factor κB and the IFN pathway (Seet et al. 2003; Haga and Bowie 2005; Soday et al. 2019). Because MVA doesn't express many of these inhibitors, the signaling pathway can take place and stimulate the immune response (Hinthong, Jin, and Shisler 2008). In addition to interfering with cytokine production, poxviruses are also able to inhibit the molecules directly. Secretion of viral receptors leads to binding of cytokines and chemokines (Alcamí and Smith 1992; Symons, Alcamí, and Smith 1995; Smith et al. 2013). Similar to the other immune evasion factors, MVA lacks the ability to produce receptors targeted against IFNs and TNF (Blanchard et al. 1998). Additionally to MVAs lack in immune evasion properties it can further stimulate an immune responses. Triggering chemokine production and activation of the complement system facilitates both the migration and the function of monocytes and leukocytes (Lehmann et al. 2009; Lehmann et al. 2015).

### 7.5 Vaccines based on MVA against infectious diseases

Infectious diseases are a threat to public health and constantly require attention and new strategies to combat them. Vaccines have always played an important part in this. Different diseases have been combated successfully in the last century with the help of vaccines. Most notably the eradication of smallpox was achieved by a global vaccination program (Simonsen and Snowden 2022). Polio is another pathogen, that is on the brink of eradication due to vaccines (Thomas et al. 2022). The recent and still ongoing pandemic caused by SARS-CoV-2 has once again proven the devastating effect a pathogen can have. Not only does it pose a threat to human life, but it has also inflicted immeasurable economic

and social damage. The development of a safe and effective vaccine is essential and can only be achieved with capable vaccine platforms and technologies. MVA has been utilized as a vector for a multitude of different pathogens with many vaccine candidates reaching preclinical and clinical trials (Gilbert 2013; Gómez et al. 2013).

The first vaccine based on recombinant MVA targeted influenza and delivered the HA and the NP antigen (MVA-HA-NP) (Sutter et al. 1994). Immunization with MVA-HA-NP induced specific antibodies and CD8+ T cells in mice, and was able to deliver protection against a lethal influenza challenge (Sutter et al. 1994). Similarly protective efficacy against an influenza virus challenge was shown in different animals, such as ponies (Breathnach et al. 2006). Considering the high zoonotic potential of influenza, especially the N5 subtype, a vaccine targeting this antigen was a valuable approach (Yang et al. 2015). Recombinant MVA delivering HA antigens was indeed able to induce protection against a challenge infection in mice and macaques (Kreijtz et al. 2007; Kreijtz, Suezer, de Mutsert, van den Brand, et al. 2009). The protection also extended to a different clade of influenza (Kreijtz, Suezer, de Mutsert, van Amerongen, et al. 2009). On the basis of this data, a clinical study with recombinant MVA delivering HA antigens was conducted. The vaccine showed no severe side effects and was able to induce promising antibody response in healthy adults (Kreijtz et al. 2014; de Vries et al. 2015). A different approach focused on the induction of T cells by utilizing the Matrix 1 and NP antigens of influenza also showed promising results. The vaccine based on recombinant MVA successfully induced CD8+ T cells in a clinical trial (Berthoud et al. 2011; Lillie et al. 2012). Even more important the vaccine was proven to be safe and immunogenic in adults over 50, the major target group of an influenza vaccine (Antrobus et al. 2012).

In addition to influenza, MVA has been used to develop candidate vaccines against HIV and malaria (Cosma et al. 2003; Webster et al. 2006). All clinical trials showed no severe adverse effects and proved the value of MVA in vaccine development. Established vaccine platforms are needed to combat newly emerging infections. This was once again demonstrated in recent years by the viruses of the *coronaviridae* family. With the emergence of the Middle East respiratory syndrome coronavirus (MERS-CoV) different approaches for

vaccine development started. The candidate vaccine MVA-MERS-S was immunogenic and demonstrated protective efficacy in preclinical animal models (Song et al. 2013; Haagmans et al. 2016). In a Phase I clinical trial, the vaccine was shown to be safe and immunogenic in humans (Koch et al. 2020). The emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was combated once again with reliable and established vaccine platforms. Vaccines based on MVA were developed quickly and preclinical evaluation showed immunogenicity in different animal models (Tscherne et al. 2021; García-Arriaza et al. 2021; Chiuppesi et al. 2020). Further evaluation showed protective capacity in animal challenge models including transgenic mice, Syrian hamsters and non-human primates (Liu et al. 2021; Routhu et al. 2021; Kulkarni et al. 2021; Chiuppesi et al. 2022). Additionally to the more standard i.m. route of immunization, Bošnjak et al. studied intranasal delivery as an alternative way to induce pulmonary immunity (Bošnjak et al. 2021).

# IV. OBJECTIVES

ZIKV is a threat to public health and holds the potential for newly emerging epidemics due to the lack of approved vaccines and therapeutics. Considering the need for insight in the interplay between vaccines and fetuses, this work describes:

1. **Generation of a recombinant MVA vaccine delivering the ZIKV proteins prM and E (MVA-prME)**

2. **In vitro characterization of the recombinant MVA-prME vaccine**

   - genetic analysis
   - analysis of protein expression
   - analysis of replication capacity

3. **In vivo characterization of the recombinant MVA-prME vaccine in pregnant IFNAR -/- mice**

   - protective capacity in dams and fetuses after challenge infection
   - analysis of humoral and cellular immune response

# V. MATERIAL AND METHODS

## 1. Materials

### 1.1. Antibodies

**Table 1: Primary antibodies used for Western Blot**

| Antibody | Specifications | Dilution | Supplier |
| --- | --- | --- | --- |
| ZIKV prM | Polyclonal, Rabbit | 1:1000 | GeneTex, Irvine, USA |
| ZIKV E | Monoclonal, Mouse | 1:2000 | GeneTex, Irvine, USA |

**Table 2: Secondary Antibodies used for Western Blot**

| Antibody | Specifications | Dilution | Supplier |
| --- | --- | --- | --- |
| Anti-rabbit IgG | Polyclonal, HRP, Goat | 1:5000 | Cell signaling Technology, Leiden, The Netherlands |
| Anti-mouse IgG | Polyclonal, HRP, Goat | 1:5000 | Agilent, Waldbronn, Germany |

**Table 3: Primary Antibodies used for Immunostaining**

| Antibody | Specifications | Dilution | Supplier |
| --- | --- | --- | --- |
| Anti ZIKV E | Monoclonal, Mouse | 1:1000 | GeneTex, Irvine, USA |

**Table 4: Secondary Antibodies used for Immunostaining**

| Antibody | Specifications | Dilution | Supplier |
| --- | --- | --- | --- |
| Anti-mouse | Fluorescent (Alexa), Goat | 1:1000 | Life Technologies, Darmstadt, Germany |

**Table 5: Antibodies used for ELISA**

| Antibody | Specifications | Dilution | Supplier |
| --- | --- | --- | --- |
| Anti-mouse IgG | Monoclonal, Rabbit | 1:1000 | Agilent Dako, Glostrup, Denmark |

**Table 6: Antibodies used for Histochemistry**

| Antibody | Specifications | Dilution | Supplier |
| --- | --- | --- | --- |
| Anti-Flavi-E | Rabbit | 1:1000 | Abcam, Oxford, UK |
| Anti-Rabbit | Goat | 1:200 | Burlingame, CA, USA |

## 1.2. Oligonucleotides

**Table 7: Oligonucleotide primers used for PCR**

| Name | Sequence 5´ → 3´ |
|---|---|
| Del I forw. | CTTCGCAGCATAAGTAGTATGTC |
| Del I rev. | CATTACCGCTTCATTCTTATATTC |
| Del II forw. | GGGTAAAATTGTAGCATCATATACC |
| Del II rev. | AAAGCTTTCTCTCTAGCAAAGATG |
| Del III forw. | GATGAGTGTAGATGCTGTTATTTTG |
| Del III rev. | GCAGCTAAAAGAATAATGGAATTG |
| Del IV forw. | AGATAGTGGAAGATACAACTGTTACG |
| Del IV rev. | TCTCTATCGGTGAGATACAAATACC |
| Del V forw. | CGTGTATAACATCTTTGATAGAATCAG |
| Del V rev. | AACATAGCGGTGTACTAATTGATTT |
| Del VI forw. | CTACAGGTTCTGGTTCTTTATCCT |
| Del VI rev. | CACGGTCAATTAACTATAGCTCTG |
| Deletion 3 forw. | GTACCGGCATCTCTAGCAGT |
| Deletion 3 rev. | TGACGAGGTTCCGAGTTCC |
| C7L forw. | CATGGACTCATAATCTCTATAC |
| C7L rev. | ATGGGTATACAGCACGAATTC |
| Insert 1 forw. | GCCATTCTCTTGGCACCTCT |
| Insert 1 rev. | TTCCATTACCTTGGCACGCT |
| Insert 2 forw. | ATGTCACCAGGCTCCCTTTG |

| Insert 2 rev. | GTGTACGGAACCTGCCATCA |
|---|---|

## 1.3. Peptides

**Table 8: Peptides used for ELISpot**

| **Peptide Name** | **Sequence** |
|---|---|
| E294 | IGVSNRDFV |
| E646 | GRLITANPVITESTE |

## 1.4. Plasmids

**Table 9: Plasmids used to generate MVA-prME**

| **Plasmid** | **Experiment** | **Supplier** |
|---|---|---|
| pUC57-ZIKV-prME | cloning | Genewiz, Leipzig, Germany |
| pIIIH5red | cloning | Gerd Sutter, LMU |
| pIIIH5red-ZIKV-prME | cloning | |

## 1.5. Bacterial strains

**Table 10: Bacteria used to generate MVA-prME**

| **Plasmid** | **Experiment** | **Supplier** |
|---|---|---|
| NEB 10 beta bacteria (*Escherichia coli*) | amplification plasmid DNA | Gen New England Biolabs, Frankfurt, Germany |

# 2. Methods

## 2.1. Cell culture

### 2.1.1. Cells

Primary chicken embryo fibroblasts (CEF) were attained from ten-day-old chicken embryos (SPF eggs, VALO, Cuxhaven, Germany) and maintained in Minimum Essential Medium (MEM) (SIGMA-ALDRICH, Taufkirchen, Germany) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (SIGMA-ALDRICH, Taufkirchen, Germany) and 1 % MEM non-essential amino acid solution (SIGMA-ALDRICH, Taufkirchen, Germany).

Vero cells (African green monkey kidney; ATCC CCL-81) were cultured in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 5 % heat-inactivated FBS and 1 % MEM non-essential amino acid solution (SIGMA-ALDRICH, Taufkirchen, Germany). Human HaCat cells (CLS Cell Lines Service GmbH, Eppelheim, Germany) were maintained in DMEM with 10 % FBS, 2 % HEPES-solution and 1 % MEM non-essential amino acid solution (SIGMA-ALDRICH, Taufkirchen, Germany). All cells were cultured at 37°C and with 5 % CO2 atmosphere.

### 2.1.2. Passaging and counting of cells

After removing the medium for cell cultivation, cells were washed with phosphate buffered saline and then trypsinized for 5-15 min at 37°C. The enzymatic reaction was stopped by adding 5-10ml of cell culture medium and the resulting cell suspension was used for new culture flasks or counted and plated for following experiments. Cells were counted in a Neubauer Chamber after mixing the cell suspension 1:10 with a Trypan blue solution containing 0.2 % sodium azide and 0.1 % trypan blue.

## 2.2. Molecular biology

### 2.2.1. Polymerase chain reaction (PCR)

Specific sections of plasmid or viral DNA were amplified using PCR and corresponding oligonucleotides (see Table 7: Oligonucleotides). The following composition was used per sample.

**Table 11: PCR Master mix**

| | **µl per sample** |
|---|---|
| ddH2O | 14 |
| Polymerase buffer | 2,5 |
| MgCL2 (50 mM) | 0,75 |
| dNTP-mix (2,5 mM) | 1,5 |
| Forward primer (10µM) | 1 |
| Reverse primer (10µM) | 1 |
| Taq-polymerase | 0,2 |
| Template | 5 |

**Table 12: Cycle protocol for Deletion and C7L PCRs**

| **Step** | | **Temperature** | **Time** |
|---|---|---|---|
| Denaturation | | 94°C | 5 min |
| 30 Cycles | Denaturation | 94°C | 30 sec |
| | Annealing | 57°C | 45 sec |
| | Extension | 72°C | 45 sec |

| Extension | | 72°C | 5 min |
| --- | --- | --- | --- |
| Storage | | 4°C | - |

**Table 13: Cycle protocol for Insert PCRs**

| Step | | Temperature | Time |
| --- | --- | --- | --- |
| Denaturation | | 94°C | 5 min |
| 30 Cycles | Denaturation | 94°C | 30 sec |
| | Annealing | 54°C | 45 sec |
| | Extension | 72°C | 45 sec |
| Extension | | 72°C | 5 min |
| Storage | | 4°C | - |

### 2.2.2. Gel electrophoresis

PCR products were fractionized according to their size by Gel electrophoresis. A 1 % Agarose gel with Gel RedTM was prepared. 5x DNA loading dye was added to the samples and the mix loaded onto a gel with a marker (1kD). Gels were run in a TAE buffer at 100 V for 90 min and then analyzed with a ChemiDoc Imaging System (Bio-Rad, Munich, Germany).

## 2.3. Generation of recombinant virus

### 2.3.1. Generation of rMVA

To generate recombinant MVA, a well-established protocol (Kremer et al 2012) was followed. CEF cells were plated 90-95 % confluent on 6-well tissue culture plates (Sarstedt, Nürnbrecht, Germany) and infected with non-recombinant MVA at a low multiplicity of infection (MOI) of 0.05. After 45 min the cells were transfected with 1 µg of pIIIH5red-ZIKV-prME plasmid DNA using XtremeGENE DNA Transfection Reagent Lipofectamine (Roche Diagnostics, Penzberg, Germany). Cells were harvested after 48 h incubation at 37 °C. To isolate recombinant MVA virus, serial rounds of plaque purification were screened for transient co-expression of the red fluorescent marker mCherry on CEF cell monolayers.

### 2.3.2. Plasmid construction

In silico modified cDNA encoding the amino acid (aa) sequences of ZIKV-prM and ZIKV structural protein E (Zika virus isolate Yap 2007, GenBank accession no. EU545988.1) was used. Silent codon replacements were used to remove G/C nucleotide runs and two termination signals (TTTTTNT) for vaccinia virus early transcription. The modified cDNA was then synthesized (Genewiz, Leipzig, Germany) and inserted by cloning into the transfer plasmid pIIIH5red for MVA (Kremer et al. 2012). Transcription was controlled by using the vaccinia virus early/late promoter PmH5 (Wyatt et al. 1996) and thus creating the MVA vector plasmids pIIIH5red-ZIKV-prME (Figure 4).

### 2.3.3. Virus amplification and purification

Recombinant MVA were amplified in CEF cells grown in T175 tissue culture flasks. Infected cells were incubated for 3-4 days at 37 °C and then frozen at -20 °C. The resulting cell-virus suspension was then collected, freeze-thawed three times and centrifuged three hours at 15,000 rpm and 4 °C (Avanti J-26XP, Beckman Coulter). The generated pellets were then collected and resuspended in 35 ml buffer (19 mMTris-HCL, pH9.0) and sonicated four times for 15 seconds. After this sonication, the suspension was centrifuged for five minutes at 1,200 rpm, the supernatant was collected and the remaining pellet was resuspended in Tris-buffer. This process was repeated four to five times or until

the pellet was completely dissolved. In the last step for purification the virus containing supernatant was centrifuged through a 36 % sucrose gradient at 15,000 rpm for 90 min at 4 °C (OptimaTM LE-80K Ultracentrifuge, Beckman Coulter). The generated pellets were resuspended in Tris-buffer for the final virus stock.

### 2.3.4. Virus handling

Recombinant and wild-type MVA were stored at -80 °C in 300 µl aliquots in cryo-tubes. Before experimental use virus stocks were slowly thawed on ice and then sonicated three times for one minute in an ice-bath.

### 2.3.5. Virus titer determination as plaque forming units (PFU)

Plaque-forming units were determined by virus titration. For this purpose, viruses were diluted in 10-fold dilutions steps. The dilutions from 10^4 to 10^9 were used to infect 90-95 % confluent CEF cells in 6-well tissue culture plates. Each infection was prepared in triplets. Infected cells were incubated at 37 °C for 48 h and subsequently fixed with acetone-methanol (1:1) for 4 min. The fixated cells were blocked with PBS containing 3 % FBS for 1 h. The primary antibody (anti-Vaccinia virus) and the secondary antibody (HRP conjugated goat anti-mouse) were both also diluted in PBS mixed with 3 % FBS. Plates were incubated at RT for 1 h and washed two times with PBS after each incubation step. For staining plates were incubated with TrueBlue™ Peroxidase Substrate on a rocking platform shaker. Titers were then expressed by counting the stained spots.

## 2.4. Biochemistry

### 2.4.1. Western Blot analysis of recombinant proteins

To generate recombinant protein, vero-cells were seeded in 6-well tissue culture plates and, after reaching 90-95 % confluence, infected with recombinant MVA-prME or MVA wild-type. Uninfected cells were used as a control. A high multiplicity of infection (MOI) of 5 was used and plates were incubated at 37 °C. At indicated time points cells and medium were harvested. To generate lysates the samples were centrifuged at 13,000 rpm for 1,5 min and the generated supernatant was discarded. The cell pellet was washed with chilled PBS before being reconstituted in lysis buffer. The suspension was incubated for 20 min on Ice, thoroughly vortexed and incubated once more for 20 min. After 12 min centrifugation at 13,000 and 4 °C the pellets were discarded and only the supernatants collected and stored at -80 °C until further experiments.

Investigation of the protein samples was done by Western Blot analysis. The samples were prepared by mixing them with reducing agent containing mercaptoethanol and boiling them at 95 °C for 5 min. Together with a standard protein marker (Pageruler protein ladder, Thermo Fisher Scientific, Planegg, Germany) the samples were loaded on a sodium dodecyl sulfate (SDS) Gel and separated by electrophoresis. After 90 min at 100 V the proteins were transferred onto a nitrocellulose membrane using 100 V for 30 to 90 min depending on the protein size. Afterwards the membrane was blocked in a PBS buffer with 5 % non-fat dried milk and 0,05 % Tween (SIGMA-ALDRICH, Taufkirchen, Germany) for 1 h. Primary antibodies were diluted in the same buffer and incubated with the membranes over night at 4 °C on a rotator. For detection of ZIKV-M a polyclonal rabbit anti-prM antibody (1:1000, GeneTex, Irvin, USA) and for detection of ZIKV-E a monoclonal anti E antibody (1:2000, GeneTex, Irvin, USA) was used. After incubation with the primary antibodies the membrane was washed three times for respectively 3 min with PBS containing 0,05 % Tween and then incubated with the secondary antibody (anti-mouse or anti-rabbit IgG; Cell Signaling Technology, Leiden, The Netherlands; 1:5000) for 1h at RT. A last washing step was performed and afterwards the membrane was developed with SuperSignal West Dura Extended Duration substrate (Thermo Fisher Scientific, Planegg, Germany). The signal was detected by

using chemiluminescent western blot imaging system (Image Lab, Bio-Rad, Munich, Germany).

### 2.4.2. Immunostaining

Vero cells were infected with recombinant MVA-prME at a MOI of 0.05 and incubated for 24 h at 37 °C. Cells were then fixed with 4 % paraformaldehyde for 8 min and then washed with PBS. Afterwards cells were permeabilized with 0,1 % Triton X-100 (SIGMA-ALDRICH, Taufkirchen, Germany) and incubated with a monoclonal mouse antibody against ZIKV-E (1:1000, GeneTex, Irvine, USA). To detect the first antibody, a fluorescent (Alexa) polyclonal rabbit anti-mouse antibody (Life technologies, Darmstadt, Germany) was used, and nuclei were stained with a solution of 4,6-diamidino-2-phenylindole (DAPI, SIGMA-ALDRICH, Taufkirchen, Germany). Cells were then analyzed with a fluorescence microscope (Keyence BZ-X700, Keyence, Neu-Isenburg, Germany).

## 2.5. Zika virus

### 2.5.1. Virus handling

For all assays ZIKV Isolate HPF/2013 (EVAg, clinical isolate, French Polynesia 2013, GenBank Sequence Accession: KJ776791) was used. The virus was amplified on Vero cells and stored at -80 °C.

Due to the fragility of RNA viruses, stocks were thawed slowly on ice and not sonicated.

### 2.5.2. Virus titration

Virus titres were determined by infecting Vero cells with serial dilutions of the sample, ranging from 10^3 to 10^8. After 1 h adsorption at 37 °C and 5 % CO2 the infection medium was discarded, plates were washed with PBS and the wells then overlayed with a solution of DMEM and carboxymethyl cellulose sodium salt (CMC). After 4 days of incubation at 37 °C and 5 % CO2 the CMC-DMEM overlay was removed and wells were washed thoroughly with PBS. Crystal violet solution was added and after 5 min plates were washed again until plaque staining was visible.

### 2.5.3. Plaque Reduction Neutralizing Assay

Sera of mice were taken 4 days and 11 days after immunization (= 2- and 9-days post challenge (dpc)) and examined by plaque reduction neutralization test (PRNT) for ZIKV neutralizing antibodies. The sera were heat inactivated at 56 °C for 30 min, diluted in 96-well plates and then mixed with 200 PFU of ZIKV. The mixture was incubated for 2 h at 37 °C and then used to infect 85 % confluent Vero cells in 24 well cell culture plates. The plates were incubated for 1 h, then overlayed with 2 % CMC diluted in demineralized water and incubated for another 48 h at 37 °C. For Staining the overlay was removed and cristal violet was added.

## 2.6. In vivo experiments

### 2.6.1. Mice

IFNAR-/- mice with a type I IFN deficiency (Müller et al. 1994) were backcrossed more than 20 times with C57BL/6 mice. Mice were housed and bred in isolated cage units (IsoCage, Tecniplast, Hohenpeißenberg, Germany) and had free access to water and food. All experiments were approved by the Government of Upper Bavaria, Munich and were carried through in abidance with the German Animal Welfare Act.

### 2.6.2. Study design

Female 6–10-week-old IFNAR -/- mice were paired one on one with males for 24 h for mating. Mice were then checked for a genital plug to confirm successful mating and positive females were separated. Mice were examined via ultrasound four days later to check for pregnancy (Forster et al. 2020). Pregnant mice (n=3-5) were then vaccinated on the same day via intramuscular (i.m.) injection into the left hind leg with 100 µl of virus suspension or PBS. A dose of 10^8 PFU of recombinant MVA was used while MVA wild-type and PBS served as control. 2 days after the immunization, mice were infected with 10^3 PFU of ZIKA virus (H/PF/2013; EVAg, clinical isolate, French Polynesia 2013, GenBank Sequence Accession: KJ776791) in 50 µl physiological saline via footpad injection. Control mice received 50 µl PBS. Mice were checked daily for survival and clinical condition as well as weight loss.

**Table 14: Clinical score sheet for ZIKV infection**

| | | |
|---|---|---|
| **1. General condition** | shiny, smooth fur | 0 points |
| | normal posture | |
| | clear, clean eyes | |
| | good nutritional status | |
| | | |
| | slightly dull fur | 5 points each |
| | eyes moderately glued | |
| | squatting position | |
| | reduced nutritional status on palpation | 10 points each |
| | weight loss up to 20% starting weight | |
| | ruffled fur | |
| | sticky, closed eyes | |

| | | |
|---|---|---|
| | emaciation visually visible | 15 points each |
| | prone position | |
| | weight loss> 20% starting weight | |
| 2. **Behavior** | curious | 0 points |
| | attentive | |
| | Interested | |
| | reaction to external stimulus | |
| | normal social behavior | |
| | | |
| | unusual behavior | 5 points each |
| | hyperactive, nervous | |
| | afraid, hidden in little house | |
| | reduced, slowed response to external stimuli | |
| | | |
| | behavior caused by illness (coordination disorders, reflexes) | 10 points |
| | apathy / lethargy | 15 points |
| 3. **ZIKV clinic** | reduced motor skills | 5 points each |
| | reduced reflexes | |
| | tiptoe | |
| | | |
| | hindquarters weakness / hindquarters lameness ; signs of paralysis on one of the two hind legs | 10 points each |
| | | 15 points each |
| | pronounced paralysis of the hind limbs | |
| | tremor or staggering | |

| **rating** | **With total points** |
|---|---|
| Score 0 | 0 |
| Score 1 | 5-10 |
| Score 2 | 10-15 |
| Score 3 | 15+ |
| Score 4 | moribund or dead |

11 days after immunization (= 9 days after ZIKV infection) mice were euthanized

and fetuses and maternal organs (placenta, ovaries, spinal cord, and brain) were harvested and measured (size and weight). The study was performed in two experiments and conducted in a biosafety level 2 laboratory.

### 2.6.3. Determination of ZIKV organ loads

Preparation of organ samples and determination of ZIKV Loads was conducted as described earlier (Forster et al. 2020). In short, organ samples were harvested and put in 2 ml tubes (Sarstedt, Nürnbrecht, Germany) containing 300 µl PBS and penicillin-streptomycin (1:5; SIGMA-ALDRICH, Taufkirchen, Germany). After weighting the samples were homogenized with a tissue lyser (Retch Tissue Lyser MM300, Qiagon GmbH, Hilden, Germany) and then centrifuged at 1500rpm and 4 °C for 1 min. Supernatants were then used for determining ZIKV organ loads or stored at –80 °C for later investigation. Titers were determined by titration as described before (Agbulos et al. 2016) and expressed in PFU per gram of the original organ material.

### 2.6.4. Enzyme-linked immune sorbent assay (ELISA)

ZIKV-E specific IgG titers were analyzed by Enzyme-linked immune sorbent assay (ELISA). To investigate the antigen-specific IgG response of immunized mice before and after ZIKV infection, sera of mice were taken 4 days and 11 days after immunization. 96-well ELISA plates (Nunc™ MaxiSorp™ Plates, Thermo Scientific) were coated with 1ng/well recombinant ZIKV-E protein (abeomics San Diego, CA USA) over night at 4 °C. Plates were then washed 3 times with PBS-Tween (0,1%) and blocked for 1 h at 37 °C with 200µl/well PBS + 1 % bovine serum albumin (BSA; SIGMA-ALDRICH, Taufkirchen, Germany) + 0.15 M sucrose (SIGMA-ALDRICH, Taufkirchen, Germany). Plates were then washed again 3 times with PBS-Tween and subsequently incubated with the samples. Sera were prepared by serial dilution in PBS + 1 % BSA starting at 1:30. After 1 h and three washing steps, the first antibody, goat anti-mouse IgG conjugated HRP (Agilent Dako, Denmark; 1:2000 in PBS/BSA), was incubated for 1 h at 37 °C. A last series of 3 washing steps was performed, before 100 µl substrate for developing was added (3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA; SIGMA-ALDRICH, Taufkirchen, Germany). Plates were incubated at room temperature until color change was visible. The

reaction was then stopped by adding 100 µl stop reagent (450 nm, SIGMA-ALDRICH, Taufkirchen, Germany). An ELISA plate reader (Tecan, Sunrise reader, Männedorf, Switzerland) was used for readout of the absorbance at 450 nm with a reference wavelength of 620 nm.

#### 2.6.5. Enzyme-linked immunosorbent Spot assay (ELISpot)

IFN-γ-producing T cells were measured by performing ELISpot assay as described before (Veit et al. 2018). At day 11 after immunization mice were sacrificed and spleens were removed to harvest splenocytes. After singularizing splenocytes with a mesh (70 µm) and Red Blood Cell Lysis Buffer the cells were resuspended in RPMI 1640 medium with 10 % FCS, 1 % penicillin/streptomycin and 1 % HEPES (SIGMA-ALDRICH, Taufkirchen, Germany). The IFN-γ ELISpotPLUS kit (Mabtech, Nacka Strand, Sweden) was then used as described in instructions provided by the manufacturer.

Briefly 2x10^5 splenocytes were seeded into the 96-well plates (Sarstedt, Nürnbrecht, Germany) and peptides with a concentration of 2 µg/ml were added. The vaccinia virus T cell epitope B8R20-27 (Tscharke et al. 2005) served as positive control and phorbol myristate acetate (PMA) in combination with ionomycin (SIGMA-ALDRICH, Taufkirchen, Germany) was used as stimulation control. The mixture of splenocytes and peptides was then transferred onto plates precoated with IFN-γ antibody and incubated for 48 h at 37 °C. Staining was then performed according to the manual and counting of spots was done with an automated ELISpot plate reader (A. EL. VIS Eli.Scan and A. EL. VIS ELISPOT Analysis Software, Hannover, Germany).

#### 2.6.6. Histopathology

Organ sections of euthanized mice were fixed in paraformaldehyde for 24 h and afterwards embedded in paraffin. Slices with a thickness of 4 µm were cut and used for immunohistochemistry. The paraffin was removed and the section slices were then blocked with hydrogen peroxide. For staining a rabbit anti Flavi-E antibody (1:1000, Oxford, UK) was incubated for 60 min at RT. Subsequently a biotinylated goat anti-rabbit antibody (1:200, Burlingam, CA USA) was used as the secondary antibody ant incubated for 60 min. In the last step Peroxidase-complexed avidin biotin (ABC-HRP, Vector, PK-6100) and diaminobenzidine

(DAB) was used together with hemalaun for final imaging. The Keyence BZ-X700 microscope (Keyence, Neu-Isenburg, Germany) was used to take images.

### 2.6.7. Statistical analysis

Statistical analysis was performed with GraphPad prism 5.0 (GraphPad Sodtware Inc., San Diego, CA, USA) and data was expressed as mean ± standard error of the mean (SEM). One-way ANOVA and Kruskal-Wallis Test was used to compare four groups. P values for statistically significance was set at 0.05.

# VI. RESULTS

## 1. Construction of recombinant MVA expressing the prM and E protein of ZIKV

### 1.1. Construction of recombinant MVA-prME

The recombinant MVA-prME was generated via infection of chicken embryo fibroblasts (CEF) with MVA and subsequent transfection with the vector plasmid pIIIH5red-prME. The encoding sequences of the precursor M and E protein of ZIKV (GenBank accession no. EU545988.1, ZIKV isolate Yap 2007) were optimized on genomic level and put under the transcriptional control of the early/late Vaccinia virus promotor PmH5 (Wyatt et al. 1996) in the vector plasmid. Furthermore, the plasmid contained the reporter gene for mCherry and flank regions (see Figure 4).

The encoding sequences were inserted into the deletion III site of MVA by homologous recombination and the reporter gene mCherry was then used for detection of recombinant MVA-prME and plaque purification. Intragenomic homologous recombination was utilized for deletion of the marker gene and to generate the recombinant virus MVA-prME.

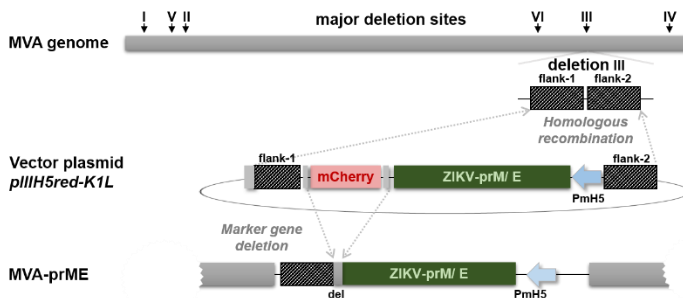


**Figure 4: Generation of recombinant MVA-prME.** Major deletion sites are indicated as I-VI and surrounding flanks are shown as black boxes. The encoding sequence of ZIKV proteins prM and E were inserted into the deletion site III and put under the control of the vaccinia virus early/late promotor PmH5 as shown. Deletion of the Marker gene mCherry was done by intragenomic homologous recombination.

## 2. Characterization of recombinant MVA-prME

### 2.1. Genetic characterization

The genetic characterization was done by standard quality control as described previously (Kremer et al. 2012). We isolated viral DNA and analyzed it by PCR for correct insertion and genetic identity of the inserted ZIKV sequences. By amplifying the deletion lll site of MVA we could confirm the correct length and insertion of the sequences in the recombinant MVA-prME. The comparison of MVA-prME with the vector plllH5red-prME also showed that the marker gene mCherry was removed completely. To confirm the genomic integrity and stability, we also analyzed the deletion sites I to VI. Additionally we tested the gene region C7L, which translates a protein of Vaccinia viruses with regulatory functions during the infection of mammalian cells (Meyer, Sutter, and Mayr 1991; Nájera et al. 2006; Backes et al. 2010). The PCR showed no difference between MVA and the recombinant MVA-prME (Figure 5).

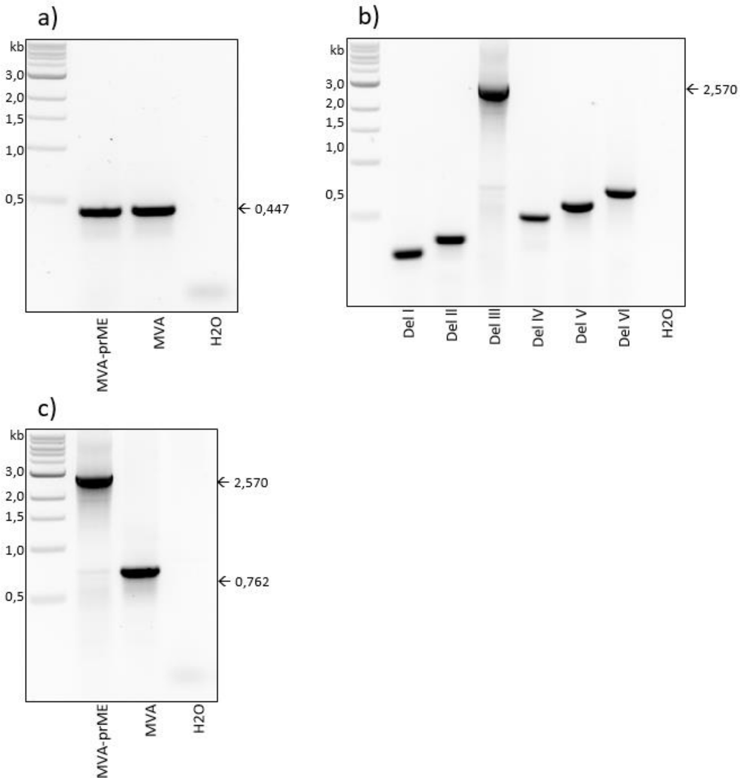


**Figure 5: Genetic stability of MVA-prME (a-c):** Isolated viral DNA was analyzed for genetic integrity of the C7L gene locus **(a)** and the six major deletion sites of MVA **(b)** Correct insertion was demonstrated by specific PCR for the deletion site lll **(c)**.

## 2.2. Growth kinetics of recombinant MVA-prME on permissive and non-permissive cell lines

The next element in our quality control was the assessment of replication properties and the growth characteristics of the recombinant virus on different cell lines. We infected CEF cells, which are routinely used to amplify MVA, and mammalian HeLa cells with the recombinant MVA-prME and non-recombinant MVA. We then determined the virus titer after indicated time points. (Figure 6).

The recombinant MVA-prME showed replication capacity in CEF cells and no observable replication in HeLa cells. In both cell lines titers were comparable to the non-recombinant MVA, showing that the recombinant MVA-prME maintained the characteristics of wild type MVA which are the ability to replicate in avian cells and replication deficiency in mammalian cells.

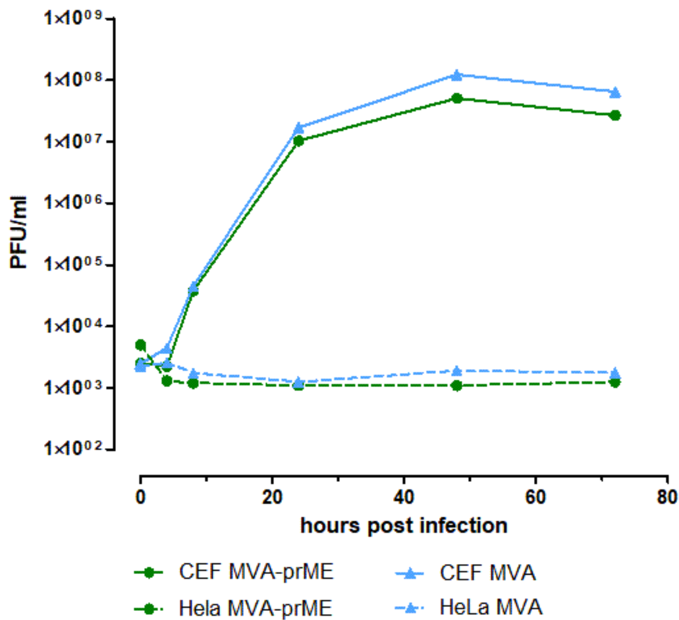


**Figure 6: Multiple-step growth analysis of recombinant MVA-prME.** MVA-prME and non-recombinant MVA show permissive growth in cells of avian origin (CEF) but fail to replicate in cells of mammalian origin (HeLa).

## 2.3. Protein expression of recombinant MVA-prME

For evaluation of protein expression of recombinant MVA-prME we infected Vero cells and analyzed cell lysates collected at different time points by Western Blot analysis. After separating proteins by SDS-PAGE we detected a ~49 kDa protein using a polyclonal antibody directed against the E protein of ZIKV. The

earliest detection was observed after eight hours and the intensity of the band increased over time. This can be expected due to the expression of proteins that was controlled by the early/late transcription promotor PmH5 (Figure 7). Similarly a 19 kDa protein could be detected with a polyclonal antibody directed against the prM protein of ZIKV (Figure 7).

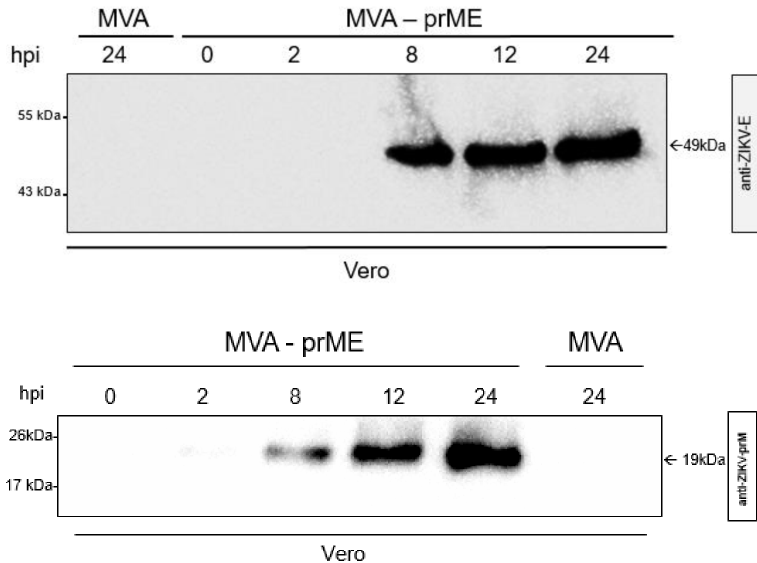


**Figure 7: Analysis of recombinant ZIKV E and prM protein by Western blot.** Vero cells were infected at a MOI of 5 with recombinant MVA-prME and non-recombinant MVA as control. Cells were harvested at indicated time points, lysed and separated by SDS-PAGE. A ZIKV-E specific monoclonal mouse antibody and a ZIKV-prM specific polyclonal rabbit antibody were used for detection.

For additional analysis we investigated the expression of recombinant protein in infected Vero cells by fluorescence microscopy. The antibody directed against the E protein of ZIKV showed a signal in permeabilized cells, while in non-permeabilized cells the signal was weaker (Figure 8).

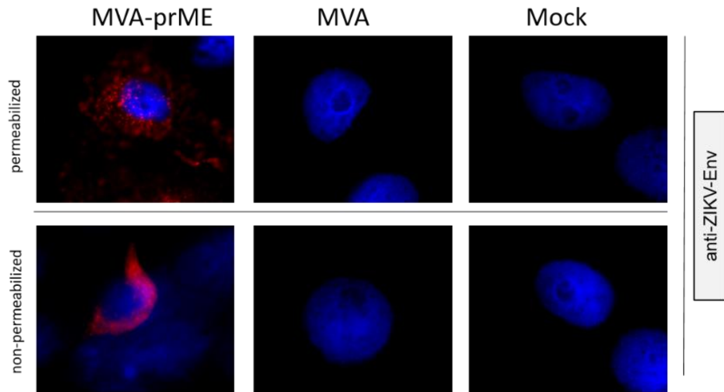


**Figure 8: Immunostaining of ZIKV-E in MVA-prME infected vero cells.** Cells were infected with MVA-prME or non-recombinant MVA. After 16h cells were fixed with paraformaldehyde and permeabilized. Staining was done with a rabbit polyclonal antibody directed against ZIKV E protein (red) and DAPI solution for nuclei (blue).

# 3. Protective Efficacy and immunogenicity

## 3.1. A single dose of MVA-prME protects pregnant mice against a ZIKV infection

We studied the protective efficacy and immunogenicity of the candidate vaccine MVA-prME in IFNAR-/- mice. These mice lack the alpha/beta interferon receptor and can serve as a reliable mouse model for ZIKV infection and disease (Müller et al. 1994; Lazear et al. 2016; Forster et al. 2020). We identified successfully mated female mice with a plug check and separated them for the experiments. Two days after the plug check, we confirmed a pregnancy by ultrasound. Pregnant mice that showed proliferation of both uterus horns and the mucosa were included in the study.

Directly after confirming pregnancy, positive mice were immunized intramuscularly with $10^8$ PFU of the candidate vaccine MVA-prME or non-recombinant MVA as control. Additional control mice received saline (PBS). Two days later, the pregnant dams were infected with $10^3$ FFU of ZIKV (H/PF/13) subcutaneously in the foot pad, while control mice received PBS. Mice were weighted daily and monitored for signs of illness. All dams were euthanized nine days later, and organs from both dams and fetuses were harvested for analysis. Blood was taken before challenge and on the day of euthanization (Figure 9).

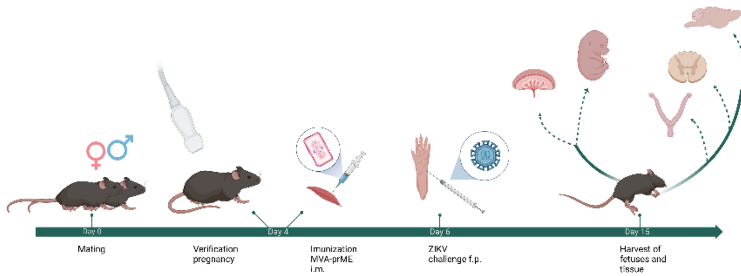


**Figure 9: Schematic of the experimental schedule.** 6-10 week old female IFNAR-/- mice were mated. Successful mating was confirmed by plug check and pregnancy was determined by US. Positive mice were separated and immunized i.m. with $10^8$ PFU of recombinant MVA-prME or non-recombinant MVA or PBS as control. Mice were then challenged with $10^3$ PFU ZIKV via footpad and monitored for symptoms and weight loss. Nine days after challenge all dams were euthanized and maternal and fetal organs were collected for analysis. Blood was taken on day four and at the end of the experiment. Created with BioRender.com

All dams immunized with MVA-prME were protected against the ZIKV infection and showed no ZIKV specific clinical signs. (Figure 10 b). Only mild symptoms could be detected after the challenge infection, which disappeared after a few days. In contrast, challenged mice from the wild-type MVA and the PBS group showed strong signs of illness and neurological disorder. Symptoms could be observed three days after challenge and were later accompanied by neurological signs like reduced motility, limb paralysis or Torticollis. All challenged control mice showed strong weight loss up to 15% at the end point of the experiment. The MVA-prME vaccinated mice gained approximately 8% weight, while the mock challenged mice gained with 10% slightly more. Thus all in vivo data indicate, that MVA-prME offered protection against ZIKV.

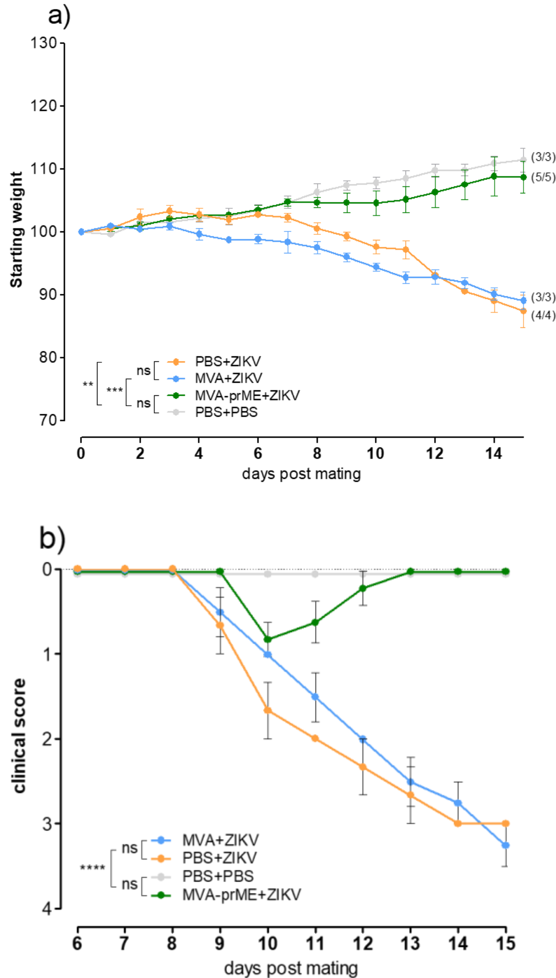


**Figure 10: MVA-prME protects pregnant mice against ZIKV challenge infection.** 6-10 week old pregnant IFNAR -/- mice were immunized with $10^8$ PFU of recombinant MVA-prME. Control groups received non-recombinant MVA or PBS. Six days after mating mice were challenged with $10^3$ PFU of ZIKV and monitored. **(a)** Mice were weighted daily and body weight is expressed as percentage of starting weight. Surviving/total animals are given in parentheses and SEMs are indicated by error bars. **(b)** Clinical symptoms are shown as score between 0 (no symptoms) and 4 (moribund/dead). SEMs are indicated by error bars. Groups were analyzed by determining area under the curve (AUC) and one-way ANOVA test. Asterisks represent statistically significant differences between groups: ns = non-significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$.

To examine the extend of protection, we analyzed viral ZIKV loads in different organs. Conditioned by the neurotropism of ZIKV, the brain, spinal cord and ovaries are particularly suited for analyses and give accurate info about the viral load in the subject (Miner and Diamond 2017).

We could not detect any ZIKV in the organs of MVA-prME vaccinated mice, consequently suggesting that all infectious ZIKV was entirely reduced by the immunization. In contrast we detected high titers of ZIKV in the organs of MVA wild type vaccinated and unvaccinated dams (Figure 11). Titers reached levels of $10^5$ PFU ZIKV per gram in the ovaries and $10^7$ PFU ZIKV per gram in the spinal cord and brain, which is comparable to levels observed in previous studies with ZIKV infected mice (Lazear et al. 2016; Elong Ngono et al. 2017; Marzi et al. 2018; Forster et al. 2020).

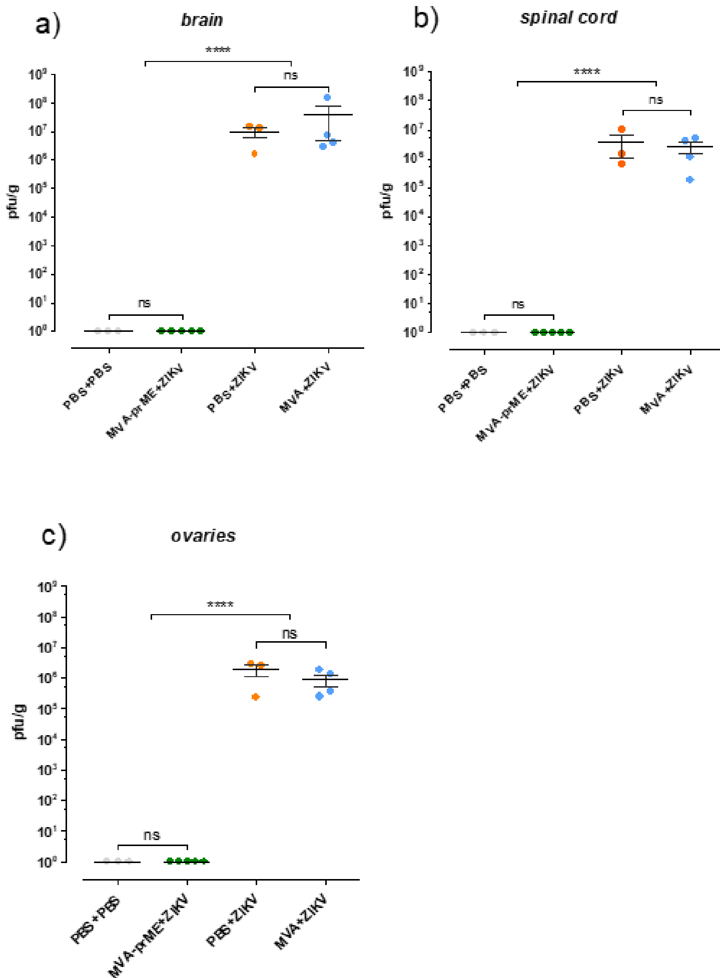


**Figure 11: ZIKV organ loads are entirely reduced in MVA-prME vaccinated mice after ZIKV infection (a-c).** Maternal organs were harvested at day 9 after challenge. **(a)** Brains **(b)** spinal cord and **(c)** ovaries were collected, homogenized and analyzed for viral loads. Titers were determined by plaque assay and are shown as plaque-forming-units (PFU). Data has been log transformed and differences between individual groups has been analyzed by one-way ANOVA and Bonferroni post-hoc test. Asterisks represent statistically significant differences between groups: ns = non-significant, **** $p < 0.0001$.

Additionally, we conducted histological analyses to further investigate the ZIKV infection and clearance, with focus on the brain. We detected considerable ZIKV-specific staining in the brains of unprotected mice, while the MVA-prME vaccinated mice showed no signs of ZIKV infected cells (Figure 12).

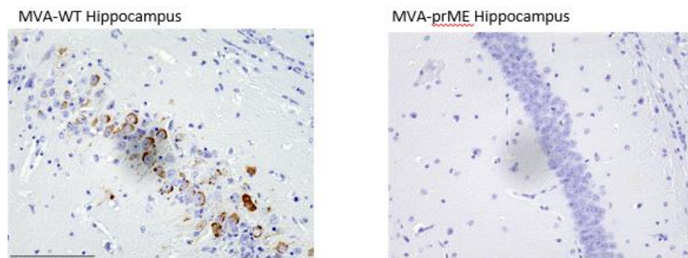


**Figure 12: Brains of MVA-prME vaccinated mice show no ZIKV signal:** Immunohistochemical analysis of brains of MVA-prME and MVA wild-type vaccinated mice. 9 days after challenge brain tissue was fixated and immunostained with a monoclonal antibody against ZIKV E. Images show sections of the Hippocampus representative for both groups.

### 3.2. Fetuses of MVA-prME vaccinated dams are protected against a ZIKV infection

We analyzed uteri, placenta, and fetuses of pregnant dams after the ZIKV infection to figure out whether the protection provided by MVA-prME extends to the fetuses. Uteri of MVA-prME vaccinated mice were physiological developed and comparable to uteri of mock challenged mice. The Fetuses also showed the same level of development and physiological growth. A clear difference could be observed in the groups of unprotected mice. Uteri were smaller and seemed to have less developed vascularization, while being shaped different and irregular (Figure 13). Fetuses were clearly underdeveloped and even missed limbs. Additionally numerous remnants of partially resorbed or atrophied fetuses could be found in the uteri of unprotected mice (Figure 13 a and b). These withered fetuses made up 52% of all fetuses of unvaccinated mice (n=19) and 62% of fetuses of MVA vaccinated mice (n=24). On the contrary, only 8,6% of fetuses in the group of MVA-prME vaccinated mice (n=35) were partially resorbed and could be considered dead before euthanization of the dams.

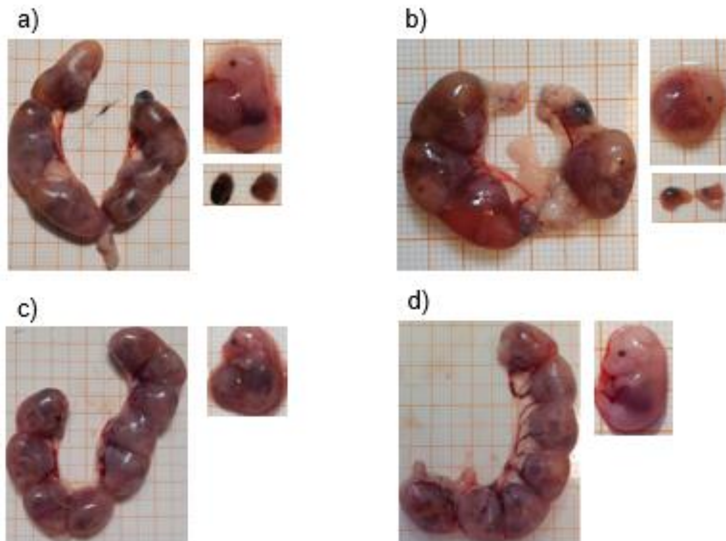


**Figure 13: Uteri and fetuses of MVA-prME vaccinated dams are protected against ZIKV infection.** Uteri and fetuses of **(a)** PBS, **(b)** MVA wild-type, **(c)** MVA-prME immunized and **(d)** non-challenged dams. Depicted uteri and fetuses are representative for groups. One big square equals 1cm.

When comparing the overall size and weight of the fetuses, a similar picture can be seen. Fetuses of MVA-prME vaccinated and mock challenge mice reached comparable size and weight, while unprotected fetuses showed a great reduction in both regards (Figure 14 a and b). Placental weight was also affected by this growth reduction and was significantly lower in the unprotected groups (Figure 14 c).

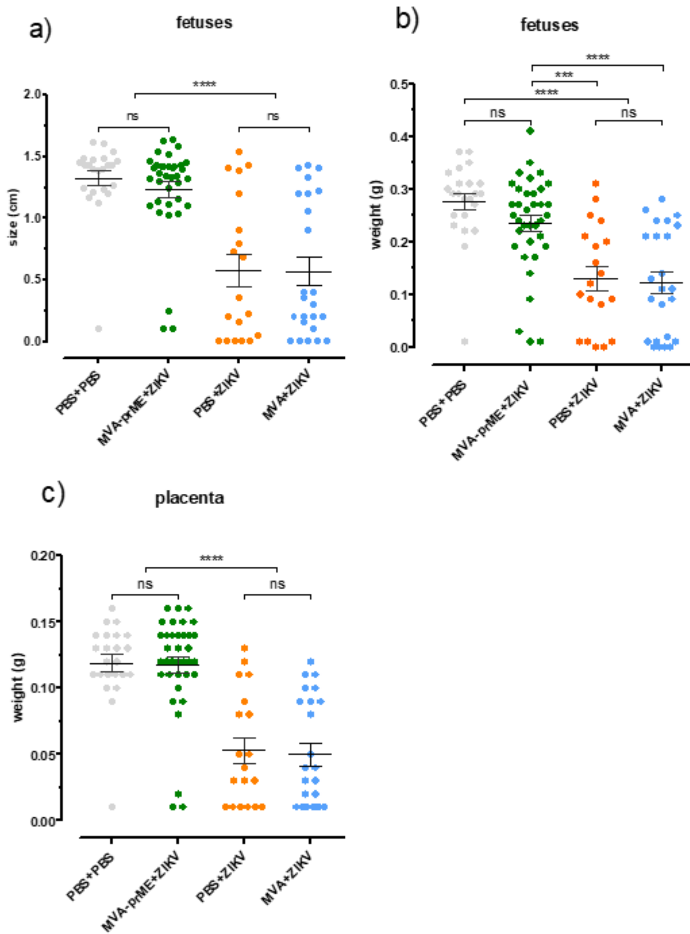


**Figure 14: Fetal and placental growth are protected in MVA-prME immunized dams after ZIKV infection (a-c).** Samples were collected 9 days after ZIKV challenge. **(a)** size and **(b)** weight of fetuses were measured together with **(c)** weight of placentas. Error bars indicate mean with SEMs. Differences between individual groups has been analyzed by one-way ANOVA and Bonferroni post-hoc test. Asterisks represent statistically significant differences between groups: ns = non-significant, **** $p < 0.0001$, *** $p < 0.001$.

To figure out the extend of ZIKV elimination in fetuses, we analyzed viral loads in the fetal heads, fetal liver, and the placenta. We could not detect any infectious virus in the fetal organs or the placenta of MVA-prME vaccinated

dams. This suggests a complete elimination of ZIKV in the dams and fetuses, or a prevention of viral spread from the dams to the fetuses altogether. On the contrary, we found high viral loads in the fetuses of unprotected dams. (Figure 15).

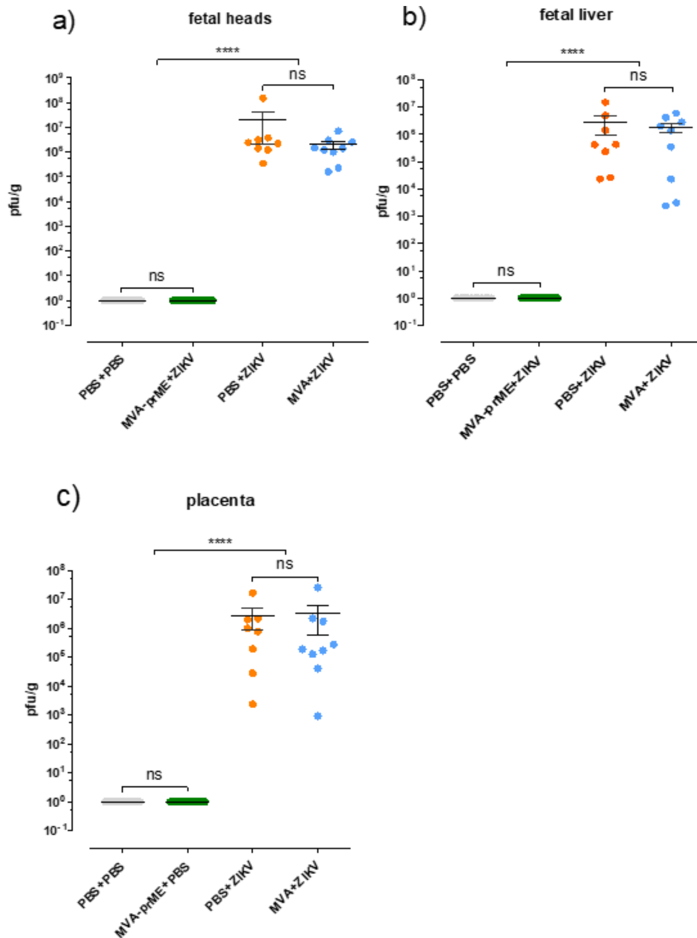


**Figure 15: Fetal and placental ZIKV loads are entirely reduced in MVA-prME immunized dams after ZIKV infection (a-c):** Pregnant dams were euthanized 9 days after ZIKV infection. **(a)** Fetal heads, **(b)** fetal liver and **(c)** placenta were harvested, homogenized analyzed for ZIKV loads. Titers were determined by plaque assay and shown as PFU. Error bars indicate mean with SEM. Differences between individual groups has been analyzed by one-way ANOVA and Bonferroni post-hoc test. Asterisks represent statistically significant differences between groups: ns = non-significant, **** $p < 0.0001$.

### 3.3. Antibody and T cell response in pregnant mice induced by MVA-prME and ZIKV challenge

To investigate possible correlates of protection induced by the candidate vaccine MVA-prME, we tested sera of pregnant mice by ZIKV-E-specific ELISA. Blood was taken four days and eleven days after immunization, which equals two and nine days post challenge (dpc) with ZIKV.

As expected, we could not detect significant E-specific antibodies four days after immunization (2 dpc) in any of the groups (Figure 16). Eleven days after immunization we detected a rise in ZIKV-E-binding antibodies in all challenged mice. The PBS and MVA vaccinated mice showed IgG titers of 1:50 with one mouse in the MVA group reaching a titer of 1:150. The MVA-prME vaccinated mice also showed titers between 1:50 and 1:150 (Figure 16).

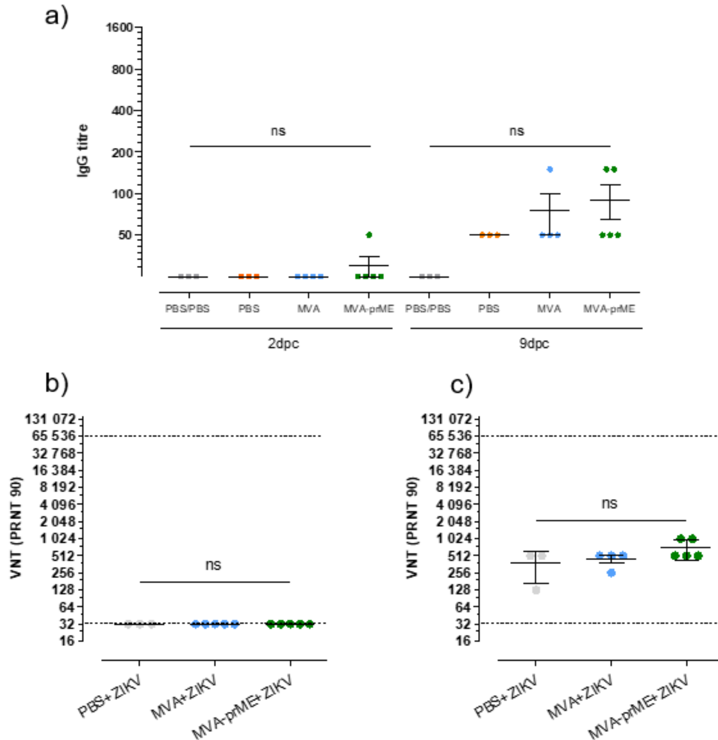


**Figure 16: Humoral immune response induced by MVA-prME and ZIKV infection (a-c)**. Sera of MVA-prME, MVA and sham (PBS) immunized dams two and nine days post challenge (four and eleven days post immunization) were analyzed by ELISA for ZIKV E specific antibodies **(a)**. A non-infected PBS group served as additional control. Virus neutralization titers of sera two days **(b)** and nine days **(c)** after challenge. Determined by VNT and expressed as the ratio denominator only. Differences between individual groups has been analyzed by one-way ANOVA and Bonferroni post-hoc test. Asterisks represent statistically significant differences between groups: ns = non-significant.

To examine the neutralization capacity of the antibodies, we performed a plaque reduction neutralization assay. As expected, sera from four days after immunization showed no detectable neutralization capability (Figure 16 b). In line with the increase of antibodies after challenge, we saw neutralization nine days after challenge. Titers of challenged mice reached values of 1:512, while two mice in the MVA-prME vaccinated group showed a titer of 1:1024 (Figure 16 c). These results are consistent with previous studies by other groups and are presumably attributed to the ZIKV infection (Aid et al. 2017; Brault et al.

2017; Pérez et al. 2018).

Besides humoral immune response, T cells play an important role in viral protection and are significantly involved in the immune response against flaviviruses (Shresta et al. 2004; Hildner et al. 2008; Elong Ngono et al. 2017). Therefore, we analyzed the T cell response specific to ZIKV-E.

Splenocytes isolated from spleens collected at the end of the experiment (11 days after immunization/9 dpc; see Figure 9) were analyzed by IFN-γ-ELISpot (Figure 17). To analyze CD8+ T cells, we used the previously described peptide epitope E294 (Elong Ngono et al. 2017) and could report elevated stimulatory activities in all challenged groups. MVA and unvaccinated mice showed levels between 415 and 1054 ZIKV-specific IFN-γ-producing T cells per $10^6$ spleen cells, while MVA-prME vaccinated mice reached significantly higher levels up to 1961 ZIKV-specific IFN-γ-producing T cells per $10^6$ spleen cells. Furthermore, we investigated the incidence of ZIKV-E specific CD4+ T cells with the already described peptide epitope E646 (Hassert et al. 2018). As was the case for CD8+ T cells, all challenged mice showed elevated levels of IFN-γ-producing T cells, while the MVA-prME vaccinated group surpassed the others (Figure 17).

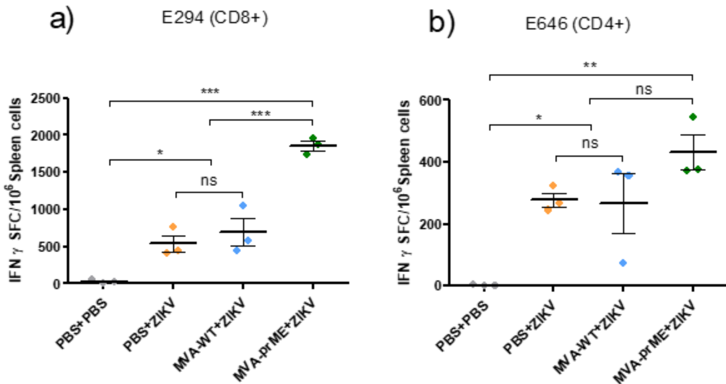


**Figure 17: Cellular immune response induced by MVA-prME (a+b).** Splencoytes were taken eleven days after immunization and T cell analyses was carried out. ZIKV-E-specific CD8+ **(a)** and CD4+ **(b)** T cell response in pregnant IFNAR -/- dams. IFN-γ spot forming T cells (IFN-γ SFC) were quantified by ELISpot. Differences between individual groups have been analyzed by one-way ANOVA and Bonferroni post-hoc test. Asterisks represent statistically significant differences between groups: ns = non-significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$.

These results show that MVA-prME induces an antibody response but is not able to reach neutralization capacity in this short time frame. Furthermore, MVA-prME induces a significant number of ZIKV-specific IFN-γ-producing CD8+ and CD4+ T cells, which surpass levels induced solely by ZIKV infection. This suggests the protective capacity induced by MVA-prME has its source in the T cells.

# VII. DISCUSSION

ZIKV poses a concerning threat due to its severe disease pattern including complications for pregnancies and neuropathies. The epidemic in South America proved its ability to spread and the potential devastating consequences of an epidemic. Although the cases of ZIKV infections have decreased in recent years, there is still need for a safe vaccine, which is especially suitable and effective for pregnant women.

In this study we developed a candidate vaccine expressing the prM and E proteins of ZIKV, using the well-established vaccine platform MVA. By utilizing the exceptional safety profile of MVA and its capacity to reliable express proteins, we developed an experimental vaccine suitable for the most important target group: pregnant women. We investigated its protective capacity in the susceptible interferon 1 deficient IFNAR-/- mice and showed the induction of a strong cellular immune response. The previously established ZIKV mouse model for pregnancy (Forster et al. 2020) allowed us to show the protective efficacy reaching from the pregnant dams to their fetuses.

**Requirements for a ZIKV vaccine**

Every pathogen requires a unique and specific approach when it comes to vaccine design and ZIKV is no exception. One of the first steps is choosing a suitable antigen and while it always needs to be immunogenic, an antigen for ZIKV needs to be chosen with caution because ADE is a potential risk. This adverse effect is known to occur in flaviviruses when an infection meets levels of non-neutralizing or only partially neutralizing antibodies and can result in a severe course of the disease. Strong antigens are often structural proteins located at the surface of pathogens. For flaviviruses the E-protein is the most prominent structure and because of its function in receptor binding and virus entry, it is a major target for vaccines. Consequently, most current vaccine candidates against ZIKV, which entered phase I and II clinical trials, are focused on structural proteins such as E and prM (Dejnirattisai et al. 2016; Kawiecki and Christofferson 2016; Bardina et al. 2017; Wilder-Smith et al. 2018). These approaches promise strong immune responses, but the risk of ADE still needs to be addressed in future studies.

While the humoral immune response is essential for ADE, the cellular parts of immune responses are not known to cause it. Instead, CD8+ T cells are confirmed to play a major role in combating flavivirus infections through elimination of pathogens (Shresta et al. 2004; Elong Ngono et al. 2017). Therefore, focusing on a T cell-specific immune response with a vaccine might be a good strategy. The antiviral activities of T cells are on the other hand known to trigger ZIKV-associated paralysis in the brain (Jurado et al. 2018). Therefore it is crucial to further study the different components and their role in the protection and pathogenesis of ZIKV. This could enable the identification of a viral antigen that is best suited for a vaccine, with the smallest possible risk of adverse side effects.

In conclusion, a ZIKV vaccine needs to be efficacious while not causing ADE. The vaccine needs to be safe for vulnerable groups, like elderly, immunocompromised and most importantly pregnant women. The offered protection has to include unborn child, given that they can suffer devastating effects by ZIKV. Considering the practical side, the ideal vaccine needs to be competitive in production, storable and effective after a single dose application.

**MVA as a vaccine platform for ZIKV**

A broad variety of vaccine platforms are used today, including different vector viruses, peptide vaccines and the new mRNA technologies. MVA distinguishes itself through its decade long use in vaccine development for many infectious diseases and convinces with strong properties.

As an attenuated live virus, MVA can infect cells and initiate expression of the desired antigens (Draper and Heeney 2010), which are then presented in their native conformation (de Vries and Rimmelzwaan 2016). The de novo synthesis leads to intracellular processing during the viral replication cycle and presentation by MHC-class I molecules. Together with MVA predominantly infecting antigen presenting cells, this facilitates a specific CD8+ T cell response (Altenburg et al. 2017). The ability to induce strong cell-mediated immune response could offer robust protection against ZIKV, while avoiding ADE complications which have not been associated with the cellular immune response. Furthermore, T cell immunity caused by VACV are known to be stable

and long lasting (Frey et al. 2003; Hammarlund et al. 2003; Crotty et al. 2003; Amara et al. 2004) and therefore contribute to an effective vaccination.

The pathogenesis of ZIKV, with its ability to cause severe congenital abnormalities and complications during pregnancy, requires vaccines to meet particularly high safety standards, which MVA can fulfill. The replication deficiency of MVA based vaccines in mammalian cells contributes to their safety without losing immunogenicity when compared to replication competent VACV strains (Sutter et al. 1994; Ramírez, Gherardi, and Esteban 2000). The safety of MVA has been confirmed in multiple animal models, such as immunosuppressed non-human-primates which suffered no pathological effects (Stittelaar et al. 2001). As a conventional smallpox vaccine, MVA proved to be immunogenic and safe for patients with atopic dermatitis or HIV infection (Greenberg et al. 2015; Overton et al. 2015; Vollmar et al. 2006). Furthermore MVA based vaccines have entered several clinical phase I to III studies and showed no severe side effects associated with the vaccination (Harrer et al. 2005). These studies also included elderly people and HIV patients. The ability of MVA to elicit strong cellular immune response further helps to avoid ADE complications caused by ZIKV.

Many vaccines, for example the mRNA-based SARS-CoV-2 vaccines, require a constant cold chain and are sensitive to higher temperatures. In contrast MVA retains most of its titer at room temperature and can even be freeze dried and stored without special cooling (Chen et al. 2021). This poses a great advantage for a ZIKV vaccine that would be predominantly used in warmer regions with less developed infrastructure. A vaccine also must be eligible for large-scale production and feasible from an economic viewpoint. MVA has already shown to meet these requirements on several occasions. For example, a recombinant MVA-based vaccine has been approved for immunization against Ebola (Ewer et al. 2016) and MVA itself is approved as a vaccine against smallpox (Vollmar et al. 2006).

**PrM and E as antigens for a ZIKV vaccine**

ZIKV offers two different kinds of antigens for vaccine development. Structural and non-structural proteins both have different properties and strength when being considered as immunogenic antigens. The biggest strength of non-structural proteins lies in their reduced risk to trigger ADE. In this context it is important to note, that antibodies in some circumstances are responsible for ADE, but nevertheless play a very important role in protection against flaviviruses and are therefore correlated with the efficacy of ZIKV vaccines (Pardi et al. 2017; Richner et al. 2017; Li, Dong, et al. 2018; Medina-Magües et al. 2021). When considering the non-structural proteins, the NS1 shows the most promising properties as a vaccine antigen. Different studies have proven the capability to elicit strong humoral and cellular responses and even showed protective capacity (Li, Yu, et al. 2018; Brault et al. 2017; Lai et al. 2017; Nazerai et al. 2021).

The envelope protein is a noteworthy structural protein of ZIKV and plays a crucial role in the pathogenesis. Virus entry in human cells is achieved through endocytosis and fusion caused by the E protein, while the prM protein mediates the process (Agrelli et al. 2019). As the most prominent structure, the E protein is also the major antigen and thus a target for neutralizing antibodies (Dai et al. 2018; Shi et al. 2018). Interestingly, while the neutralizing antibodies are considered a key correlate of protection against ZIKV infection, the prM plays a vital role in folding the immunogenic E (Liu et al. 2018). Studies have shown, that in contrast to using E alone, a combination of prM and E result in higher neutralizing antibody response against ZIKV (Liu et al. 2018). Consequently, most vaccine designs against ZIKV use prME as the target antigen (Lin et al. 2018; Shan, Xie, and Shi 2018; Poland, Ovsyannikova, and Kennedy 2019; Pielnaa et al. 2020). By doing so, prME has been proven to elicit strong cellular and humoral immune response with protective capacity against ZIKV (Muthumani et al. 2016; Pérez et al. 2021). Different platforms like recombinant viruses, mRNA or DNA vaccines have been utilized (Richner et al. 2017; Bullard et al. 2018; Griffin et al. 2017; Liu et al. 2018; Zhong et al. 2019) with two of the plasmid-based DNA vaccines reaching the phase II clinical trial stage (Dowd et al. 2016; Gaudinski et al. 2018). The protective capacity is therefore sufficiently proven, but only little data suggests an extended protection to fetuses after two

vaccinations (Jagger et al. 2019; Hazlewood et al. 2020; Choi et al. 2021). MVA has so far only been tested in a combined vaccination protocol for T and B cell induction (Pérez et al. 2021).

**IFNAR -/- mice as a pregnancy model to study ZIKV vaccine candidates**

The most detrimental effects of a ZIKV infection are seen in pregnant women and their child, including congenital abnormalities, microcephaly and fetal death. The epidemic in 2015 and 2016 showed the importance of a ZIKV vaccine, which can protect pregnant women. Today there is still no licensed ZIKV vaccine or therapy available even though some have reached clinical trials. Considering that pregnant women are generally excluded from vaccine clinical trials, testing new ZIKV vaccines in pre-clinical pregnancy models is undoubtedly necessary. While NHP are a model close to humans, they are unfavorable from an ethical and practical standpoint. Additionally to these factors, a mouse model has to be susceptible for the ZIKV infection and also suitable in regards of pregnancy. IFNAR -/- mice reliably reflect ZIKV pathogenesis and work as a lethal challenge model (Lazear et al. 2016). There are however differences between mouse and human pregnancy concerning physiology, placental layers and gestation time that need to be addressed. The much shorter gestation time in mice makes them even more suitable for research purposes, but also leads to a shorter window for ZIKV infection and hinders comparability. The placentas are however both hemochorial which causes a direct contact between maternal blood and fetal epithelium (Georgiades, Ferguson-Smith, and Burton 2002; Maltepe, Bakardjiev, and Fisher 2010). While studies have shown the susceptibility of fetuses to a ZIKV infection, the pathogenesis differs in some aspects. Fetuses in IFNAR -/- mice don't show microcephaly, calcifications in the brain or pathological brain development (Miner et al. 2016). Contrariwise, great effects can be observed in the placenta. The most striking effect is the overall reduced size and weight of ZIKV infected placentas, with first noticeable changes seen after ED 13,5 by US (Forster et al. 2020). These effects are hypothesized to be caused by viral cytotoxicity and could lead to ischemia (Miner et al. 2016; Forster et al. 2020). In conclusion it can be said that, although there are differences to human pregnancies, the susceptibility for ZIKV and the resulting pathogenesis make

the IFNAR-/- mouse model a suitable option for vaccine research in pregnancy.

**MVA-prME fully protects pregnant dams and their fetuses against ZIKV challenge infection**

In our study we showed, that a single dose of recombinant MVA-prME protects pregnant mice in a ZIKV challenge model. Pregnant dams were fully protected and showed no clinical symptoms or weight loss. Furthermore, there was no ZIKV detectable in any maternal organs nine days after infection with ZIKV, suggesting that the virus had been totally cleared. The control groups in contrast suffered from ZIKV related symptoms, a heavy course of disease and furthermore showed high loads of ZIKV in all analyzed organs. The fetuses of dams immunized with MVA-prME were also protected and showed no signs of growth reduction or congenital damage. There was no ZIKV detectable in these fetuses or the placenta of dams immunized with MVA-prME. These results are in line with other studies, where prME induced protective capacity when delivered by different technologies (Pattnaik, Sahoo, and Pattnaik 2020). Two studies, using different DNA and RNA vaccines, reported fetal protection with small viral breakthrough in fetal heads in a two dose immunization regime (Jagger et al. 2019; Choi et al. 2021). A chimeric vaccine was furthermore able to proof efficacy in NHP and protect mice fetuses 210 days after immunization (Li, Dong, et al. 2018). In continuance of these results, we were able to show protective efficacy in pregnant mice using recombinant MVA-prME in a single dose application.

**Strong cellular immune response is induced by MVA-prME**

Neutralizing antibodies targeted against structural proteins, especially against E, are long proven to be a correlate of protection in flavivirus infections (Colombage et al. 1998; Throsby et al. 2006). Vaccines eliciting neutralizing antibodies offer reliable protection and have confirmed this through serum and antibody transfers (Nürnberger et al. 2019; Medina-Magües et al. 2021). The cellular immune responses on the other hand can offer equally important factors in vaccines design (Sallusto et al. 2010; Pulendran and Ahmed 2011). In the case of ZIKV, studies have shown that CD8+ T cells play an important role in clearing the virus from the CNS and CD4+ T cells could contribute through

cytokine production and antibody maturation (Pérez et al. 2018; Pierson and Graham 2016). The risk of ADE and the fact that antibody response in the elderly declines further underline the potential of T cell induction through vaccines. Considering the schedule of our experiments, neutralizing antibodies were not the targeted correlate of protection. The short time frame between immunization and challenge instead showed that T cells could deliver protection. Indeed we only saw neutralizing antibodies after the challenge with no significant difference between vaccinated and unvaccinated mice, suggesting they were caused by the infection itself. On the other hand, we could observe a strong ZIKV-E-specific CD8+ and CD4+ response induced by MVA-prME, which surpassed levels caused solely by a ZIKV challenge.

**Future perspective**

A vaccine against ZIKV has to prove not only its efficacy and safety like any other vaccine, but also meet high requirements for the most vulnerable target group, pregnant women. The structural proteins prM and E of ZIKV have already been shown to induce protective capacity and therefore proven their potential as a vaccine antigen for adults. A more detailed look on their effect on pregnant and fetuses is now warranted. In this context, the humoral immune response plays an important role, particularly in reducing the viremia (Hurtado-Monzón et al. 2020). Nevertheless the cellular immune response should also be an equally important target for a vaccine. This could unlock important potential for vaccine efficacy as studies with the recently licensed DENV vaccine suggest (Scherwitzl, Mongkolsapaja, and Screaton 2017) and also help diminish the risk of ADE (Rivino and Lim 2017; Ngono and Shresta 2018).

The protective efficacy induced by MVA-prME confirms the suitability of this vaccine approach. Our study demonstrated the capability of prME to induce sufficient protection against ZIKV infection based on T cells. Although the CD8+ T cell response in pregnant mice is lowered (Winkler et al. 2017), it was still sufficient to fully protect dams and fetuses. However further studies should be conducted to investigate the correlate of protection in detail. Additional vaccine schedules are needed to investigate the role of neutralizing antibodies and potential impairments of delivered newborn mice should be considered.

# VIII. SUMMARY

The Zika virus was discovered over half a century ago in Uganda. After years of remaining mostly unnoticed the virus spread to multiple countries and caused several outbreaks. After spreading to Asia in 1966 and Oceania in 2007, ZIKV reached the Americas in 2015. At this time point severe ZIKV cases had started to accumulate. Cases of Guillain-Baré-Syndrome were reported and ZIKV infections were connected to congenital birth defects for the first time. This new aspect of severe complications lead to the declaration of a "Public Health Emergency of International Concern" by the WHO. Even though cases of ZIKV infections have declined in recent years, the possibility of newly emerging epidemics still exists. Factors like urbanization, increased global connection and climate change foster the possibility of virus spreading. The expanding distribution of ZIKV vectors, like *Aedes albopictus*, could enable outbreaks even in the western hemisphere.

To combat the thread of ZIKV a safe and efficacious vaccine is the best instrument. Despite joined efforts of the scientific community, no vaccine has been licensed yet. There are however promising approaches focusing on the structural proteins prM and E of ZIKV as the antigen of choice. Vaccine candidates based on different platforms are able to induce protective efficacy in animal models. Some candidates, such as two DNA-based vaccines, have reached first clinical trials. Due to the fact that they only have been tested in healthy adults and not pregnant women, only little is known about fetal protection. The role of T cell immunity must also be studied in more detail, especially considering the possibility of ADE. Due to declining cases and the lack of pregnant women in clinical trials, assessment of vaccine candidates in animal models is necessary.

This work investigates the potential of ZIKV proteins prM and E to induce protective capacity in a pregnancy mouse model. This objective was achieved by construction and characterization of recombinant MVA expressing prME and subsequent testing of the candidate vaccine in a ZIKV challenge model. The in-vitro characterization of MVA-prME proved genetic stability and demonstrated reliable expression of the prM and E protein as well as replication deficiency in mammalian cells. A single vaccination with MVA-prME protected dams and their

fetuses against a ZIKV challenge infection. Further assessment demonstrated the induction of ZIKV specific T cells in absence of detectable neutralizing antibodies at the time of infection.

The results of this study demonstrate the potential of T cells in protection against ZIKV infection for dams and fetuses and therefore support a focus on T cell immunity in further vaccine research.

# IX. ZUSAMMENFASSUNG

Das Zika Virus wurde bereits vor über einem halben Jahrhundert in Uganda entdeckt. Nach Jahren in denen es größtenteils unbeachtet blieb, verbreitete sich das Virus in mehreren Ländern und verursachte etliche Ausbrüche. Nach der Verbreitung in Asien 1966 und Ozeanien 2007, erreichte ZIKV den amerikanischen Kontinent im Jahr 2015. Zu diesem Zeitpunkt häuften sich bereits ZIKV Fälle mit schwerem Verlauf. Fälle des Guillain-Baré-Syndroms traten auf und ZIKV Infektionen wurden erstmals mit angeborenen Geburtsfehlern in Verbindung gebracht. Aufgrund dieser schweren Komplikationen erklärte die WHO schließlich die „gesundheitliche Notlage von internationaler Tragweite". Obwohl Fälle von ZIKV Infektionen in jüngster Zeit zurückgegangen sind, besteht weiterhin die Gefahr einer neu ausbrechenden Epidemie. Faktoren wie Urbanisation, verstärkte globale Verknüpfung und Klimawandel begünstigen eine virale Verbreitung. Die Ausweitung des Verbreitungsgebietes der Vektoren von ZIKV wie *Aedes albopictus*, könnte Ausbrüche in der westlichen Hemisphäre ermöglichen.

Das beste Instrument um die Gefahr von ZIKV zu bekämpfen stellt eine sichere und wirksame Impfung dar. Trotz vereinter Anstrengung der wissenschaftlichen Gemeinschaft wurde bisher kein Impfstoff zugelassen. Es gibt jedoch vielversprechende Ansätze die sich auf die Strukturproteine prM und E von ZIKV als Antigen konzentrieren. Impfstoffkandidaten basierend auf unterschiedlichen Plattformen sind in der Lage Schutzwirkung in verschiedenen Tiermodellen zu erzeugen. Einige Kandidaten, wie zwei DNA-basierte Impfstoffe, haben erste klinische Versuche erreicht. Aufgrund der Tatsache, dass sie nur in gesunden Erwachsenen und nicht in schwangeren Frauen getestet wurden, ist wenig über fetale Schutzwirkung bekannt. Die Rolle der T-Zell Immunität muss ebenso genauer untersucht werden, besonders aufgrund der Möglichkeit von durch Antikörper verstärkter Infektion (ADE). Der Rückgang von Infektionen und der Ausschluss von schwangeren Frauen in klinischen Untersuchungen macht die Untersuchung von Impfstoffkandidaten in Tiermodellen notwendig.

Diese Arbeit untersucht das Potential der Zika-Virus Proteine prM und E Schutzwirkung in einem Tiermodell für Schwangerschaft hervorzurufen. Dieses

Ziel wurde durch die Konstruktion und Charakterisierung von rekombinanten MVA welches prME exprimiert, sowie dessen Untersuchung, erreicht. Die in-vitro Charakterisierung von MVA-prME zeigte genetische Stabilität, verlässliche Expression der Proteine prM und E, sowie Vermehrungsunfähigkeit in Säugetierzellen. Eine einmalige Impfung mit MVA-prME schützte Muttertiere und Feten gegen eine ZIKV Infektion. Weitere Untersuchungen zeigten die Anregung von ZIKV spezifischen T Zellen in Abwesenheit von neutralisierenden Antikörpern zum Zeitpunkt der Infektion.

Die Ergebnisse dieser Studie zeigen das Schutzpotential von T Zellen in Muttertieren und Feten bei einer ZIKV Infektion und tragen somit zum besseren Verständnis der Impfstoffforschung zu ZIKV bei.

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# XI. APPENDIX

## 1.1. Chemicals and Reagents

| Chemical/Reagent | Supplier |
| --- | --- |
| Acetone | Carl Roth, Karlsruhe, Germany |
| Biozym LE Agarose | Biozym, Hessisch Oldendorf, Germany |
| DAPI | Thermo Fisher Scientific, Planegg, Germany |
| DMSO (C2H6OS) | Sigma-Aldrich, Taufkirchen, Germany |
| ELISA Stop Reagent | Sigma-Aldrich, Taufkirchen, Germany |
| ELISA TMB | Sigma-Aldrich, Taufkirchen, Germany |
| Ethanol (96 %) | Carl Roth, Karlsruhe, Germany |
| GelRed (10.000x) | Biotrend, Cologne, Germany |
| Glycine (C2H5NO2) | PanReac AppliChem, Darmstadt, Germany |
| Nonfat dried milk powder | PanReac AppliChem, Darmstadt, Germany |
| Red Blood Cell Lysis Buffer | Sigma-Aldrich, Taufkirchen, Germany |
| Roti-Load, 4x, (reducing) | Carl Roth, Karlsruhe, Germany |
| SDS (C12H25NaO4S) | Carl-Roth GmbH, Karlsruhe, Germany |

| | |
|---|---|
| Sucrose | Sigma-Aldrich, Taufkirchen, Germany |
| Tris buffer | BioRad, Munich, Germany |
| Trypan-blue | Sigma-Aldrich, Taufkirchen, Germany |
| Tween 20 | Sigma-Aldrich, Taufkirchen, Germany |
| 2-Propanol | Carl Roth, Karlsruhe, Germany |

### 1.2. Materials and Consumables

| Material | Supplier |
|---|---|
| Cell culture flask 25 | Sarstedt, Nümbrecht, Germany |
| Cell culture flask 75 | Sarstedt, Nümbrecht, Germany |
| Cell culture flask 175 | Sarstedt, Nümbrecht, Germany |
| Criterion TGX stain free gel (4-20%) | Bio-Rad, Munich, Germany |
| Coverslips | Thermo Fisher Scientific, Planegg, Germany |
| CryoPure tube | Sarstedt, Nümbrecht, Germany |
| Disposal bags | Sarstedt, Nümbrecht, Germany |
| Ep T.I.P.S 10-1000 µl | Eppendorf AG, Hamburg, Germany |
| Filter tips (10 µl) | Sarstedt, Nümbrecht, Germany |
| Filter tips (100 µl) | Sarstedt, Nümbrecht, Germany |
| Filter tips (1000 µl) | Sarstedt, Nümbrecht, Germany |
| Microtest plate (96-well) | Sarstedt, Nümbrecht, Germany |
| Microscope Slides | Engelbrecht, Edermünde, Germany |
| MiniCollect vials | Greiner Bio-One, Frickenhausen, Germany |
| Nitrocellulose membrane | GE Healthcare Europe, Munich, Germany |

| | |
|---|---|
| Nunc-Immuno Plate | Thermo Fisher Scientific, Planegg, Germany |
| SafeSeal tube (1.5 ml) | Sarstedt, Nümbrecht, Germany |
| SafeSeal tube (2 ml) | Sarstedt, Nümbrecht, Germany |
| Serological pipette 5 ml | Sarstedt, Nümbrecht, Germany |
| Serological pipette 10 ml | Sarstedt, Nümbrecht, Germany |
| Serological pipette 25 ml | Sarstedt, Nümbrecht, Germany |
| Tissue culture plates (6 well) | Sarstedt, Nümbrecht, Germany |
| Tissue culture plates (24 well) | Sarstedt, Nümbrecht, Germany |
| Tissue culture plates (96 well) | Sarstedt, Nümbrecht, Germany |
| Tube (15 ml) | Sarstedt, Nümbrecht, Germany |
| Tube (50 ml) | Sarstedt, Nümbrecht, Germany |

### 1.3. Media and supplements

| **Material** | **Supplier** |
|---|---|
| DMEM (VLE Dulbecco's) | Merck Millipore, Biochrom GmbH |
| DPBS | Thermo Fisher Scientific, Planegg, Germany |
| FBS | Thermo Fisher Scientific, Planegg, Germany |
| MEM | Sigma-Aldrich, Taufkirchen, Germany |
| Non-essential amino acid solution for MEM | Sigma-Aldrich, Taufkirchen, Germany |
| Penicillin-Streptomycin (10mg/ml) | Sigma-Aldrich, Taufkirchen, Germany |
| RPMI-1640 medium | Sigma-Aldrich, Taufkirchen, Germany |
| TrypLE™ Select Trypsin | Thermo Fisher Scientific, Planegg, Germany |

### 1.4. Commercial Kits

| Material | Supplier |
|---|---|
| MINI-Protean TGX | Bio-Rad, Feldkirchen, Germany |
| Mouse IFN-y ELISpot kit (ALP) | Mabtech, Nacka Strand, Germany |
| NucleoBond Xtra Midi | Macherey-Nagel, Düren, Germany |
| NucleoSpin Blood QuickPure | Macherey-Nagel, Düren, Germany |
| NucleoSpin Gel and PCR Clean-up | Macherey-Nagel, Düren, Germany |
| NucleoSpin Plasmid | Macherey-Nagel, Düren, Germany |
| SuperSignal West Dura Extended Duration Substrate | Thermo Fisher Scientific, Planegg, Germany |

### 1.5. Buffer

| | |
|---|---|
| PBS (10x) | 80 g/l NaCl<br>2 g/l KCl<br>2 g/l KH2PO4<br>11,5 g/l Na2HPO4 |
| LB-Medium (pH= 7.5 ) | 10 g/l Trypton<br>5 g/l NaCl<br>5 g/l Yeast extract |
| Lysis Buffer | 1 % Triton X-100<br>25 mM Tris<br>1 M NaCl |
| Running buffer (5x) | 72,5 g Glycin<br>15,2 g Tris<br>25 ml 20 % SDS |
| TAE buffer (50x; pH= 7.4) | 242 g Tris<br>57,1 ml acetic acid<br>18,6 g EDTA |
| Transfer Buffer concentrate (Towbin) | 24 g Tris<br>114,6 g Glycin |

| | |
|---|---|
| Transfer Buffer for WB | 80 ml Towbin (conc.)<br>200 ml Methanol<br>720 ml ddH2O |
| SDS Gels 10%) | 9,9 ml acrylamide (30%)<br>7,5 ml (1.5M Tris-HCl; pH 8.8)<br>0,15 ml SDS (20%)<br>0,9 ml ammoniumpersulfate (10%)<br>24 µl TEMED<br>30 ml ddH2O |
| LB-Medium (pH= 7.5 ) | 10 g/l Trypton<br>5 g/l NaCl<br>5 g/l Yeast extract |
| Vaccine buffer (pH=7.4) | 10 mM Tris<br>140 mM NaCl |

## 1.6. Laboratory equipment

| Equipment | Supplier |
|---|---|
| A.EL.VIS Universal plate reader | A.EL.VIS GmbH, Hannover, Germany |
| Avanti® J-26 XP Centrifuge | Beckman Coulter, Krefeld, Germany |
| Biofuge fresco | Heraeus, Hanau, Germany |
| ChemiDocTMMP, Imaging System | Bio-Rad, München, Germany |
| Galaxy 170S Incubator | New Brunswick, Hamburg, Germany |
| Linear transducer SL3116 (20MHz) | Esaote, Genoa, Italy |

| | |
|---|---|
| KEYENCE BZ-X710 Fluorescence Microscope | KEYENCE Deutschland GmbH, NeuIsenburg, Germany |
| Microplate reader Sunrice | Tecan Group Ltd. Männedorf, Switzerland |
| MJ Research PTC-200 Thermal Cycler Peltier | GMI, Ramsey, USA |
| MyLab Delta | Esaote, Genoa, Italy |
| Olympus CKX41 | Olympus Life Sciences, Hamburg, Germany |
| OptimaTM LE-80K Ultracentrifuge | Beckman Coulter, Krefeld, Germany |
| Sonoplus | Bandelin electronic, Berlin, Germany |

## 1.7. Software

| **Software** | **Provider** |
|---|---|
| Adobe Reader | Adobe Systems, San Jose, USA |
| A.EL.VIS V6.1 | A.EL.VIS GmbH, Hannover, Germany |
| BioRender | BioRender, Toronto, USA |
| DNASTAR Lasergene | DNASTAR, Inc., Madison, Wisconsin, USA |
| GraphPad prism | GraphPad Software, San Diego, USA |
| Image Lab 5.0 | Bio-Rad, Feldkirchen, Germany |
| Microsoft Office 2016 Professional | Microsoft Corporation, Redmond, USA |

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