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Evaluation of vector-specific immune responses following a homologous prime boost immunization with the vaccine candidate MVA-MERS-S

Dissertation

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1.1.1 Coronaviruses as emerging pathogens

To date, seven human pathogenic coronaviruses have been identified. Four human coronaviruses (HCoV) are globally endemic and cause up to 30 % of mild and self-limiting upper respiratory tract infections (Paules et al., 2020).

Among them, HCoV-229E and HCoV-OC43 were first discovered in the 1960s and extensively studied (Bradburne et al., 1967). HCoV-NL63 and HCoV-HKU1, were discovered in 2004 and 2005, respectively (Pyrc et al., 2007).

By contrast, the three remaining HCoV are highly pathogenic and cause severe acute respiratory illness in humans. They are responsible for several past and ongoing outbreaks.

First, Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) caused an epidemic in 2002/2003 in China (Drosten et al., 2003). It led to over 8,000 infections in 26 countries and had a case-fatality rate of 9.6 % (WHO, 2003).

Roughly ten years later, another new HCoV was isolated and named Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (de Groot et al., 2013, Corman et al., 2014). Unlike SARS-CoV, MERS-CoV is continuing to circulate and cause infection in humans.

Currently, the world is facing a pandemic caused by another newly emerging HCoV named SARS-CoV-2 due to phylogenetic proximity to SARS-CoV. It is the causative pathogen of coronavirus disease 2019 (COVID-19), which was first discovered in December 2019 in Wuhan, China (Coronaviridae Study Group of the International Committee on Taxonomy of, 2020). Since then, over 24.0 million cases and more than 820,000 deaths were reported in 216 countries (WHO, 2020b) as of August 2020.

All three newly identified HCoV are respiratory viruses of zoonotic origin (Wang et al., 2020).

Considering the high epidemic potential of these HCoV and that there are no medical countermeasures available fast and efficient development of protective vaccines is needed.

Therefore, the World Health Organization (WHO) has listed Middle East respiratory syndrome (MERS), Severe Acute Respiratory Syndrome (SARS) and COVID-19 on the research and development (R+D) blueprint list of priority diseases (**Table 1**). The list was first launched in 2015 in the aftermath of the West African Ebola epidemic

which was declared a Public Health Emergency of International Concern (PHEIC) in 2014. The aim is to accelerate and facilitate research and development of medical countermeasures against emerging pathogens. The list is updated on a regular basis and when a new disease emerges. “Disease X” refers to a currently unknown pathogen that can cause an epidemic outbreak (WHO, 2020c, Mehand et al., 2018).

Table 1: WHO R+D priority diseases (updated 2020)

Priority disease	Listed since
COVID-19	2020
Ebola virus disease and Marburg virus disease	2015
Lassa fever	2015
Middle East respiratory syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS)	2015
Nipah and henipaviral diseases	2015
Rift Valley fever	2015
Zika virus disease	2017
“Disease X”	2017

1.1.2 Epidemiology and transmission

MERS-CoV circulates mainly in the Arabian Peninsula, i.e. Saudi Arabia, Jordan, United Arab Emirates. However, sporadic predominantly travel-associated MERS-CoV infections have occurred in other countries.

The first case of MERS caused by the MERS-CoV was reported in Saudi Arabia in June 2012. The novel virus was isolated from the sputum of a 60-year-old man with acute severe pneumonia and renal failure (Zaki et al., 2012).

In September 2012, another case of severe respiratory illness caused by the same virus was diagnosed in a 49-year-old man in a hospital in London. He had been transferred from a hospital in Qatar. Interestingly, this patient had a history of travel to Saudi Arabia (Bermingham et al., 2012).

Later, a retrospective analysis of cases with unknown respiratory illness in a hospital in Jordan confirmed a cluster of 13 MERS-CoV infections that had occurred already in April 2012 (Hijawi et al., 2013).

According to the WHO, 2,519 laboratory-confirmed cases and 866 deaths (case-fatality rate: 34.4 %) in 27 countries had been reported until the end of January 2020 with 84.2 % of cases in Saudi Arabia (WHO, 2020a).

In search for the animal reservoir of this zoonotic disease it was first hypothesized that bats are responsible for animal-to-human transmission. In fact, there is evidence that MERS-CoV, like other alpha- and beta-coronaviruses, originated in bats, as sequences similar to MERS-CoV were isolated from several bat species (Wang et al., 2014, Memish et al., 2013). Woo et al. showed that MERS-CoV is phylogenetically related to the bat coronaviruses HKU4 and HKU5 (Woo et al., 2012).

Nevertheless, recent research points towards dromedary camels as main animal reservoir for primary MERS-CoV infection in humans. MERS-CoV specific antibodies were found in dromedary camels from Saudi Arabia, the United Arab Emirates, Jordan, Oman, Qatar, Spain, Egypt, among others (Reusken et al., 2014b).

Furthermore, MERS-CoV has probably been circulating in dromedary camels for 20 – 30 years, since retrospective analyses showed that the earliest MERS-CoV sample was probably from 1992 (Corman et al., 2014).

Of note, MERS-CoV infection in dromedary camels is transient and causes mild upper respiratory tract symptoms such as rhinitis (Adney et al., 2014).

The theory that dromedary camels may be the animal reservoir is underlined by the study of Azhar et al. who observed camel-to-human transmission. They isolated MERS-CoV from a camel worker and from his MERS-CoV infected camel with rhinorrhea. They compared the full genome sequences of both isolates and found matching genome sequences (Azhar et al., 2014).

Another study by Reusken et al. demonstrated the persistence of virus ribonucleic acid (RNA) in the milk of lactating camels, indicating that the consumption of non-pasteurized camel milk and products might also be a risk factor for MERS-CoV infection (Reusken et al., 2014a).

Alshukairi et al. found a high prevalence of MERS-CoV in camel workers in Saudi Arabia. 50 % of the 30 tested camel workers had serological evidence for prior MERS-CoV infection (Alshukairi et al., 2018).

However, animal-to-human transmission is not the only source of infection. Human-to-human transmission is possible and related to household- and hospital-acquired infections. Nosocomial MERS-CoV infections pose a threat to health-care workers and patients (Al-Tawfiq and Auwaerter, 2019). In a retrospective analysis of laboratory

confirmed cases reported to WHO from 2012 to June 2018, 18.6 % of all MERS-CoV cases were health-care workers (Elkholy et al., 2020).

Several outbreaks in health-care facilities have been reported. In the Republic of Korea in May 2015, one patient who had developed fever and cough after returning from the Middle East initiated a nosocomial outbreak of 186 confirmed MERS cases and 38 related deaths. The traveler had visited several health-care facilities in South Korea before he was diagnosed with MERS-CoV. This way, the index patient had infected 28 individuals and through consecutive nosocomial transmission the virus was spread throughout the country. This is considered the largest outbreak outside of the Arabian Peninsula (Cho et al., 2016).

Drosten et al. studied 26 MERS-CoV positive index patients and their 280 household contacts and found 12 (approximately 4 %) probable cases of secondary transmission from the index patients to household contacts (Drosten et al., 2014).

Human-to-human transmission is possible but limited, requires close contact and occurs via respiratory droplet infection (Zumla et al., 2015).

1.1.3 Taxonomy and structure

According to the International Committee on Taxonomy of Viruses (ICTV) coronaviruses are enveloped, large (26.4 – 31.7 kb), single-stranded positive-sense RNA viruses. They belong to the order *Nidovirales* in the family *Coronaviridae.* The subfamily *Orthocoronavirinae* is comprised of four genera: *alpha-, beta, gamma-* and *deltacoronavirus* (ICTV, 2019). A*lpha-* and *betacoronaviruses* are known to infect mammals like bats, pigs, cats, mice and humans (Wang et al., 2020).

MERS-CoV is a betacoronavirus which belongs to the lineage C together with *Tylonycteris* bat coronavirus HKU4 and *Pipistrellus* bat coronavirus HKU5 (Woo et al., 2012).

The single-stranded RNA genome of MERS-CoV has a size of approximately 30 kb and 10 open reading frames (ORF). 16 non-structural proteins are encoded at the 5’ end (ORFs 1a and 1b). Further, the RNA encodes for the structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N). S, E and M are viral membrane proteins (**Figure 1**). The trimeric S protein is involved in attachment and entry into the host cell. It is highly immunogenic and therefore seen as therapeutic and vaccine target. M and E proteins are necessary for viral assembly. The N protein is important for RNA synthesis (Song et al., 2019).

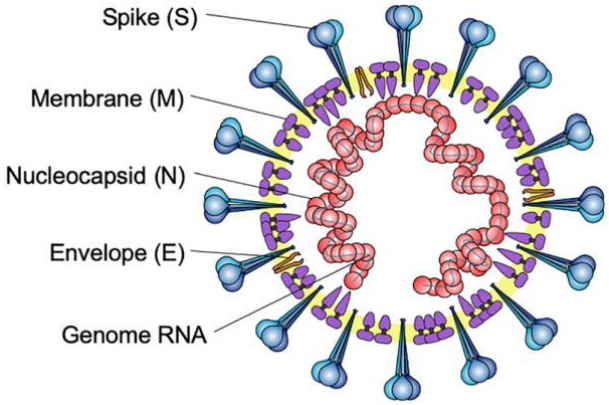


Figure 1: Cartoon model structure of MERS-CoV (Xu et al., 2019)

1.1.4 Pathogenesis and clinical features

After inoculation, MERS-CoV first infects the upper and lower respiratory tract in humans. Raj et al. discovered that MERS-CoV enters host-cells through binding of the receptor binding domain (RBD) of the S1 subunit of S-glycoprotein to dipeptidyl-peptidase 4 (DPP4) of the host-cell.

DPP4 is a 766 amino acid (aa) long transmembrane glycoprotein. It is expressed on the epithelial cells in the kidney, the small intestine, the liver and the prostate in humans. It was also found at high levels in lung tissue on non-ciliated bronchial epithelial cells. DPP4 has several functions, such as the cleavage of dipeptides from hormones and chemokines to regulate their bioactivity and the degradation of incretins in glucose metabolism. Interestingly, MERS-CoV infection could not be impaired by DPP4 inhibitors sitagliptin, vildagliptin or saxagliptin (Raj et al., 2013).

The clinical picture of MERS is diverse: it ranges from asymptomatic or mild respiratory symptoms with cough and fever to severe acute pneumonia with acute respiratory distress syndrome (ARDS) and multi-organ failure and death. The incubation period ranges from five days to two weeks. Symptoms of disease onset include fever, chills, cough, sore throat, myalgia, and arthralgia. Additionally, gastrointestinal symptoms were present in a third of MERS-CoV infected patients. Patients with dyspnea and rapidly progressing severe pneumonia often require intensive care and mechanical ventilation (Assiri et al., 2013b).

Risk factors for a severe course of disease are a compromised immune status and comorbidities such as obesity, respiratory disease, cardiac disease and diabetes (Assiri et al., 2013a). Age ≥ 65 years is also associated with a higher mortality (Saad et al., 2014).

1.1.5 Therapy and prevention

There is no specific treatment available against MERS-CoV infection. Few therapeutic options are being evaluated in clinical trials, such as ribavirin/interferon, lopinavir/ritonavir and the fully human polyclonal antibody SAB-301 (Momattin et al., 2019).

To prevent camel-to-human and human-to-human transmission the implementation of hygiene measures is important, as well as the development of a vaccine.

To date, there are worldwide efforts to develop a protective vaccine against MERS-CoV. Several vaccine candidates are being tested in pre-clinical and clinical trials. The Spike(S)-glycoprotein on the surface of MERS-CoV is immunogenic and therefore a target for vaccine development. So far, three vaccine candidates, using S-glycoprotein as antigen, completed clinical phase 1 trials.

Modjarrad et al. conducted a phase 1 trial with the vaccine candidate GLS-5300, a deoxyribonucleic acid (DNA)-based vaccine expressing the full-length S-glycoprotein. The vaccine was safe and immunogenic with a S1-specific Enzyme-linked Immunosorbent Assay (ELISA) seroconversion in 61 (94 %) of 64 study participants after three vaccinations (Modjarrad et al., 2019).

Furthermore, two viral-vectored vaccine candidates are under evaluation. Folegatti et al. studied the vaccine candidate ChAdOx1 MERS expressing S-glycoprotein. ChAdOx1 is a replication-deficient simian adenovirus vector. Here, study participants received a single intramuscular immunization at three different doses. The vaccine was safe and well tolerated and elicited both humoral and cellular MERS-CoV-specific immune responses (Folegatti et al., 2020).

Koch et al. assessed safety and immunogenicity of the vaccine candidate MVA-MERS-S (Koch et al., 2020). MVA-MERS-S is discussed in more detail in chapter 1.4.3.

1.2 A brief history of smallpox and vaccination

1.2.1 History of smallpox

Smallpox likely first appeared around 10,000 BC at the time of first agricultural settlements in Africa. The earliest evidence for smallpox was found on Egyptian mummies who presented typical skin lesions, e.g. on the mummified head of the Egyptian pharaoh Ramses V who died in 1156 BC (Barquet and Domingo, 1997).

Smallpox was introduced to Europe between the fifth and seventh century. Frequent smallpox epidemics had been reported in the Middle Ages. Overall, smallpox has greatly affected the development of Western civilization (Riedel, 2005).
For example, the decline of the Roman Empire coincided with a large-scale epidemic called the plague of Antonine, which accounted for almost seven million deaths and was presumably due to smallpox (Littman and Littman, 1973).
It was introduced to the New World by Spanish and Portuguese conquistadors, where smallpox had been instrumentalized and lead to the fall of the Aztec and Inca empires. Also, in North America early settlers spread smallpox to decimate the American Indian population (Barquet and Domingo, 1997).
In the 18th century in Europe, 400,000 people died annually of smallpox. Case-fatality rates ranged from 20 % to 60 %, the mortality rate in infants was even higher and survivors where often disfigured by scars (Riedel, 2005).

1.2.2 Variolation

An early attempt to protect from smallpox was variolation, meaning the subcutaneous instillation of virus into a nonimmune person. Therefore, a lancet with fresh poxvirus material from a pustule of a smallpox infected person was introduced into the arms or legs of the nonimmune person (Barquet and Domingo, 1997, Riedel, 2005).
Variolation was brought to Europe in the early 18th century from Istanbul. Long before, it was practiced in Africa, India, Turkey, and China. However, variolation became popular in Europe, although 2 % to 3 % of variolated people died. Also, variolation sometimes resulted in smallpox outbreaks and the procedure itself helped transmit other diseases such as syphilis. Still, the case-fatality rate after variolation was about 10 times lower than after naturally occurring smallpox (Riedel, 2005).

1.2.3 Vaccination

Based on the so-called milkmaid-myth saying that milkmaids were naturally protected from smallpox after suffering from cowpox disease, Edward Jenner, an English physician, performed his first vaccination (from *vacca,* Latin for cow) in 1796. He therefore used material from fresh cowpox lesions on the hands of a dairymaid and introduced it to an 8-year-old boy. A few months later he challenged the boy with material from a fresh smallpox lesion and the boy did not develop smallpox. He concluded that vaccination was protective against smallpox (Riedel, 2005).

Since then vaccination has been widely used to protect against smallpox. Interestingly, it was shown that, instead of cowpox virus, vaccinia virus was mostly used for vaccination. However, the exact origin of vaccinia virus is unknown (Jacobs et al., 2009).

Smallpox was the first and, so far, only human infectious disease that has been eradicated. After successful worldwide vaccination campaigns the world was declared free of smallpox in 1980 (Breman and Arita, 1980).

During eradication the replication-competent vaccinia virus strain Lister from Lister Institute in Elstree, England was recommended by the WHO and widely used as smallpox vaccine (Garcel et al., 2007, Kennedy et al., 2009b).

In the Federal Republic of Germany as well as in the German Democratic Republic immunization against smallpox was mandatory until the late 1970s for children in their second year of life (Klein et al., 2012).

In the United States, Dryvax® was licensed by the Food and Drug Administration (FDA) and broadly used as smallpox vaccine. It derived from the replication-competent New York City Board of Health (NYCBOH) strain of vaccinia. Lister and Dryvax® vaccine were first-generation smallpox vaccines produced on the skin of claves or other large animals (Kennedy et al., 2009a).

Later, first-generation vaccines were replaced by second-generation vaccines produced in tissue culture. For instance, the second-generation smallpox vaccine ACAM2000® was licensed by the FDA in 2007 and replaced Dryvax® as smallpox vaccine. It derived from a purified clone of the Dryvax® vaccine and is produced in Vero cells (Kennedy et al., 2009b).

Both, first- and second-generation smallpox vaccines were based on live-replicating vaccinia viruses that were administered by intradermal scarification with a bifurcated needle. This resulted in the formation of a characteristic pustule at the vaccination site, which was considered a correlate of protection against smallpox.

Although very effective, the vaccines had high rates of adverse events which included headache, joint and muscle pain, lymphadenopathy, and self-limiting rashes. (Kennedy and Poland, 2007). There was also evidence for postvaccinal myopericarditis (Arness et al., 2004). Rare but potentially life-threatening adverse events like eczema vaccinatum or generalized vaccinia infection have been reported after vaccination with replicating vaccinia virus. Another feared serious adverse event was Postvaccinal Encephalitis (PvE) leading to a lasting impairment or even death.

High risk group for PvE were children initially vaccinated at the age of 2 years or older. In Europe, incidence of PvE in this late vaccinated group was 1:5,000 to 1:100,000. Death occurred in 1 – 2 vaccinees per million vaccinations (Mayr, 2003, Kennedy et al., 2009b).

Furthermore, there were numerous contraindications which precluded individuals with immunodeficiencies, cancer, organ transplant recipients, patients with immunosuppressive therapies, individuals suffering from skin diseases such as atopic dermatitis, eczema, psoriasis or those with heart conditions (Kennedy and Poland, 2007).

In 2004, it was estimated that in case of a novel smallpox outbreak, i.e. due to bioterrorism, immunization with Dryvax® would have been contraindicated in up to 20% of the US population (Harrop et al., 2004).

Considering this and also several imported monkeypox outbreaks in the US, vaccines with better safety profiles were of interest (Kennedy et al., 2009b).

The third-generation smallpox vaccines are based on highly attenuated vaccinia viruses. MVA is a third-generation vaccine that was licensed by the Europe Medicines Agency (EMA) as IMVANEX® in 2013 and by the FDA as JYNNEOS® in 2019. It follows a two-dose regimen and is administered intramuscularly on day 0 and day 28. JYNNEOS® is also licensed against monkeypox (Vaughan et al., 2020).

1.2.4 Vaccine immunology

Briefly, vaccines protect by inducing humoral and cellular immune responses directed against the pathogen. Vaccine-elicited antibodies produced by B lymphocytes are essential for the humoral immune response. There are five different types of immunoglobulins (Ig): IgM, IgG, IgA, IgD and IgE. The primary antibody response is conferred by IgM following Ig class-switch towards IgG, IgA, or IgE during B cell differentiation. IgG is the main Ig during secondary immune responses and can neutralize and opsonize antigens (Siegrist, 2018).

Cellular immune responses are mediated by antigen-specific T lymphocytes. CD8+ cytotoxic T lymphocytes (CTL) are able to recognize and kill infected cells or tumor cells either directly via secretion of granzymes and perforins inducing apoptosis or indirectly by producing antimicrobial cytokines. CD4+ T-helper lymphocytes “help” mediate cellular and humoral immune responses through direct cell contact (cognate

function) or the secretion of specific cytokines. CD4+ T-helper cells have multiple effector mechanisms, such as activation of innate immune cells, B lymphocytes and CTL. Also, some CD4+ T cells function as regulatory T cells with the capacity to suppress immune activation (Chaplin, 2010, Luckheeram et al., 2012, Siegrist, 2018).

1.3 Modified Vaccinia virus Ankara

1.3.1 Origin

In search for a safer smallpox vaccine, the highly attenuated laboratory vaccinia virus strain MVA was developed (Mayr et al., 1975).

It derived from the wild type Chorioallantois Vaccinia virus Ankara (CVA). CVA was used as smallpox vaccine in Turkey and maintained by donkey – calf – donkey passages. It was brought to Munich, Germany in 1953 (Meisinger-Henschel et al., 2007).

Mayr et al. passaged it over 570 times in Chicken Embryo Fibroblasts (Mayr et al., 1975). After passage 516 the virus was renamed to MVA.

MVA was safety-tested during the smallpox eradication in the 1970s in Germany, where it was given to over 120,000 people (Mayr et al., 1978).

1.3.2 Taxonomy and structure

Poxviridae are large double-stranded DNA viruses. Vaccinia viruses belong to the subfamily *chordopoxvirinae* and to the genus *orthopoxvirus* which includes variola virus, the causative pathogen of smallpox, cowpox virus and vaccinia virus, among others. Structural proteins are highly conserved among *orthopoxviruses*. Immunization with vaccinia virus provides cross-protection against variola and other *orthopoxviruses*, such as monkeypox (Jacobs et al., 2009).

The extremely complex genomes of poxviruses encode for several hundred proteins. The viral life cycle is peculiar and includes vaccinia virus-specific replication-enzymes. It consists of three phases: early, intermediate, and late viral gene expression (Lefkowitz et al., 2006).

Due to attenuation of MVA, approximately 15 % of its parental genome is lost. Further, MVA suffered six major deletions and several mutations which affect host-range restriction, immunomodulation, and some structural proteins. The genome size is 178

kb and it has 193 ORF probably corresponding to 177 genes, 25 of which resulted in truncated proteins due to mutations and fragmentation of genes (Antoine et al., 1998). MVA is unable to replicate in human and most mammalian cells. Replication is blocked at a late stage of virion assembly. However, viral gene expression of early, intermediate and late genes is accomplished allowing for recombinant antigens to be expressed under the control of vaccinia virus-specific promoters (Sutter and Moss, 1992).

1.3.3 Immunogenicity

Historically, it was believed that immunity to poxviruses was antibody mediated. However, it seems that T cell memory is also important for protection considering the observation that smallpox vaccine recipients with abnormal T cell functions developed generalized vaccinia virus infections, whereas vaccine recipients suffering from agammaglobulinemia did not (Moss, 2011).

Several studies showed that MVA is highly immunogenic and induces robust humoral and cellular immune responses comparable or superior to other vaccinia viruses, despite its replication-deficiency, although a higher vaccine dose may be required (Chahroudi et al., 2006).

In a phase 3 efficacy trial of MVA, the vaccine was proven safe and immunogenic as smallpox vaccine. Neutralizing antibody titers after MVA immunization were noninferior to neutralizing antibody titers of the control group which had received one vaccination with ACAM2000, which is FDA-approved and based on the replicating NYCBOH strain of vaccinia virus (Pittman et al., 2019).

Unlike other poxviruses, MVA lost the ability to evade innate immune responses leading to chemokine and interferon induction and making it an immunostimulant (Price et al., 2013). Further, Altenburg et al. showed that MVA preferentially targets antigen presenting cells such as dendritic cells (Altenburg et al., 2017).

1.3.4 Longevity of anti-vaccinia virus immunity

A study of Hammarlund et al. showed that > 90 % of volunteers maintained measurable humoral or T cell-mediated immunity for up to 75 years after smallpox vaccination. T cell responses declined over time with a half-life of 8 – 15 years. Humoral responses

remained stable up to 75 years after smallpox vaccination. Therefore, smallpox vaccination could provide long-lived and, in some cases, life-long immunity (Hammarlund et al., 2003).

Combadiere et al. evaluated time effects of T cell memory after smallpox vaccination in humans. In the group with previous smallpox vaccination (last immunization 13 -25 years ago), IFN-*γ* secretion by vaccinia virus-specific memory T cells was measurable above threshold in only 20 % of vaccinees. Further, they observed that effector memory T cell responses vanished ≥ 45 years after prime immunization and were comparable to unvaccinated individuals.
They also showed that this was not only due to aging, e.g. lower frequencies of IFN-*γ*-producing cells, by comparing frequencies of IFN-*γ*-producing cells against control vaccine antigens, such as tuberculin. Some of the study participants had received the Bacille de Calmette et Guérin (BCG) vaccine against tuberculosis in their childhood. Tuberculin-specific IFN-*γ*-producing T cells were still detectable in the vaccinees aged 45 - 63 years.
However, proliferative vaccinia virus-specific memory T cell responses were still present ≥ 45 years after priming (Combadiere et al., 2004).

1.3.5 Use as viral vector

Despite the eradication of smallpox in 1980, the interest in smallpox vaccines never ceased, due to the use of MVA and other vaccinia viruses as gene expression vectors. Recombinant poxviruses can be engineered using homologous DNA recombination. In fact, this occurs naturally with a frequency of 0.1 % between different viral genomes present in an infected cell during the poxviral life cycle. Like other poxviruses, vaccinia viruses replicate in the cytoplasm and use their own transcription systems. For the construction of recombinant vaccinia virus vectors, a recombinant plasmid insertion vector is used that contains the foreign gene as well as a vaccinia specific promoter and vaccinia DNA (**Figure 2**) (Moss, 2013).

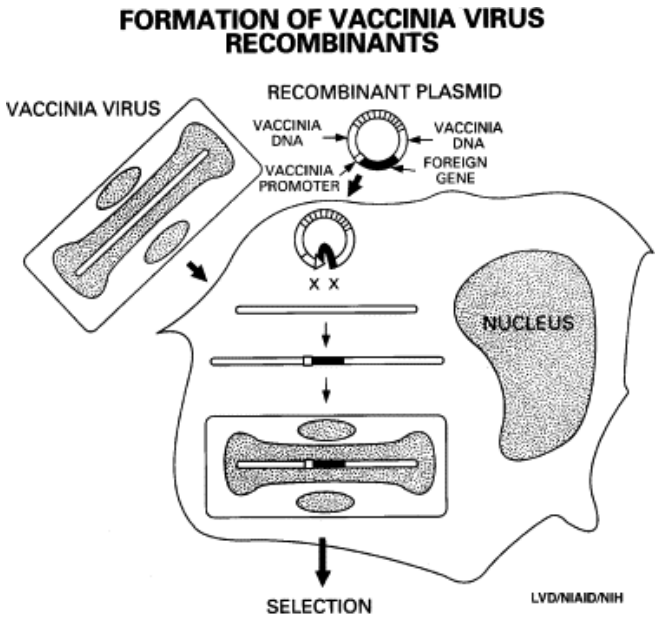


Figure 2: Formation of vaccinia virus recombinants (Moss, 2013)

The use of recombinant MVA as viral vector has advantages. First, it has an excellent safety profile. In contrast to replication-competent vaccinia virus strains, it can be administered even to immunosuppressed patients (Mayr and Danner, 1978).
Also, MVA has lost various immunomodulatory factors. Therefore, it induces rapid local immune responses similar to an adjuvant (Price et al., 2013).
Another advantage of poxviruses is the ability to carry large amounts of heterologous DNA as well as multiple foreign genes. Also, integration and expression of heterologous DNA is reliable. Furthermore, recombinant gene expression is under virus-specific control. The virus does neither integrate into the host genome nor persist in the host. Efficacy and immunogenicity were demonstrated in various vaccine trials (a selection is displayed in **Table 3**). Large-scale vector and vaccine production is also possible (Volz and Sutter, 2017).
In summary, recombinant MVA vectors are valuable tools for the development of novel vaccines and therapeutics.

In 2020, the EMA licensed the first recombinant MVA vaccine MVA-BN-Filo (Mvabea). It is administered in a heterologous prime boost regimen with Ad26.ZEBOV (Zabdeno) following MVA-BN-Filo (Mutua et al., 2019).

Table 3: Selection of recombinant MVA vaccine studies in humans

Vaccine candidate(s)	Regimen	Pathogen	Phase	Reference
Ad26.ZEBOV (Zabdeno) and MVA-BN-Filo (Mvabea)	Hetero-logous	Ebola	Licensed (EMA)	(Mutua et al., 2019)
FP9 ME-TRAP and MVA ME-TRAP	Hetero-logous	Malaria	II	(Bejon et al., 2006)
GeoVax pGA2/JS7 DNA and MVA/HIV62	Homo- and hetero-logous	Human immunodeficiency virus (HIV)	II	(Goepfert et al., 2011, Goepfert et al., 2014)
HIV-1 DNA and HIV-1 MVA	Hetero-logous	HIV	I/II	(Gudmundsdotter et al., 2009, Bakari et al., 2011)
MVA-H5-sfMR	Homo-logous	Influenza	I/II	(Kreijtz et al., 2014)
Triplex	Homo-logous	Cytomegalovirus (CMV)	II	(La Rosa et al., 2017, Aldoss et al., 2020)

1.4 Vector immunity

Vector immunity describes the induction of immune response directed against the viral vector. The role of vector-specific immune responses in vaccine immunogenicity is controversial. Yet, vector immunity is often seen as a possible drawback of vector vaccines.

1.4.1 Recombinant adenovirus and vesicular stomatitis virus vectors

For adenovirus vectors, the effect of pre-existing immunity on the formation of humoral and cellular immune responses to the antigenic insert was evaluated. In a phase 1

adenovirus type-5 vector-based Ebola vaccine trial conducted by Zhu et al. study participants received one immunization with either high dose, low dose, or placebo. Pre-existing adenovirus-specific immune responses were present in 75 – 85 % of study participants. Vaccine-elicited antigen-specific neutralizing antibody titers were reduced in study participants with pre-existing anti-vector neutralizing antibodies. They noted an inverse correlation of -0.39 (p= 0.014) between the baseline adenovirus type-5 neutralizing antibody titers and the vaccine-triggered Ebola glycoprotein-specific antibody titers. Further, they found that anti-vector neutralizing antibodies significantly diminished the antigen-specific T cell responses (Zhu et al., 2015).

Vesicular stomatitis virus (VSV) is another replication-competent viral vector. A problem here is the high proportion of pre-existing immunity to VSV in some areas.
Poetsch et al. reported the induction of vector-specific immune responses to VSV in VSV-naïve individuals. In their study, one-third of study participants developed VSV vector-specific T cell responses and non-neutralizing antibodies after one immunization with VSV-EBOV. They hypothesized that cell-mediated responses to the vector might inhibit the induction of antigen-specific immune responses.
Interestingly, there was a strong positive correlation ($r = 0.7$; $P < .0001$) between VSV vector-specific and Ebola-GP-specific antibodies measured on day 56. This indicates that immune responses are induced against the viral vector itself but also against the antigenic insert (Poetsch et al., 2019).

1.4.2 Recombinant MVA vectors

While MVA is increasingly used as a viral vector in vaccine development against infectious diseases and cancer, questions about the influence of vector-specific immunity are left unanswered. On the one hand, there are concerns about limited vaccine immunogenicity within the scope of pre-existing immunity. In contrast to adenovirus and VSV viral vectors, vaccinia virus infections are not naturally occurring in humans. However, there is the possibility of pre-existing immunity to vaccinia viruses in adults with prior smallpox vaccination in the era before smallpox eradication.
On the other hand, induction of MVA vector-specific immune responses after repeated recombinant MVA immunizations to re-boost immune responses to the antigenic insert could also interfere negatively with vaccine immunogenicity. In the past, MVA vector vaccine studies often showed diverging results (Cottingham and Carroll, 2013).

The influence of pre-existing vaccinia virus-specific immune responses after smallpox vaccination on vaccine immunogenicity has been evaluated in previous studies. Gudmundsdotter et al. studied the effects of previous smallpox vaccination on the immunogenicity of a recombinant MVA vaccine candidate expressing several HIV-antigens. They showed that the recombinant MVA vaccine candidate effectively boosted DNA-primed HIV-specific immune responses in humans, despite previous smallpox vaccination. However, the magnitude of humoral and cellular antigen-specific immune responses was lower in previously smallpox vaccinated study participants compared to study participants without pre-existing vaccinia immunity (Gudmundsdotter et al., 2009).

In mice, the effect of pre-existing vaccinia virus-specific immunity was addressed in a study of Altenburg et. al in 2018. An MVA-based influenza vaccine was tested in the presence and absence of pre-existing vaccinia virus-specific immunity. They showed that pre-existing immunity limited the induction of antigen-specific antibodies and almost completely inhibited the induction of antigen-specific T cells. However, pre-existing vector immunity did not reduce survival of rMVA-H5 vaccinated mice after a lethal H5N1 influenza virus challenge (Altenburg et al., 2018).

Further, the formation of MVA vector-specific immunity after repeated immunizations with recombinant MVA has been addressed. Kreijtz et al. conducted a phase 1/2a homologous prime-boost vaccine trial with an MVA-based H5N1 influenza vaccine (MVA-H5-sfMR) in healthy individuals. Despite induction of vector-specific immune responses, they observed strong antigen-specific antibody responses after second and third immunizations (Kreijtz et al., 2014).

Additionally, the influence of vector immunity was studied in heterologous vaccine regimens. Bejon et al. conducted a phase 1 malaria vaccine trial in using a heterologous prime-boost regimen with two recombinant viral vectors in adults and children in Kenia. Both recombinant viral vectors encoded for the pre-erythrocytic antigen ME-TRAP. They performed either one or two immunizations with the recombinant fowlpox vector FP9 ME-TRAP followed by one immunization with MVA ME-TRAP, each immunization was given three weeks apart. They observed that anti-

vector T cell responses negatively correlated with T cell responses against the antigenic insert (r= -0.33, p= 0.03) (Bejon et al., 2006).

Goepfert et al. compared homologous and heterologous vaccination regimens with recombinant MVA and DNA vaccine candidates expressing HIV-1 virus-like particles. They observed higher antigen-specific antibody titers in the homologous regimen with three recombinant MVA immunizations than in the heterologous regimen, despite the presence of MVA vector-specific T cells (Goepfert et al., 2011).

1.4.3 MVA-MERS-S

MVA-MERS-S is a viral vector vaccine using the highly attenuated and replication-deficient poxvirus strain Modified Vaccinia virus Ankara (MVA). Recombinant MVA expresses the full-length S-glycoprotein of MERS-CoV which is under transcriptional control of the vaccinia virus early/late promoter mH5 and is introduced into the MVA genome at the deletion site III (Song et al., 2013).

It was safe and immunogenic in animal models. In mice, Song et al. demonstrated the induction of high-levels of MERS-CoV neutralizing antibodies after MVA-MERS-S immunization (Song et al., 2013).

Haagmans et al. challenged MVA-MERS-S vaccinated and non-vaccinated dromedary camels with MERS-CoV and observed a significant reduction of excreted infectious virus and viral RNA transcripts in the vaccinated animal group. As a positive side effect, MVA vector-specific antibodies provided cross-protection to camelpox virus (Haagmans et al., 2016).

The open-label, phase 1, first-in-human vaccine trial „Safety, Tolerability and Immunogenicity of Vaccine Candidate MVA-MERS-S" (NCT03615911) against Middle East Respiratory Syndrome Coronavirus was conducted between November 2017 and May 2019 at the University Medical Center Hamburg-Eppendorf (UKE) in Hamburg, Germany. 26 healthy study participants aged between 18 – 55 years were recruited. Prior MVA vaccination was a key exclusion criterion. The results recently have been published (Koch et al., 2020).

Briefly, study participants were vaccinated intramuscularly on day 0 and day 28 with either a low dose (1 x 10^7 plaque-forming units (PFU)) or a high dose (1 x 10^8 PFU) of the vaccine candidate MVA-MERS-S. A subgroup received a second booster

immunization with 1 x 10^8 PFU twelve months (+/- four months) after the prime immunization (Fathi et al., separate paper in preparation).

Primary endpoints were safety and tolerability, secondary endpoint of the study was immunogenicity. Humoral immune responses were measured in ELISA and neutralization tests. Further, an interferon-*γ* (IFN-*γ)* Enzyme-linked ImmunoSpot (ELISpot) assay was performed to evaluate T cell immunity.

Three of the 26 recruited study participants discontinued the trial after the first immunization due to personal reasons or a urinary tract infection, respectively. Repeated immunizations with MVA-MERS-S were well tolerated and showed only transient mild-to-moderate, dose-dependent reactogenicity. No severe or serious adverse events were reported. Most common adverse events were local pain and swelling at the injection site and headache, fatigue or malaise.

In the MERS-S1-ELISA, seroconversion was observed in nine (75 %) of twelve participants in the low-dose group and eleven (100 %) participants in the high-dose group after the second immunization. There was a statistically significant, positive correlation between binding antibody titers and MERS-CoV-specific neutralizing antibodies (Spearman correlation r= 0,86, p= 0,0001). Ten (83%) of twelve immunized participants in the low-dose group and ten (91%) of eleven immunized participants in the high-dose group showed MERS-S-specific T cell responses after the second immunization.

In the subsequent boost study, all study participants showed seroconversion after the third vaccination with MVA-MERS-S (manuscript in preparation by Fathi et al.).

In summary, Koch et al. demonstrated that vaccination with MVA-MERS-S was safe and immunogenic (Koch et al., 2020). A subsequent phase1b/2 trial will start at the end of 2020.

1.5 Hypothesis and study objectives

MVA vector-specific immune responses after immunization with the novel vaccine candidate MVA-MERS-S have not been evaluated previously in clinical trials. Data from other recombinant MVA vaccine candidates show diverging results. While an animal study in mice indicated a negative effect of pre-existing vaccinia virus-specific immunity on vaccine immunogenicity (Altenburg et al., 2018), another study in humans reported only minimal to no influence (Gudmundsdotter et al., 2009).
Homologous and heterologous prime boost regimens with recombinant MVA vaccine candidates were highly immunogenic after repeated immunizations (Kreijtz et al., 2014, Goepfert et al., 2011). By contrast, another study reported a limiting effect on immunogenicity due to the development of vector immunity in a prime boost regimen with two poxvirus vectors (Bejon et al., 2006).
In summary, the results in the above-mentioned studies indicate that vector immunity remains poorly understood and illustrate the need for further investigations.
Therefore, this study aims to evaluate vector immunity within the scope of a phase 1 vaccine trial with the vaccine candidate MVA-MERS-S against MERS-CoV.

Hypothesis: Vaccination with the vaccine candidate MVA-MERS-S induces MVA vector-specific immune responses which may impact immunogenicity of the antigenic insert MERS-S.

To evaluate MVA vector-specific T cell immunity, the first aim was to establish an IFN-*γ* MVA vector-specific ELISpot assay to detect MVA vector-specific T cell responses. Second, the presence of MVA vector-specific T cells in the MVA-MERS-S vaccinated cohort was measured at different time points post vaccination.
The third aim was the measurement of humoral anti-vector immune responses. Therefore, an indirect immunofluorescence assay was conducted to assess anti-vaccinia virus IgG.
Lastly, MERS-S-specific cellular and humoral immune responses from the same time points and donors were correlated.
The evaluation of vector-immunity in this MVA-MERS-S vaccinated cohort could be useful to further optimize MVA vaccination schemes for future vaccine studies.

2. Materials and methods

2.1 Materials

2.1.1 Table 4: Consumables

Item	Manufacturer	Use
Biosphere® Filter Tips, sterile (0,1 – 2,5 µl, 2 – 20 µl, 2 – 100 µl, 2 – 200 µl, 100 – 1000 µl)	SARSTEDT AG & Co. KG	Multi Use (MU)
Cell culture dishes	Thermo Scientific™	MU
CELLSTAR® TUBES Polypropylene, graduated, conical bottom, sterile (15 ml, 50 ml)	Greiner bio-one GmbH	MU
ClipTip™ Pipette Tips, sterile (yellow, red)	Thermo Scientific™	MU
Color coded inserts for CryoPure tubes (white, yellow, green)	SARSTEDT AG & Co. KG	Cryopreservation
Counting Slides	Bio-Rad Laboratories GmbH	Cell counting
CryoPure tubes 1,6 ml, sterile (white, yellow, green)	SARSTEDT AG & Co. KG	Cryopreservation
Eppendorf tubes (200 µl, 1,5 ml, 2 ml)	SARSTEDT AG & Co. KG	MU
Microscopic cover glass (24 x 65 mm)	R. Langenbrinck GmbH	Immunofluorescence test (IFT)
Pre-coated 12 chamber slides (Vero cells infected with vaccinia virus strain Lister)	Virology Department, Bernhard Nocht Institute, Hamburg, Germany	IFT
Reagent reservoirs, 50 ml	VWR	ELISpot
Serological pipets, sterile (5ml, 10ml, 25ml)	SARSTEDT AG & Co. KG	MU

StrataCooler Cryo Preservation Module	Agilent Technologies, Inc.	Cryopreservation
Syringe-driven Filter Unit, 33 mm, sterile	Merck Millipore Ltd. KGaA	MU
Tissue Culture flask (T75)	SARSTEDT AG & Co. KG	Cell culture
Tissue culture plates (6, 12, 24, 48, 96 well)	SARSTEDT AG & Co. KG	Cell culture
T-Track® ELISpot kit human IFN-*γ* HiSpecificityPRO - **Pre-coated 96 well microtiter plate (12x8 strips)**	Lophius Biosciences GmbH	ELISpot

2.1.2 Table 5: Technical equipment

Item	Manufacturer	Use
Axio Lab.A1 Fluorescence microscope, equipped with: - **470nm LED module** - **filter unit 09 (excitation: 450-490 nm, emission: 515 nm)** - **AxioCam MR R3**	Carl Zeiss AG	IFT
Centrifuge 5810 R	Eppendorf AG	MU
AID ELiSpot Reader ELR07	Autoimmun Diagnostika GmbH	ELISpot
Eppendorf Research® plus mechanical pipette, 8-channel (10 – 100 µl, 30 – 300 µl)	Eppendorf AG	MU
Eppendorf Research® plus mechanical pipette, single-channel (0.5 – 10 µl, 10 – 100 µl, 20 – 200 µl, 100 – 1000 µl)	Eppendorf AG	MU

Heraeus Multifuge X3R Centrifuge	Thermo Fisher Scientific Inc.	MU
Incubation Bath	GFL GmbH	MU
Incubation chamber	Weckert Labortechnik	IFT
Inverse microscope OCM 161	KERN & SOHN GmbH	Cell culture
Pipetboy acu Pipette Controller	INTEGRA Biosciences AG	MU
Polymax 1040 platform shaker	Heidolph Instruments GmbH & Co. KG	IFT
TC 20™ Automated Cell Counter	Bio-Rad Laboratories GmbH	Cell counting
Vortex Genie 2 Vortex Mixer	Scientific Industries, Inc.	MU

2.1.3 Table 6: Media and culture reagents

Item	Manufacturer	Use
Dimethylsulfoxid (DMSO)	Sigma-Aldrich Chemie GmbH	MU
Dulbeco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich Chemie GmbH	MU
Evans blue	Sigma-Aldrich Chemie GmbH	IFT
Fetal Bovine Serum (FBS) superior (Heat-inactivated: 56°C, 1h)	Biochrom GmbH	MU
Hank's Balanced Salt Solution	Sigma-Aldrich Chemie GmbH	MU
Hepes (1M) Buffer solution	Life Technologies Corp.	MU
Histopaque 1077 (Ficoll)	Sigma-Aldrich Chemie GmbH	Isolation of PBMC
Human anti-IgG *FITC* antibody	sifin diagnostics GmbH	IFT

Human IL-2 (100 IU/µl)	Miltenyi Biotec B.V. & CO. KG	*In vitro* Stimulation (IVS)
Human IL-4 (500 IU/µl)	Miltenyi Biotec B.V. & CO. KG	IVS
Human IL-7 (10 µg/ml)	Miltenyi Biotec B.V. & CO. KG	IVS
Modified Vaccinia virus Ankara F6 (MVA F6)	Ludwig-Maximilians University Munich, Germany, kindly provided by Prof. Dr. Gerd Sutter	ELISpot
Penicillin/Streptomycin (5000 IU/5000 µg/mL)	Mediatech GmbH	MU
Pepmix™ CEF Pool (extended)	JPT Peptide Technologies GmbH	ELISpot
Pepmix™ MVA093L (0,5 mg/ml)	JPT Peptide Technologies GmbH	ELISpot
Pepmix™ MVA121L, 2 sub pools (0,5mg/ml)	JPT Peptide Technologies GmbH	ELISpot
Pepmix™ MVA189R (0,5 mg/ml)	JPT Peptide Technologies GmbH	ELISpot
Phenol red	SERVA Electrophoresis GmbH	IFT
Phytohemagglutinin (PHA)	Sigma-Aldrich Chemie GmbH	ELISpot
RPMI-1640 with Glutamine	Sigma-Aldrich Chemie GmbH	MU
Trypan Blue	Sigma-Aldrich Chemie GmbH	Cell counting
Trypsin-EDTA, 0,25%	Life Technologies Corp.	MU
T-Track® ELISpot kit human IFN-γ HiSpecificityPRO	Lophius Biosciences GmbH	ELISpot

- **mAb-AP (1:180 dilution)**
- **Dilution buffer**
- **Washing buffer 1**
- **Washing buffer 2**
- **Stain (BCIP/NBT)**

2.1.4 Table 7: Media preparation

Name	Media	Supplements
R10	RPMI-1640 with glutamine	10 % FBS superior 1 % Penicillin/Streptomycin
R10+	RPMI-1640 with glutamine	10 % FBS superior 1 % Penicillin/Streptomycin 1 % Hepes buffer

2.1.5 Table 8: Software

Product	Version	Developer
AID ELiSpot Software	7.0	Autoimmun Diagnostika GmbH
Endnote	X9	Clarivate Corp.
IBM SPSS statistics	25	IBM Corp.
Microsoft Office 365 ProPlus	1911	Microsoft Corp.
Prism	8	GraphPad Software Inc.
ZEN 2 blue edition	2	Carl Zeiss AG

2.2 Methods

2.2.1 Recruitment of study participants and specimen collection

Healthy volunteers with no prior MVA vaccination were recruited for the phase 1a clinical MVA-MERS-S vaccine trial (NCT03615911) against Middle East Respiratory Syndrome Coronavirus at the University Medical Center Hamburg-Eppendorf in Hamburg, Germany. The immunization schedule is displayed in **Figure 3**. Whole blood samples and serum were collected at defined time points before and after each immunization until day 28 after the second boost immunization. Peripheral Blood Mononuclear Cells (PBMC), plasma and serum were isolated and cryopreserved.

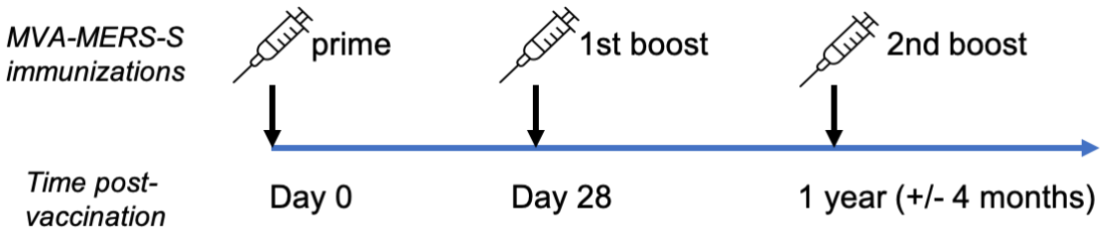


Figure 3: Immunization schedule of the MVA-MERS-S phase 1a trial.

Further, healthy volunteers who had received at least one smallpox vaccination in their childhood were recruited. Whole blood samples were collected and PBMC, plasma and serum were isolated and cryopreserved.
Cryopreserved PBMC and plasma samples from the well characterized "Immunological Norm Values Hamburg Healthy Cohort" served as control cohort.

All study participants gave their written informed consent prior to study entry. Studies were performed in accordance with the Declaration of Helsinki. All data was pseudonymized.

2.2.2 General laboratory practice

All procedures were performed under sterile conditions in a tissue culture hood in a biosafety level 2 laboratory at room temperature, if not noted otherwise.

2.2.3 Counting of cells

Cells were counted with an automated cell counter (gate: 6-17 µm). Counting slides were prepared using 10 µl of cell solution mixed 1:1 with trypan blue.

2.2.4 Thawing of cells

Cells were rapidly thawed in a 37 °C water bath until only a small pellet of ice was visible. Subsequently, cells were decanted into 15 ml tubes which contained pre-warmed culture medium.

2.2.5 Preparation and cryopreservation of plasma

Whole-blood samples from ethylenediaminetetraacetic acid (EDTA)-tubes were transferred into 50 ml tubes and centrifuged at 200 x *g* for 10 minutes (accelerate: 9; brake:9).

Plasma was transferred into a new 15 ml tube, centrifuged at 1000 x *g* for 10 minutes (accelerate: 9; brake: 9) and aliquoted into cryotubes à 1 ml. Subsequently, cryotubes were put on dry ice. Samples were stored at -80 °C.

2.2.6 Isolation and cryopreservation of PBMC

PBMC were isolated from EDTA anticoagulated venous blood samples using density gradient centrifugation (**Figure 4**).

Whole-blood samples were transferred into 50 ml tubes and filled up to 30 ml with Hank's Balanced Salt Solution. Next, the blood solution was carefully layered on top of 14 ml pre-warmed Histopaque 1077. Tubes were centrifuged at 500 x *g* for 30 minutes without brake (accelerate: 1; brake: 0). Subsequently, the PBMC layer on top of Histopaque 1077 was harvested and transferred into a new 50 ml tube. PBMC were washed 3 times with Hank's solution.

For cryopreservation, tubes were filled up to 40 ml with ice-cold R10. PBMC were resuspended and washed. Ice-cold freezing solution, consisting of 90 % FBS superior and 10 % DMSO, was added to a final concentration of 10 x 10^6 PBMC per ml. PBMC solution was aliquoted into pre-cooled and labeled cryotubes. PBMC were incubated on ice for 1 minute and afterwards cooled down in a stratacooler to ensure controlled freezing of PBMC with a freezing rate of -1 °C/minute in a -80 °C freezer overnight for 14 - 24 hours. The day after, PBMC were placed into a liquid nitrogen tank for long-time storage at -195 °C.

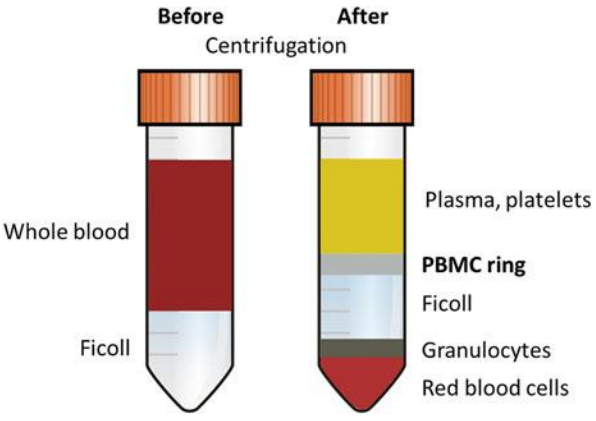


Figure 4: Isolation of PBMC with density gradient centrifugation, published in (Janetzki, 2016).

2.2.7 Preparation and cryopreservation of serum

For the isolation of serum, blood collected in serum tubes was centrifuged at 2500 x *g* for 10 minutes. Samples were aliquoted into pre-cooled and labeled cryotubes, shortly incubated on dry-ice and stored at -80 °C.

2.2.8 Preparation and inactivation of MVA

The virus stock titer was 2 x 10^{10} PFU/ml. Virus stock was diluted in RPMI-1640 medium to a concentration of 2 x 10^{7} PFU/ml, aliquoted and stored at -80 °C.

For inactivation, virus was treated with psoralen (1 µg/ml) for 10 minutes following application of long-wave UV light for 10 minutes. The method psoralen and ultraviolet A (PUVA) method to inactivate vaccinia viruses was described by Tsung et al. in 1996 (Tsung et al., 1996).

2.2.9 Use of MVA overlapping peptide pools

Via literature review three immunogenic MVA antigens were selected for MVA vector-specific T cell stimulation (Cripe et al., 2015).

The MVA antigens used were designed as Overlapping peptides (OLP), which span the entire protein sequence. The selected pools consisted of 15mer long peptides overlapping in 11 amino acids (aa) and were derived from MVA. MVA OLP were dissolved in DMSO and used according to manufacturer's instructions at a concentration of 1 µg/ml, if not noted otherwise. In **Table 9**, selected OLP as well as their corresponding ORF for MVA and for the Vaccinia Virus Copenhagen (CPN) strain as well as the encoded protein with aa length and pool size are listed. In this project the MVA nomenclature for ORF is used, the corresponding CPN ORF is in brackets.

Table 9: MVA overlapping peptide pools

Name	ORF MVA	ORF CPN strain	Protein	UniProt ID	Length (aa)	Pool size
PepMix™ VACV (MVA 121L)	MVA121L	A10L	Major core protein P4a	O57223	891	223 (111+ 112)
PepMix™ VACV (MVA093L)	MVA093L	H3L	IMV heparin binding surface protein	O57206	324	79
PepMix™ VACV (MVA189R)	MVA189R	B22R	Putative 21.7k protein	O57265	188	45

2.2.10 IFN-γ MVA vector-specific ELISpot assay

Rationale

The ELISpot assay is a standard immunological method to evaluate cytokine secretion of antigen-specific memory T cells in response to antigen stimulation. It was first described in 1983 (Czerkinsky and Svennerholm, 1983) for the detection heat-labile (LT) enterotoxin produced by Escherichia coli and later modified for the detection of cytokine-secreting cells (Czerkinsky et al., 1988).

Procedure

The ELISpot assay was performed according to manufacturer's recommendations with some laboratory adapted modifications.

Cryopreserved PBMC were used. Therefore, PBMC were thawed, washed with R10+ and rested at a concentration of 4 x 10^6 PBMC/ml overnight for 16 – 22 hours at 37 °C in a humidified air chamber containing 5 % CO_2.

The next day, PBMC were washed with R10+ and adjusted to a final concentration of 1 x 10^6 PBMC/ml.

MVA OLP, MVA and controls were prepared with R10+. For T cell stimulation MVA OLP at a concentration of 1 µg/ml as a well as live or inactivated MVA at multiplicities of infection (MOI) 1 - 5 were used. DMSO at a concentration of 2 µg/ml served as negative control. PHA at a concentration of 10 µg/ml and CEF (Cytomegalovirus,

Epstein-Barr virus and influenza virus peptide pool) at a concentration of 1 µg/ml served as positive controls.

Next, pre-coated 96 well strip plates for IFN-*γ* capture (Lophius Biosciences GmbH) were used. 50 µl of OLP, MVA and controls were added to the corresponding wells. Subsequently 100 µl PBMC (100,000 PBMC/well) were transferred to each well. All samples were tested at minimum in duplicates and in triplicates whenever possible. The plate was incubated at 37°C and 5% CO_2 overnight for 16 hours.

On the third day, cell suspension and medium were discarded. Wells were washed six times with 200 µl washing buffer 1. Subsequently, 100 µl IFN-*γ* detection antibody solution mAb-AP (monoclonal antibody conjugated with alkaline phosphatase, 1:180 dilution) was added and incubated for two hours. After incubation, detection antibody solution was discarded, plates were washed three times with 200 µl washing buffer 1 and three times with 200 µl washing buffer 2.

To visualize spots, 50 µl staining solution NBT/BCIP was added and incubated for seven minutes in the dark. The staining reaction was stopped with warm tap water. Plates were washed three times with warm tap water and then placed in the air stream of a laminar flow cabinet for one hour or dried overnight at room temperature.

Evaluation and quality control of spot counts

Scanning and spot counting was done with the AID ELISpot Reader and AID ELISpot Reader software. Spot counts were double checked manually.

Means of negative controls per assay and study participant were subtracted from spot counts of corresponding wells to normalize samples. Next, means of replicates were calculated and extrapolated to 1,000,000 PBMC.

For quality control, mean of negative controls had to be ≤ 50 spot forming cells (SFC)/million and mean PHA response had to be ≥ 1000 SFC/million.

A response to stimulation with MVA and MVA OLP was considered positive at a minimum of 50 SFC/million (empirical threshold) and at least four times of the spot count of day 0 (baseline). For example, if a study participant had 20 SFC/million (normalized mean) after stimulation with MVA on day 0, a mean positive response had to be ≥ 80 SFC/million (4 x 20 SFC/million).

The threshold of 50 SFC/million is commonly used in other vaccine trials to monitor T cell responses (Combadiere et al., 2004, Koch et al., 2020).

2.2.11 In vitro Stimulation of PBMC

Rationale

Even though the ELISpot assay is a sensitive method for the detection of antigen-specific memory T cells, their frequency might be below detection level. Therefore, using *in vitro* stimulated PBMC is seen as an option to increase their frequency (Chudley et al., 2014).

Procedure

On the first day, cryopreserved PBMC from the Smallpox vaccine cohort were thawed and washed with R10+. For stimulation, PBMC were pulsed with 1 µl of MVA OLP in ~200 µl R10+ (concentration of each peptide: 0,5 µg/200 µl) for 90 minutes at 37 °C, in a humidified air chamber containing 5 % CO_2.

After incubation, PBMC were washed and added to a 24 well plate. 4 x 10^6 PBMC/well in 1 ml culture medium were used.

As culture medium, R10+ either supplemented with IL-2 (100 IU/ml) or IL-4 (1500 IU/ml) and IL-7 (10 ng/ml) was prepared.

Next, PBMC were incubated at 37 °C, 5% CO_2 for 9 days. On days three and seven, the culture medium (R10+ and cytokines) was replaced with 0.5 ml fresh culture medium.

After 9 days, PBMC were transferred into 15 ml tubes, washed, and used for *in vitro* ELISpot assays.

2.2.12 In vitro IFN-γ MVA vector-specific ELISpot assay

For *in vitro* ELISpot assays, *in vitro* stimulated PBMC were used. Nine days post stimulation, cell numbers were adjusted to 4 x 10^6 PBMC/ml and rested overnight. The *in vitro* ELISpot assays followed the (*ex vivo)* ELISpot protocol as described earlier (see chapter 2.2.10).

2.2.13 Anti-vaccinia virus IgG indirect immunofluorescence test

Rationale

Indirect immunofluorescence is a technique for the detection of circulating antibodies in body fluids, e.g. serum or plasma. Antigen-specific antibodies, if present, bind to an antigen. This complex can be detected by a secondary antibody which is labeled with a fluorochrome.

Procedure

Cryopreserved pre-coated 12-chamber slides were used. They had been prepared with Vero (African green monkey kidney) cells infected with vaccinia virus strain Lister in 2005.

Further, cryopreserved plasma samples were tested, unless otherwise noted.

A protocol from the Virology Department of the Bernhard Nocht Institute in Hamburg, Germany was adapted for this assay.

Plasma samples, serum controls and pre-coated slides were thawed. Plasma samples were diluted 1:10 with “red PBS”, PBS supplemented with 1% phenol red, in a 96-well microtiter plate. Further serial dilutions were prepared up to a dilution of 1:320.

Usually, five samples in two dilutions were tested together with the positive and negative control on one 12-chamber slide. First, 1:10 and 1:40 dilutions were tested per sample. If both dilutions were positive, further serial dilutions were tested up to 1:320. Well characterized cryopreserved positive and negative serum controls were kindly provided by the Virology Department of the Bernhard Nocht Institute.

15 µl of diluted sample was added to the corresponding chamber on the pre-coated 12-chamber slide and incubated for one hour at 37 °C in a humidified chamber in the incubator. Next, plasma and serum samples were removed by rinsing of slides with PBS. Slides were placed into a glass cuvette containing PBS and washed while rotating on a platform shaker for 5 minutes.

Anti-human IgG FITC labeled antibody, diluted 1:350 with PBS and supplemented with 1 % Evans blue, was added. Slides were incubated for 20 minutes at 37 °C in a humidified chamber in the incubator.

After rinsing and washing, slides were covered with a microscopic cover glass and evaluated under AxioLab fluorescence microscope using a 470nm light-emitting diode (LED) module and the filter unit 09 (excitation 450-490 nm, emission 515 nm) from Zeiss. Images were taken with AxioCam MR R3.

In line with the standard operation protocol from the virology department at the Bernhard Nocht Institute, Hamburg, an IgG titer $\geq$ 1:40 was considered as positive. Evaluation of samples was performed by one operator.

2.2.14 MERS-S-specific ELISpot assay

The MERS-S-specific IFN-γ ELISpot assay was performed in our working group according to manufacturer's recommendations (ImmunoSpot, Cellular Technology, Cleveland, OH, USA; 384-well plate) with some laboratory adapted modifications.
The method has been described in the publication by Koch et al. (Koch et al., 2020).
Briefly, PBMC were stimulated for 16 h in triplicates with five overlapping peptide pools spanning the entire MERS-CoV-S amino acid sequence (JPT). PHA and CEF served as positive controls, and serum-free medium (Cellular Technology) supplemented with DMSO served as negative control.

For evaluation and quality control of spot counts see chapter 2.2.10. To correlate results to MVA vector-specific immune responses, the spot counts of all five MERS-S peptide pools were added.

2.2.15 MERS-CoV S1 IgG ELISA

The MERS-CoV S1 IgG ELISA was performed at the partner site Erasmus Medical Center (EMC) in Rotterdam, Netherlands. The method has been described in the publication by Koch et al. (Koch et al., 2020).
Briefly, serum samples of study participants were analyzed at different time points post vaccination. Therefore, 96-well microtiter plates were coated overnight with 1 µg/mL MERS-CoV S1 protein. Plates were blocked for one hour. Next, diluted sera (1:100) were added and incubated for one hour. Bound antibodies were detected using horseradish peroxidase (HRP)-labelled rabbit anti-human IgG (Dako), signal was developed with 3,3',5,5'-Tetramethylbenzidine (TMB, ThermoFisher Scientific) and sulfiric acid was used to stop the reaction. Absorbance was measured at 450 nm and an optical density of 0,5 was set as cutoff value.

2.2.16 Statistical analysis

SPSS and GraphPad Prism were used for statistical analysis. All data was examined for normal distribution. A nonparametric distribution of the data was assumed, if not noted otherwise. In general, nonparametric data was described as median with inter-quartile range (IQR). P-values of 0.05 or less were considered significant and calculated using two-tailed t-tests with a 95 % confidence interval. The Mann-Whitney-U-test was applied for comparisons between groups. For pair-wise comparisons the

Wilcoxon matched-pairs signed rank test was used. To compare more than two groups pair-wise Friedman test was applied. Correlation of two variables was calculated using the Spearman correlation.

3. Results

3.1 Study population

3.1.1 MVA-MERS-S cohort

This cohort consists of participants of the MVA-MERS-S phase 1a clinical trial. Study participants (n=26) were healthy, aged between 18 - 55 years and had no prior MVA vaccination.

Longitudinal samples of the subgroup (n=10), who received a second boost immunization, were used to assess MVA-vector specific immune responses. Of this subgroup all study participants are female and white. Three study participants received the low dose vaccine on day 0 and day 28, seven study participants received the high dose vaccine. The second booster immunization was given in the high dose. The characteristics of the MVA-MERS-S subgroup are listed in **Table 10**.

Table 10: Characteristics of MVA-MERS-S subgroup

ID	Age (years)	Sex	No. of vaccinations	Dose group
ML1	25	female	3	Low dose
ML2	18	female	3	Low dose
ML3	31	female	3	Low dose
MH1	36	female	3	High dose
MH2	32	female	3	High dose
MH3	21	female	3	High dose
MH4	31	female	3	High dose
MH5	27	female	3	High dose
MH6	25	female	3	High dose
MH7	40	female	3	High dose
	⌀ 28.6	**♀ 1.0**	**⌀ 3**	

3.1.2 Smallpox vaccine cohort

Healthy volunteers who had remembered receiving at least one smallpox vaccination in their childhood were recruited. In seven out of ten cases vaccination could be verified via vaccination certificate (**Table 11**).

The Smallpox vaccine cohort consists of ten volunteers aged between 47 – 63 years (mean age 54.9 years), 70 % are female and 30 % are male, all white. They had

received one up to three smallpox vaccinations (mean 1.6 immunizations). Time since prime immunization ranged from 46 – 62 years (mean 53.8 years). Last immunization occurred 46 – 51 years before sample collection (mean years after last immunization 48.8 years).

Table 11: Characteristics of Smallpox vaccine cohort

ID	Age (Years)	Sex	No. of vaccinations	Time since prime immunization (Years)	Time since last immunization (Years)	Verification
S1	63	male	3	62	51	Remembered
S2	52	female	1	51	51	Vaccination certificate
S3	58	male	2	57	46	Vaccination certificate
S4	61	female	2	60	49	Remembered
S5	51	female	1	50	50	Vaccination certificate
S6	62	female	2	61	51	Vaccination certificate
S7	47	female	1	46	46	Vaccination certificate
S8	51	female	1	50	50	Vaccination certificate
S9	55	male	2	54	47	Vaccination certificate
S10	49	female	1	47	47	Typical scar
	⌀ 54.9	**♀ 0.7**	**⌀ 1.6**	**⌀ 53.8**	**⌀ 48.8**	

3.1.3 Control cohort

The Control cohort consists of healthy adults aged between 23 – 42 years (mean age 29.1 years) with no prior smallpox or MVA vaccination. 70.6 % were female and 29.4 % were male participants.

3.2 Establishment of an IFN-γ MVA vector-specific ELISpot assay

3.2.1 Selection of immunogenic MVA proteins

To start, assay establishment was performed with MVA overlapping peptide pools for stimulation. They were designed for CD8+ as well as CD4+ T cell stimulation. Due to the large size of MVA, identification of immunogenic proteins is complex and there are several immunodominant antigens. However, via literature research three immunodominant antigens for the establishment were selected (Cripe et al., 2015, Jing et al., 2008, Terajima et al., 2003, Kennedy and Poland, 2007). The three selected immunogenic proteins are MVA189R (B22R), MVA093L (H3L) and MVA121L (A10L). Of note, in the literature sometimes the nomenclature for vaccinia virus Copenhagen (CPN) strain instead of MVA nomenclature is used to describe MVA antigens. Therefore, both nomenclatures are listed here, CPN strain ORF are in brackets.

3.2.2 Testing of Smallpox vaccine cohort for assay establishment

To establish an IFN-γ MVA vector-specific ELISpot assay, samples from the Smallpox vaccine cohort were planned as test cohort.

In nine out of ten study participants there were no detectable vaccinia virus-specific T cell responses in the ex vivo ELISpot assay above threshold (≥ 50 SFC/million PBMC). Study participant S8 had a positive response to OLP MVA093L with 490 SFC/million and to MVA121L_1 with 106.67 SFC/million (**Figure 5**).

To increase the frequency of IFN-γ-secreting vaccinia virus-specific memory T cells, as described previously by Combadiere et al. (Combadiere et al., 2004), an *in vitro* ELISpot assay was performed in selected samples from the Smallpox vaccine cohort (n= 4). Briefly, this led to higher T cell responses compared to *ex vivo* (see **Appendix 1**).

In the following, assay establishment was additionally performed with samples from MVA-MERS-S cohort due to low responder rates in the Smallpox vaccine cohort.

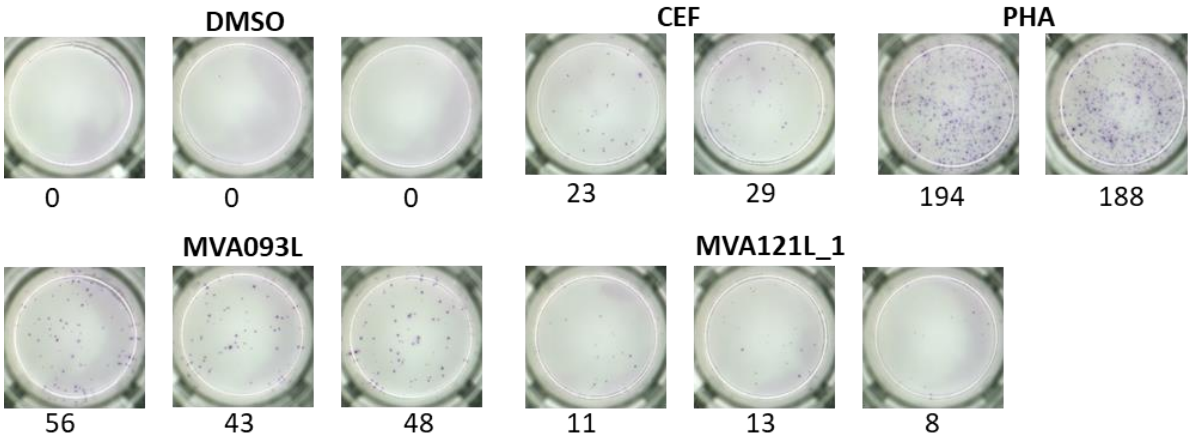


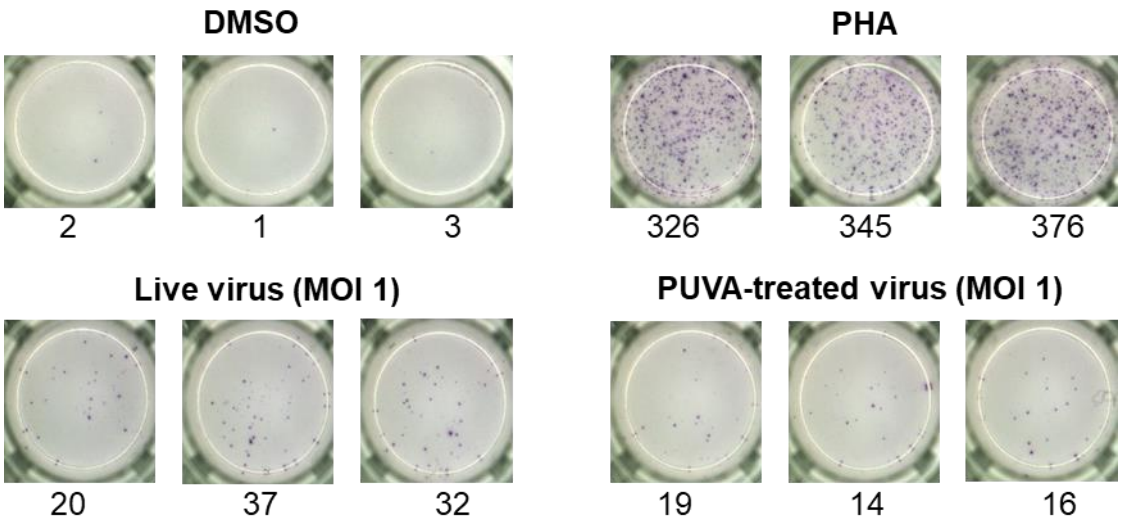
Figure 5: Quality controlled ELISpot image of S8 from Smallpox vaccine cohort. *Ex vivo, 100,000 PBMC per well, numbers indicate spot count, DMSO as negative control, OLP MVA093L and MVA121L_1 were tested in triplicates, CEF and PHA as positive controls were tested in duplicates.*

3.2.3 Testing of live and inactivated MVA

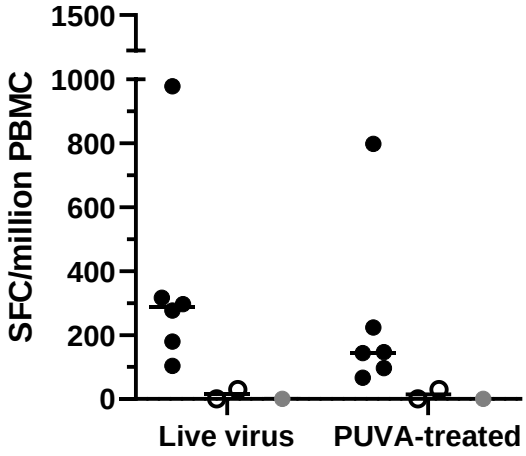
For stimulation with whole MVA several methods were tested to optimize the assay. First, live and inactivated MVA was tested. Study participant ML7 on day 56 was selected to demonstrate effect of virus treatment on spot formation **(Figure 6A)**.

Samples from different time points (but not day 0) of MVA-MERS-S cohort (n=6), as well as samples from Smallpox vaccine cohort (n=2) and Control cohort (n=1) were stimulated with either live MVA (MOI 1) or PUVA-treated virus (MOI 1) (**Figure 6B**). Median T cell responses after stimulation with MVA were 286.7 SFC/million (IQR= 160.8 – 482.1 SFC/million) for live virus and 145.0 SFC/million (IQR= 89.17 – 367.1 SFC/million) for PUVA-treated virus in the MVA-MERS-S cohort (n=6).

Differences in samples stimulated with live virus and PUVA-treated virus were statistically significant (p= 0.0313). Spot counts of samples stimulated with live virus tended to be higher.



A



B

Figure 6: Higher magnitude of T cell responses after stimulation with live virus.

A *Quality controlled ELISpot image of ML7 Day 56 (exemplary). Ex vivo, 100,000 PBMC per well, numbers indicate spot count, tested in triplicates, DMSO as negative control, PHA as positive control.*

B *Samples of MVA-MERS-S cohort (n=6, black dots), Smallpox vaccine cohort (n=2, black circles) and Control cohort (n=1, grey dot) stimulated with live and PUVA-treated MVA (MOI 1), dots and circles indicate mean spot count per sample, lines indicate median of group.*

3.2.5 Testing of different multiplicities of infection

Different MOI were tested for stimulation of PBMC with MVA: 1, 2.5 and 5. Samples from the MVA-MERS-S cohort (n=6) were tested and compared with samples from the Control cohort (n=12).

Median spot counts of MVA-MERS-S cohort were 183.3 SFC/million (IQR= 77.5 – 292.5 SFC/million) at MOI 1, 341.7 SFC/million (IQR= 179.2 – 737.5 SFC/million) at MOI 2.5 and 436.7 SFC/million (IQR= 221.2 – 720.0 SFC/million) at MOI 5.

Median spot counts of the Control cohort were 13.3 SFC/million (IQR= 0.0 – 25.8 SFC/million) at MOI 1 and 33.3 SFC/million (IQR= 6.7 – 72.5) at MOI 2.5. MOI 5 was not tested in the Control cohort (**Figure 7**).

Spot counts were significantly elevated in MVA-MERS-S cohort compared to controls ($p= 0.0004$ for MOI 1 and $p= 0.0020$ for MOI 2.5 with Mann Whitney t test).

MOI 1 showed the lowest background reactivity in the Control cohort and was therefore selected as optimal MOI for the assay.

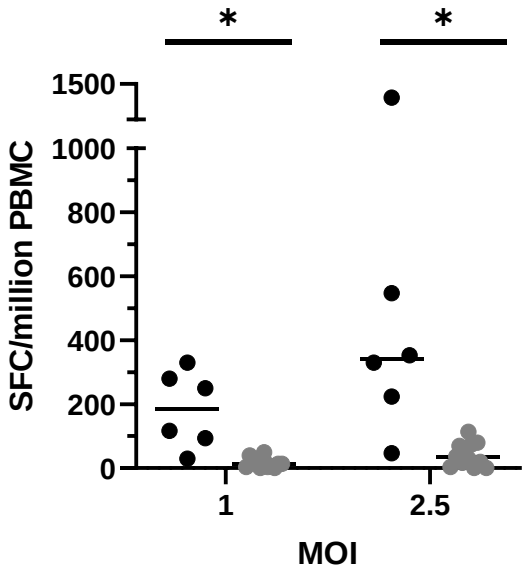


Figure 7: Virus MOI 1 was optimal for T cell stimulation in the ELISpot assay*. Samples of MVA-MERS-S cohort (n=6, black dots) and samples of Control cohort (n=12, grey dots), stimulated with MVA MOI 1 and MOI 2.5, dots indicate mean spot count per sample, lines indicate median of group, *p ≤ 0.05.*

3.2.6 Testing of different stimulation methods

Further, two different methods for stimulation of PBMC were tested. We compared spot counts of samples stimulated with MVA directly on the plate with spot counts of samples, which were first incubated with MVA for 1 h, eventually washed and added to the plate. The second method was previously described (Combadiere et al., 2004). Comparison in these two stimulation methods resulted in no statistically significant differences in spot counts (data not shown).

Because the direct method is faster and requires less steps, this method was chosen.

In summary, establishment of an IFN-γ MVA vector-specific ELISpot assay was completed. As optimal parameters, live MVA and a virus MOI of 1 were selected.

3.3 Evaluation of MVA vector-specific T cell responses via ELISpot

To evaluate MVA vector-specific immune responses after three immunizations with MVA-MERS-S, samples of a subgroup of 10 study participants of the MVA-MERS-S cohort were tested. All study participants of the subgroup had received a second booster immunization twelve months (+/- four months) after priming.

To gain longitudinal data, samples from five time points per study participant were tested. The selected time points were day 0 (D0), day 28 (D28), day 42 (D42) after prime immunization, day 0 of the second boost (B0) and 14 - 28 days after the second boost (B14/28).

Samples from one study participant at the above-mentioned time points were tested simultaneously on two 96-well plates. Either one or two study participants were tested on the same day. All samples from the control cohort (n=12) were tested simultaneously on the same day.

Of note, for study participant MH4 only three time points could be included. Here, time point B0 was not tested due to limited availability of PBMC and D28 did not show a sufficient PHA response and was therefore excluded.

3.3.1 Background reactivity

Background reactivity of selected MVA OLP and MVA was measured using samples from the Control cohort (n=12). Therefore, samples were run in triplicates.

Median background reactivity for MVA189R, MVA093L and MVA121L_1 was 0,00 SFC/million. Median background reactivity for MVA121L_2 was 1,67 SFC/million. Median background reactivity for MVA at MOI 1 was 13.33 SFC/million.
Next, background reactivity of samples from MVA-MERS-S cohort (n=10) before prime immunization with MVA-MERS-S on day 0 was measured.

In summary, both background reactivities are comparable, slight differences are not statistically significant **(Figure 8)**.

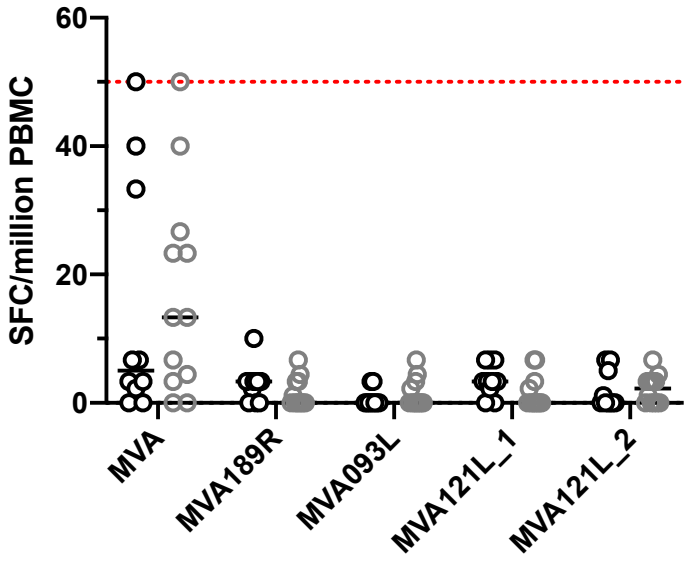


Figure 8: Comparable background reactivity of MVA-MERS-S cohort on day 0 (n=10, black circles) and Control cohort (n=12, grey circles). *ELISpot, circles indicate mean spot count per sample, lines indicate median of group, red dotted line serves as empirical threshold.*

3.3.2 T cell responses after stimulation with MVA

For the evaluation of MVA-vector specific T cell responses, samples were tested with the established IFN-γ MVA vector-specific ELISpot assay stimulated with live MVA (MOI 1).

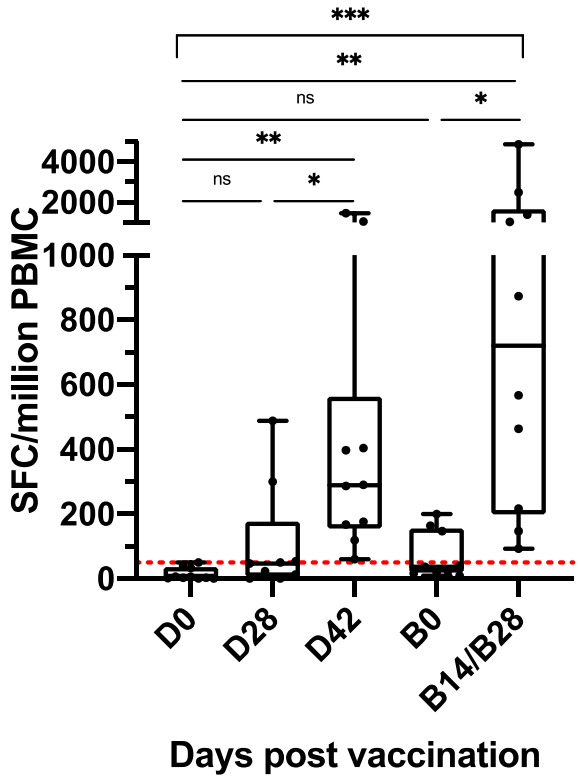
Figure 9 shows the results of the ELISpot assay. T cell responses at baseline (D0) were below threshold (≥ 50 SFC/million) in all ten study participants with a median response of 5.0 SFC/million (IQR= 1.7 – 35.0 SFC/million). Median T cell responses on D28 were 46.7 SFC/million (IQR= 6.7 – 176.7 SFC/million) with three of nine positive responders, on D42 288.3 SFC/million (IQR= 154.6 – 562.5 SFC/million) with ten positive responders, on B0 36.7 SFC/million (IQR= 18.3 – 155.0 SFC/million) with

three of nine positive responders and on B14/28 720.0 SFC/million (IQR= 199.2 – 1658.0 SFC/million) with ten positive responders (**Figures 9A, 9B and Table 11**).

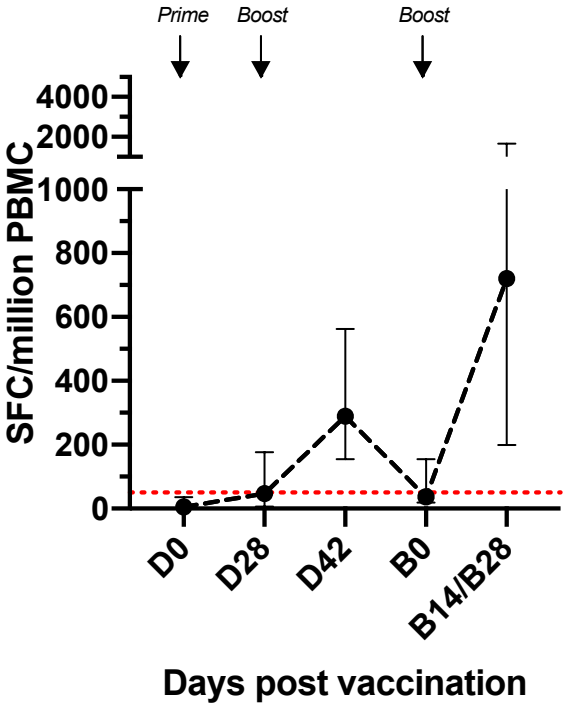
Statistical significance between two time points (groups) was tested with Wilcoxon matched pairs signed rank test. Differences between time points D0 and D28 as well as D0 and B0 were not statistically significant. Statistically significant were D0 and D42 (p= 0.0020), D0 and B14/28 (p= 0.0020), D28 and D42 (p= 0.0039), B0 and B14/28 (p= 0.0039).

The statistical significance comparing all groups was tested with Friedman test (p< 0.0001).

Figures 9C and 9D characterize longitudinal T cell responses of all ten study participants of high dose group and low dose group.



A



B

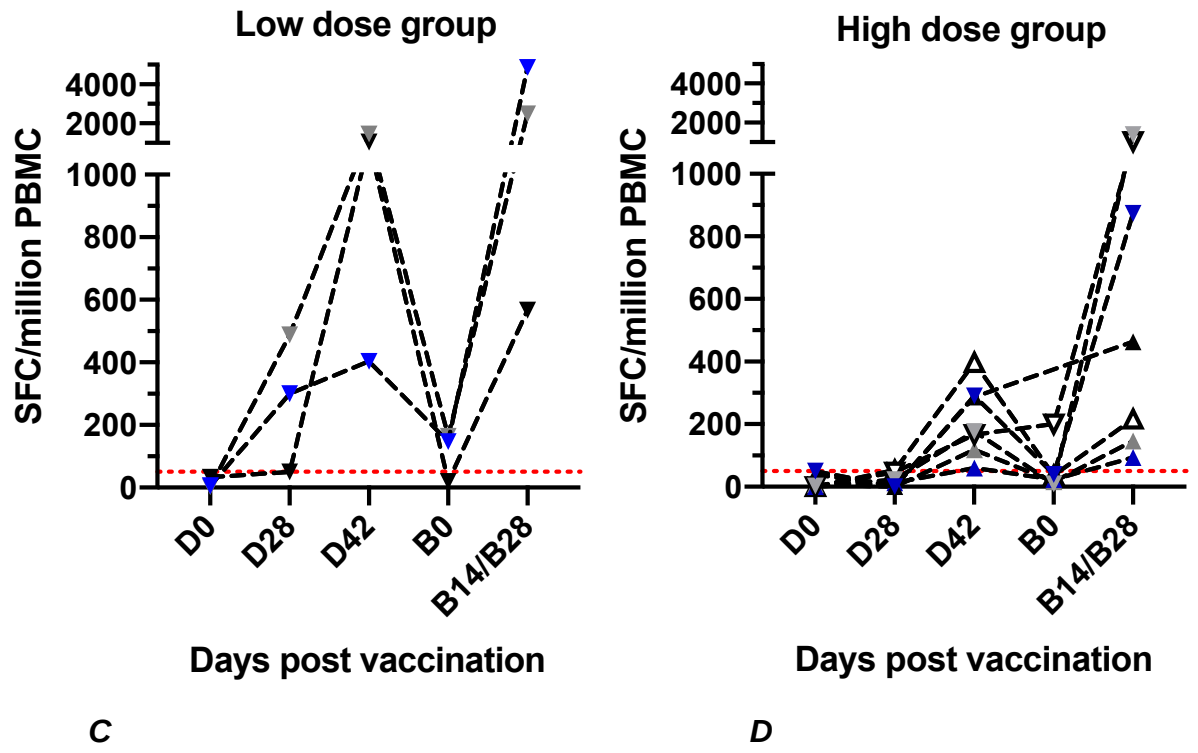


Figure 9: ELISpot results of MVA-MERS-S cohort after stimulation with live MVA (MOI 1). *Samples from subgroup with a second boost of MVA-MERS-S cohort (n=10), five time points: day 0 (D0), day 28 (D28), day 42 (D42) after priming, day 0 of the second boost (B0) and 14 to 28 days after the second boost (B14/28), red dotted line indicates threshold.*

A Boxplot. *Dots indicate mean per sample, box indicates median per group/time point with IQR, whiskers indicate Minimum and Maximum, significance between two time points (groups) was tested with Wilcoxon matched-pairs signed rank test, * p= 0.0039, ** p= 0.0020, significance between all five time points was tested with Friedman test, *** p< 0.0001.*

B Longitudinal data. *Dots indicate median per time point, error bars indicate IQR.*

C Longitudinal data from study participants of Low dose group (n=3).

D Longitudinal data from study participants of High dose group (n=7).

3.3.3 T cell responses after stimulation with MVA OLP

Study participants exhibited a diverse response to the selected MVA OLP. 30 % of study participants showed no response to the selected MVA OLP. 50 % had detectable responses to one MVA OLP and 20 % had detectable T cell responses to two MVA OLP.

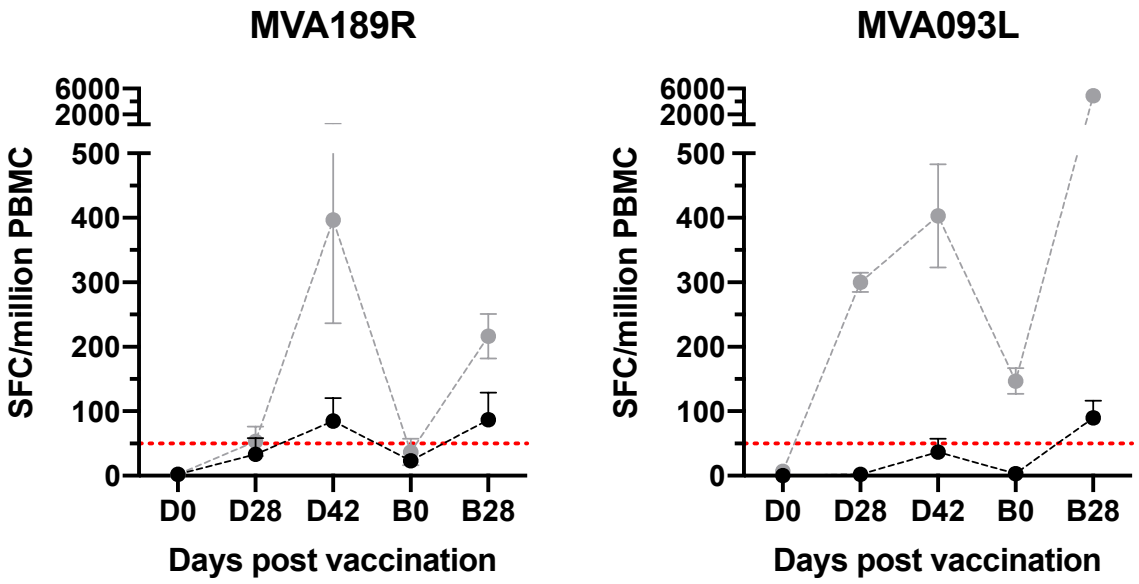
40 % had measurable T cell responses to MVA189R (B22R) which encodes for a 21.7k protein, e.g. study participant MH1 (**Figure 10A)**.

10 % of vaccinees had responses to MVA093L (H3L), which encodes for the IMV heparin binding surface protein on the membrane, e.g. study participant ML3 **(Figure 10B)**.

40 % of study participants showed a robust response to stimulation with MVA121L (A10L), which was separated in two sub pools due to the large size of the protein, e.g. study participant ML3 responded to sub pool MVA121L_1 **(Figure 10C)** and study participant MH7 responded to sub pool MVA121L_2 **(Figure 10D**). MVA121L (A10L) encodes for major core protein P4a.

The responses peaked 14 days after the first boost (day 42 post-prime) and had a second peak response 14 to 28 days after the second boost.

Further, the dynamics of T cell responses after MVA OLP stimulation measured in the IFN-*γ* ELISpot assay seem to follow the dynamics of T cell responses measured after stimulation with (whole) MVA (**Figure 10A – D,** grey dots).



A ***B***

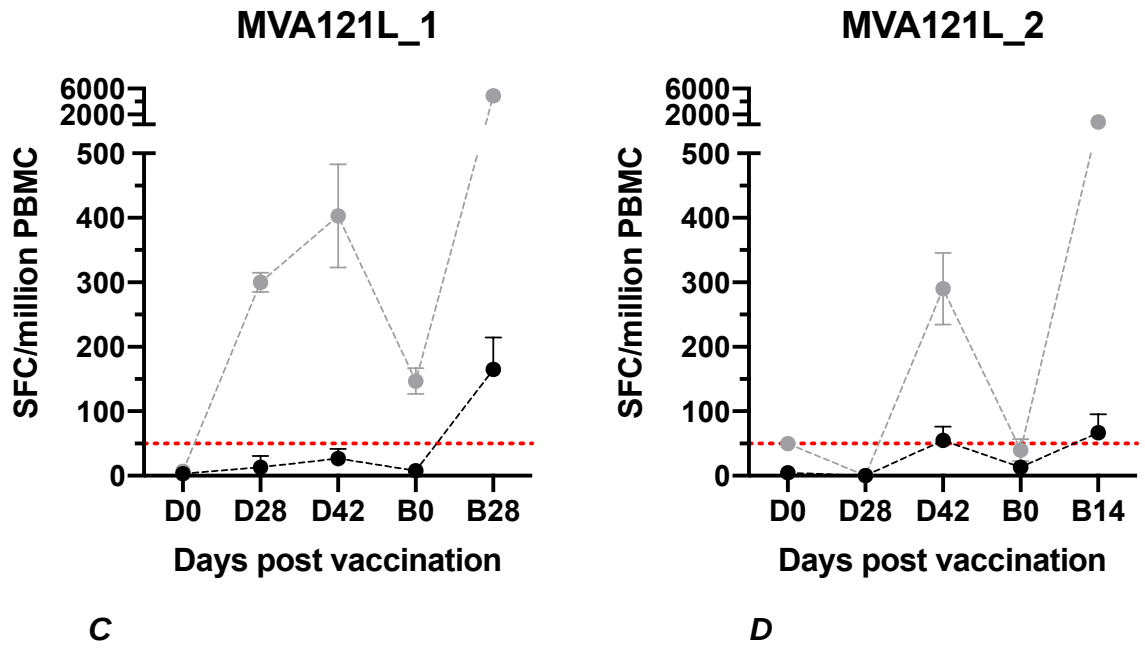


Figure 10: Top responders to MVA OLP (black dots) show comparable dynamic after stimulation with MVA (grey dots). *IFN-γ vector-specific ELISpot assay, subgroup with a second boost of MVA-MERS-S cohort (n=10), 5 time points: day 0 (D0), day 28 (D28), day 42 (D42) after priming, day 0 of the second boost (B0) and 14 to 28 days after the second boost (B14/28), tested in duplicates or triplicates, dots indicate means, error bars show standard deviation, black dots mark responses to MVA OLP, grey dots show corresponding responses to MVA, red dotted line indicates threshold. Top responders to MVA OLP were selected, study participant in brackets:* ***A*** *MVA189R (MH1);* ***B*** *MVA093L (ML3);* ***C*** *MVA121L_1 (ML3);* ***D*** *MVA121L_2 (MH7).*

In summary, **Table 12** demonstrates positive responders at different time points. MVA vector-specific T cells were above threshold in all study participants after 14 – 28 days after the second and third MVA-MERS-S immunization when stimulated with MVA.

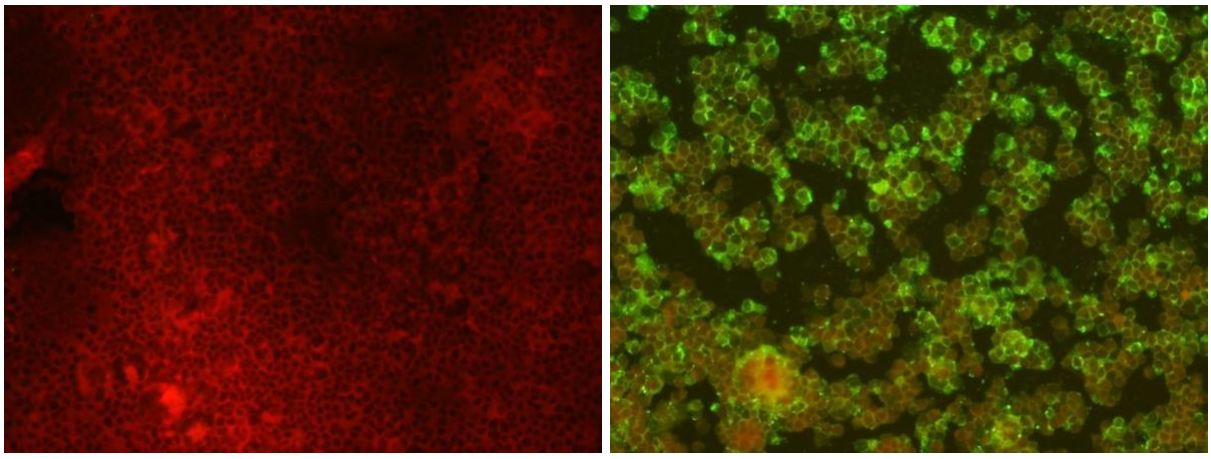
Table 12: Responders to MVA and MVA OLP at different time points

Time point	MVA	MVA189R	MVA093L	MVA121L_1	MVA121L_2
D0	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %
D28	33.33 %	0.00 %	0.00 %	0.00 %	10.00 %
D42	100.00 %	30.00 %	0.00 %	10.00 %	30.00 %
B0	33.33 %	0.00 %	0.00 %	0.00 %	0.00 %
B14/28	100.00 %	30.00 %	10.00 %	10.00 %	20.00 %

3.4 Evaluation of anti-vaccinia virus IgG responses via IFT

Anti-vaccinia virus humoral immune responses were assessed via indirect immunofluorescence test (IFT). Therefore, plasma samples were tested for the presence of anti-vaccinia virus IgG.

For evaluation of binding IgG antibodies samples were evaluated under a fluorescence microscope. Exemplary, **Figure 11** demonstrates a representative negative and positive control. The negative control displays Vero cells stained with phenol red. The positive control indicates the green-fluorescent FITC-labelled anti-IgG antibodies that bind to anti-vaccinia virus IgG.



Negative control ***Positive control***

Figure 11: Representative examples of images of anti-vaccinia virus IgG indirect immunofluorescence test (IFT). *Negative and positive control at 200x magnification.*

3.4.1 Background reactivity

Samples from the Control cohort (n=10) were tested to evaluate background reactivity of the anti-vaccinia virus IgG IFT. Nine out of ten study participants showed an antibody titer ≤ 1:10. One study participant had an antibody titer of 1:10 (data not shown).

3.4.2 Smallpox vaccine cohort

Samples from the Smallpox vaccine cohort were tested to evaluate functionality of the assay (after pre-coated slides had been stored for 15 years at – 80 °C). Study

participant S4 had positive anti-vaccinia virus IgG titer of 1:80 (**Figure 12**). Other study participants from the Smallpox vaccine cohort had either no measurable IgG titer or a titer < 1:40 and therefore did not meet criteria for positivity (data not shown).

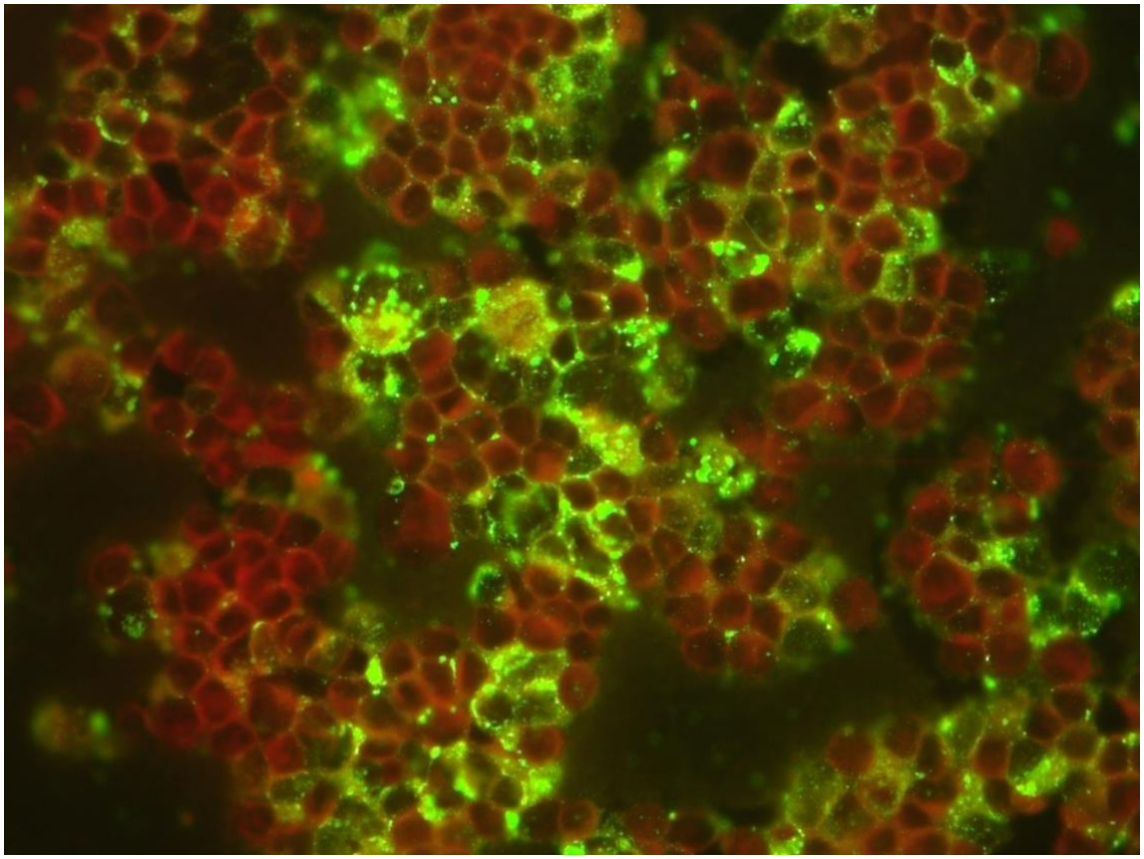
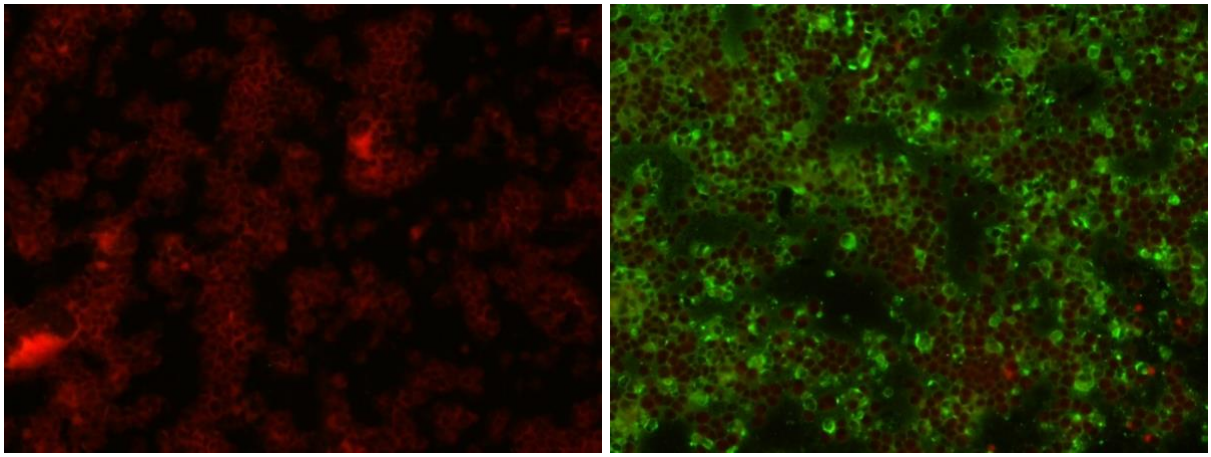


Figure 12: Exemplary image of anti-vaccinia virus IgG IFT from study participant S4 (top responder) from Smallpox vaccine cohort. *Titer 1:40, 400x magnification, green fluorescent dots around cells represent typical immunofluorescence pattern and indicate presence of anti-vaccinia virus IgG.*

3.4.3 MVA-MERS-S cohort

To evaluate anti-vector humoral immune responses, an anti-vaccinia virus IgG IFT was performed in the same subgroup (n=10) of the MVA-MERS-S cohort as for the ELISpot assay. To gain longitudinal data, IFT was performed at different time points. Time points D0, D28, D42, B0 and B28 were tested.

Figure 13 demonstrates exemplary IFT images of study participant MH6. The participant was selected because of the good IgG response on B28.



D0 ***B28***

Figure 13: Exemplary images of anti-vaccinia virus IgG IFT on D0 and B28 from study participant MH6 from MVA-MERS-S-cohort.

A: *D0, 1:10, 200x, negative* ***B:*** *B28, 1:40, 200x, positive.*

Figure 14A displays the anti-vaccinia virus IgG titers of all ten study participants over time. **Figure 14B** gives an overview of the dynamic of antibody responses. In this cohort, anti-vaccinia virus IgG responses peaked 14 days after the second immunization, eventually declined to 1:10 on B0 and peaked a second time 28 days after the third immunization with IgG titers ranging from 1:40 to 1:320.

No pre-existing humoral immune responses were found at baseline. 28 days after the first immunization 1 study participant showed a positive antibody titer, whereas 14 – 28 days after the second and third immunization all study participants had a measurable antibody response (**Table 13**).

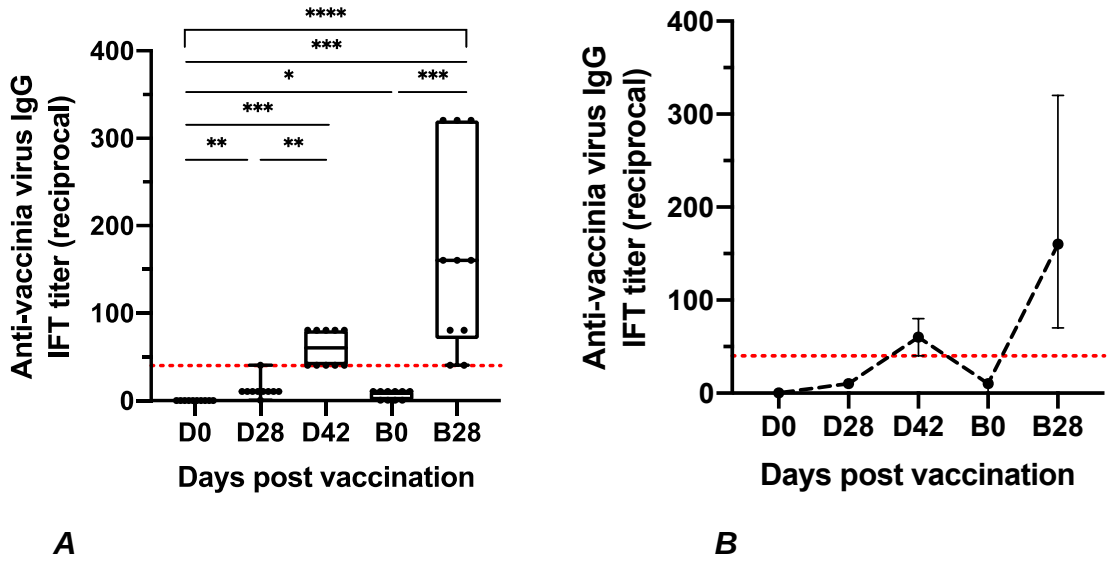


Figure 14: Anti-vaccinia virus IgG titer from MVA-MERS-S cohort (n=10), IFT.

A Boxplot. *Dots indicate single values per study participant, box shows median per group with IQR and range (whiskers), red dotted line demonstrates threshold.*

B Longitudinal data. *Dots show median titer per group with IQR.*

Table 13: Responders to anti-vaccinia virus IgG IFT at different time points.

Time point	Anti-vaccinia virus IgG
D0	0.00 %
D28	10.00 %
D42	100.00 %
B0	0.00 %
B28	100.00 %

3.5 Correlations

3.5.1 MVA vector-specific T cell responses and anti-vaccinia virus IgG responses

MVA vector-specific T cell responses and anti-vaccinia virus IgG responses were correlated using Spearman correlation.

14 to 28 days after receiving the third MVA-MERS-S immunization (B14/28) a positive (r= 0.6753) and statistically significant (p= 0.0378) correlation was found (**Figure 15**).

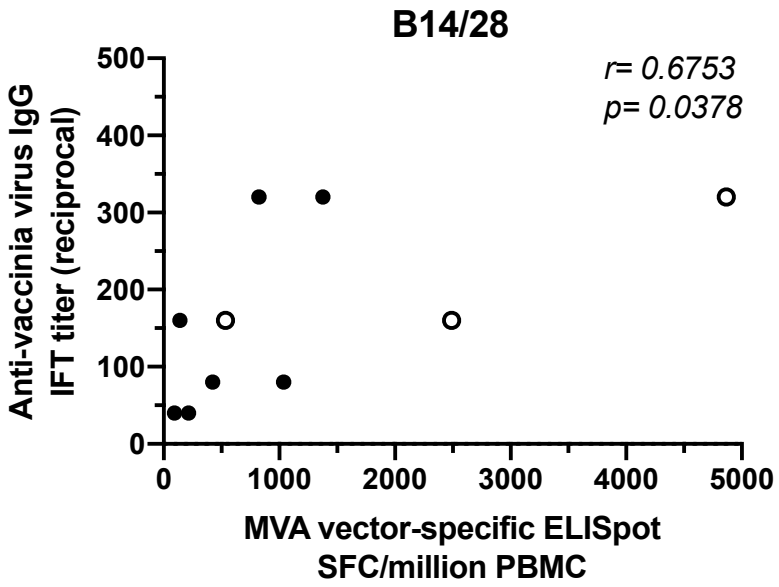


Figure 15: MVA vector-specific T cell responses and anti-vaccinia virus IgG titers correlate after three immunizations with MVA-MERS-S on B14/28. *T cell responses were measured with ELISpot assay, IgG was measured with IFT, samples from MVA-MERS-S cohort (n=10), black dots indicate high dose group (n=7), black circles indicate low dose group (n=3).*

3.5.2 MVA vector-specific and MERS-S-specific T cell responses

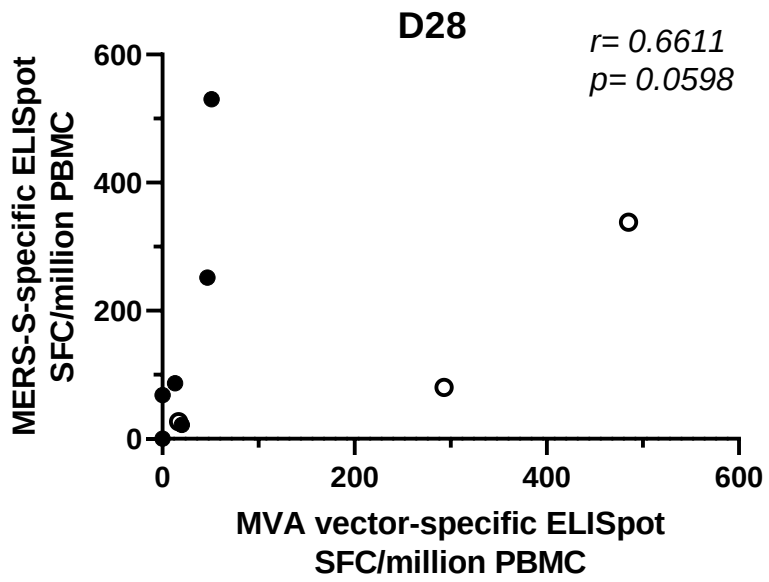
MERS-specific and MVA vector-specific T cell responses were correlated using Spearman correlation.

28 days after prime immunization (D28), there was no statistically significant correlation (r= 0.6611, p= 0.0598, **Figure 16A**). Also, 14 days after the second immunization (D42) no statistically significant correlation was found (r= 0.6000, p= 0.0734, **Figure 16B**).

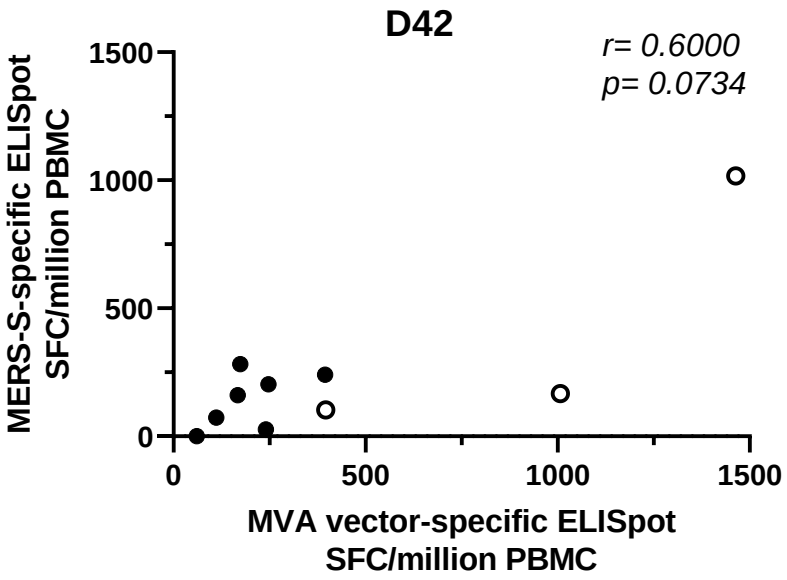
T cell responses measured on day 14 or day 28 after the third immunization (B14/28) showed a correlation (r= 0.7333, p= 0.0202, **Figure 16C**).

MVA vector-specific T cell responses that had been induced after the first immunization on D28 and MERS-S-specific T cell responses measured on D42, 14 days after receiving the second vaccination, showed a correlation (r= 0.6946, p= 0.0448, data not shown).

There was no correlation of MVA vector-specific T cell responses twelve months (+/- four months) after prime immunization (B0) and MERS-S-specific T cell responses 28 days after the third vaccination (B28) (r= 0.5788, p= 0.1096, data not shown).


D28
r= 0.6611
p= 0.0598
MERS-S-specific ELISpot
SFC/million PBMC
600
400
200
0
0
200
400
600
MVA vector-specific ELISpot
SFC/million PBMC

A


D42
r= 0.6000
p= 0.0734
MERS-S-specific ELISpot
SFC/million PBMC
1500
1000
500
0
0
500
1000
1500
MVA vector-specific ELISpot
SFC/million PBMC

B

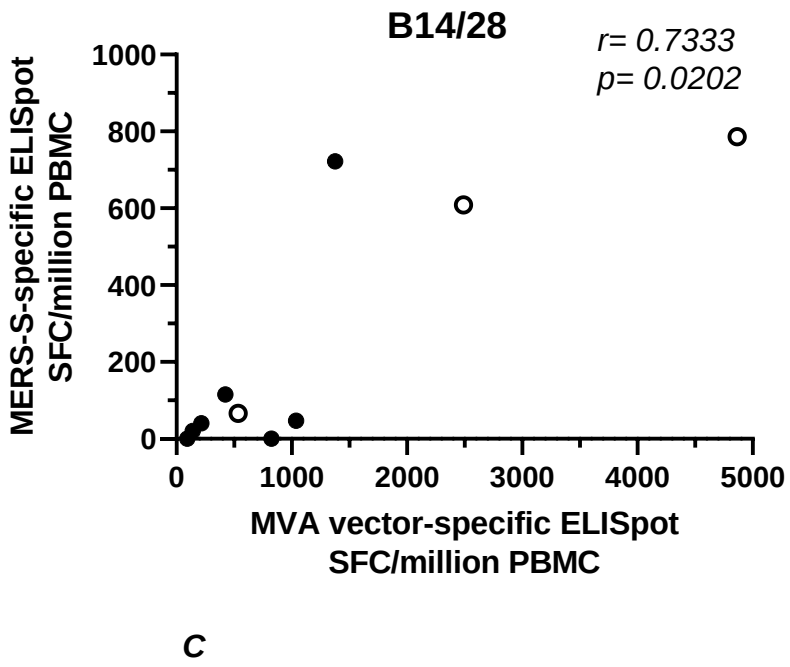


Figure 16: No statistically significant correlation between MVA vector-specific and MERS-S-specific T cell responses after one (A) and two (B) immunizations, but after three (C) immunizations with MVA-MERS-S. *Results from MVA vector-specific and MERS-S-specific ELISpot assays of MVA-MERS-S subgroup (n=10), black dots indicate high dose group (n=7), black circles indicate low dose group (n=3),* ***D28 (A), D42 (B) and B14/28 (C)****.*

3.5.3 MVA vector-specific T cell responses and anti-MERS-S1 IgG responses

Between MVA vector-specific T cell responses and anti-MERS-S1 IgG responses, there was no statistically significant correlation on D28 (r= -0.1597, p= 0.6779), D42 (r= 0.4182, p= 0.2325) and B14/28 (r= 0.4012, p= 0.2506, **Figure 17**).

Further, MVA vector-specific T cell responses on D28 and anti-MERS-S1 IgG responses on D42 did not correlate significantly (r= 0.1841, p= 0.6334), neither did MVA vector-specific T cell responses on B0 and anti-MERS-S1 IgG responses on B28 (r= 0.3560, p= 0.3532).

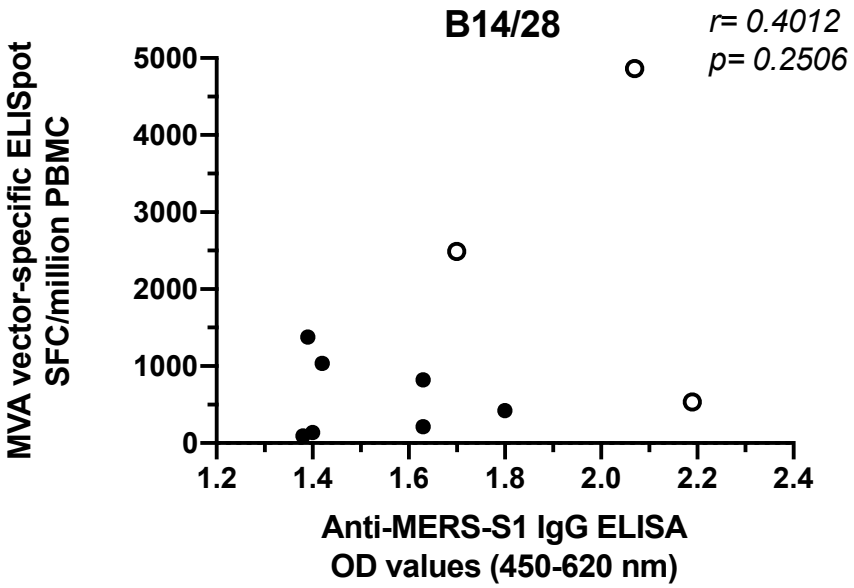


Figure 17: No statistically significant correlation between MVA vector-specific T cell responses and anti-MERS-S1 IgG responses in MVA-MERS-S cohort (n=10) 14 – 28 days after the third immunization (B14/28). *High dose group (n=7, black dots), low dose group (n=3, black circles).*

3.5.4 Anti-vaccinia virus IgG and anti-MERS-S1 IgG responses

As displayed in **Figure 18**, anti-vaccinia virus IgG measured in IFT and anti-MERS-S1 IgG measured in ELISA did not correlate (r= 0.000, p > 0.9999) on B14/28. Also, no statistically significant correlation was found on D28 (r= 0.3514, p= 0.3556) and D42 (r= 0.4526, p= 0.2222).

Further, anti-vaccinia virus IgG responses on D28 and anti-MERS-S1 IgG responses on D42 did not show a statistically significant correlation (r= -0.3114, p= 0.4667), neither did anti-vaccinia virus IgG responses on B0 and anti-MERS-S1 IgG responses on B28 (r= -0.07194, p= 0.9190).

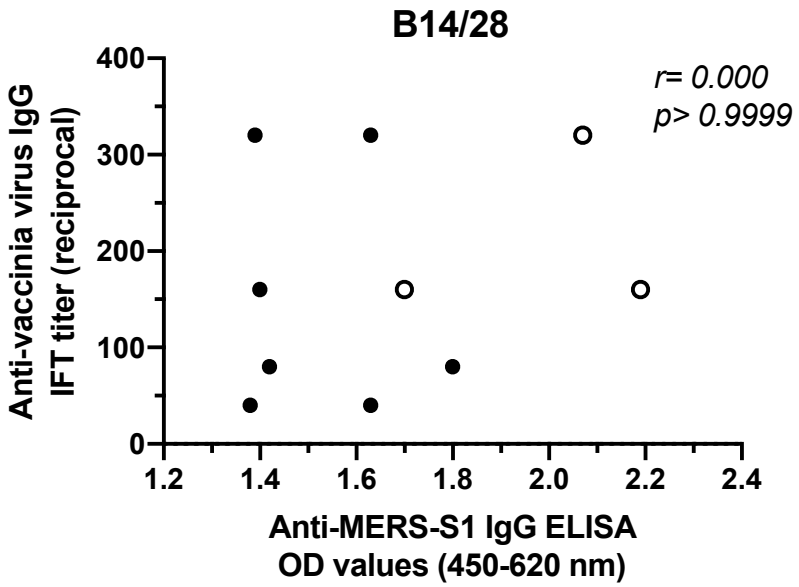


Figure 18: No correlation between anti-vaccinia virus IgG and anti-MERS-S1 IgG responses in MVA-MERS-S cohort (n=10) 14 – 28 days after the third immunization. *High dose group (n=7, black dots), low dose group (n=3, black circles).*

4. Discussion

The aim of this work was the evaluation of MVA vector immunity within the scope of a phase 1 trial with the vaccine candidate MVA-MERS-S against MERS-CoV.

MVA vector-specific T cell responses and anti-vaccinia virus IgG responses were induced after vaccination.

There was no inverse correlation between anti-vector and anti-MERS-S immune responses. In contrast, a positive correlation was found between MVA vector-specific T cell responses and MERS-S-specific T cell responses 14 to 28 days after the third immunization ($r= 0.7333$, $p= 0.0202$). No statistically significant correlation could be found between anti-vaccinia virus IgG and anti-MERS-S1 IgG responses in this study.

4.1 MVA vector-specific ELISpot assay was optimally stimulated with live MVA

To optimize the IFN-*γ* ELISpot assay, live and inactivated virus was tested for stimulation. For vaccinia viruses, a cytopathic effect on virus infected cells was described leading to possible cell damage or apoptosis (Tsung et al., 1996), which may result in low T cell responses in the ELISpot assay. In this study, T cell responses after stimulation with PUVA-treated virus were significantly lower than after stimulation with live MVA ($p= 0.0313$).

Harrop et al. described similar observations and hypothesized that the use of inactivated virus could stimulate CD8+ T cells to a lower degree than live virus due to different pathways for antigen-presentation (Harrop et al., 2004).

MOI 1 had a lower background reactivity in the control group than MOI 2.5 and was therefore selected as optimal MOI, as did other study groups (Howles et al., 2010, Combadiere et al., 2004).

4.2 Vaccination induces robust anti-vector cellular and humoral immune responses

After stimulation of PBMC with live MVA in the ELISpot assay, all study participants showed a robust induction of MVA vector-specific T cell responses after repeated immunization. However, the magnitude of the anti-vector response varied among study participants.

Our results are comparable to observations made by Smith et al., who had seen a similar kinetic of MVA vector-specific T cell responses within the scope of a recombinant MVA.MEL3 vaccine trial where one group had received up to four injections in a bi-weekly interval with a vaccine dose of 5 x 10^7 PFU intradermally (Smith et al., 2005).

In this study, only one of ten study participants had developed a positive anti-vaccinia virus-specific IgG titer 28 days after the first immunization (D28), while after second and third immunizations all ten study participants showed a positive response.

Pittman et al. conducted a phase 3 efficacy trial of MVA as a vaccine against smallpox. The study participants had no history of prior smallpox vaccination. One group was vaccinated with 1 x 10^8 TCID50 MVA on day 0 and day 28. Like in our study, peak antibody titers were observed 6 weeks (42 days) after priming. However, after the first immunization Pittman et al. observed a high rate of seroconversion, in 94.6 % in the ELISA and in 94.1 % in the Plaque reduction neutralization test (PRNT) four weeks (28 days) after priming (Pittman et al., 2019) .

These results differ from our 10 % seroconversion on day 28. One possible explanation could be the use of different immunological assays to detect IgG. Further, Pittman et al. defined seroconversion as seropositivity above baseline, in patients who were seronegative at baseline, and as at least two times the baseline titer, in patients who were seropositive at baseline (Pittman et al., 2019).

4.3 T cell responses to MVA OLP are diverse among study participants

The advantage of stimulation with overlapping peptide pools in the ELISpot assay is their independence of the human leucocyte antigen (HLA)-haplotype. Especially if specific epitopes and HLA-types are unknown (Larsson et al., 1999).
The results from this study are comparable to several other studies indicating that immunodominance to vaccinia viruses is conferred by multiple antigens and extremely

diverse in humans. As of August 2020, 59 HLA human epitopes of 36 MVA antigens are listed on the immune epitope database and analysis resource (IEDB, 2020).

Cripe et al. measured vector-specific T cell-responses after injection of the oncolytic cancer vaccine candidate Pexa-Vec in children. In their *in vitro* ELISpot assay they saw robust responses after vaccination to most of the tested MVA OLP, including MVA121L, MVA189R, MVA105L, MVA093L, MVA018L and MVA074R (Cripe et al., 2015).
Terajima et al. found several CD8+ T cell epitopes conserved among variola and vaccinia viruses. Like others, they observed a diverse CD8+ T cell response with several immunodominant antigens, which contain multiple epitopes and are recognized by different HLA class I molecules. Among them, MVA189R (B22R) had two HLA-A2 restricted epitopes and one epitope restricted to HLA-B7 (Terajima et al., 2006).
Jing et al. demonstrated that also CD4+ T cell responses to vaccinia viruses are extremely diverse in humans and that abundant structural proteins are immunodominant. Overall, they could detect CD4+ T cell responses for 122 ORF from a total of 180 ORF with a mean of 39 ORF (range 13 – 63 ORF) that were recognized per study participant. Among the most frequently recognized ORF where MVA121L and MVA093L (Jing et al., 2008).

Poxviruses are extremely complex viruses with large double-stranded DNA genomes which encode for several hundred proteins (Antoine et al., 1998). A detailed knowledge of the specific viral antigens and epitopes targeted by the immune system is needed and the search is still ongoing (Kennedy and Poland, 2007).
The data on MVA OLP in this study is helpful to further characterize MVA vector-specific T cell responses and could serve as a starting point for epitope-mapping to find new MVA-specific T cell epitopes.

4.4 Anti-vector immune responses and MERS-S-specific immune responses did not show an inverse correlation

In this study, study participants with high MVA vector-specific T cells tended to have high MERS-S-specific T cells. There was a positive correlation ($r= 0.7333$ and $p= 0.0202$) between MVA vector-specific and MERS-S-specific T cell responses 14 – 28 days after the third immunization with MVA-MERS-S.

While several studies made comparable observations (La Rosa et al., 2017, Kreijtz et al., 2014), others had diverging results (Altenburg et al., 2018, Bejon et al., 2006).

Altenburg et al. described a negative effect of pre-existing vaccinia virus-specific immune responses on the induction of antigen-specific T cell responses in mice, when testing an MVA-based influenza vaccine in the presence and absence of pre-existing vaccinia virus-specific immunity (**Figure 19**). Immune responses to the antigenic insert were significantly reduced in mice primed with MVA or vaccinia virus prior to recombinant MVA vaccination compared to unprimed mice (Altenburg et al., 2018). However, Altenburg et. al did not measure MVA-specific T cell responses.

In the phase 1 MVA-MERS-S trial, study participants had no record of prior smallpox vaccination. Therefore, the influence of previous smallpox vaccination on vaccine immunogenicity was not evaluated.

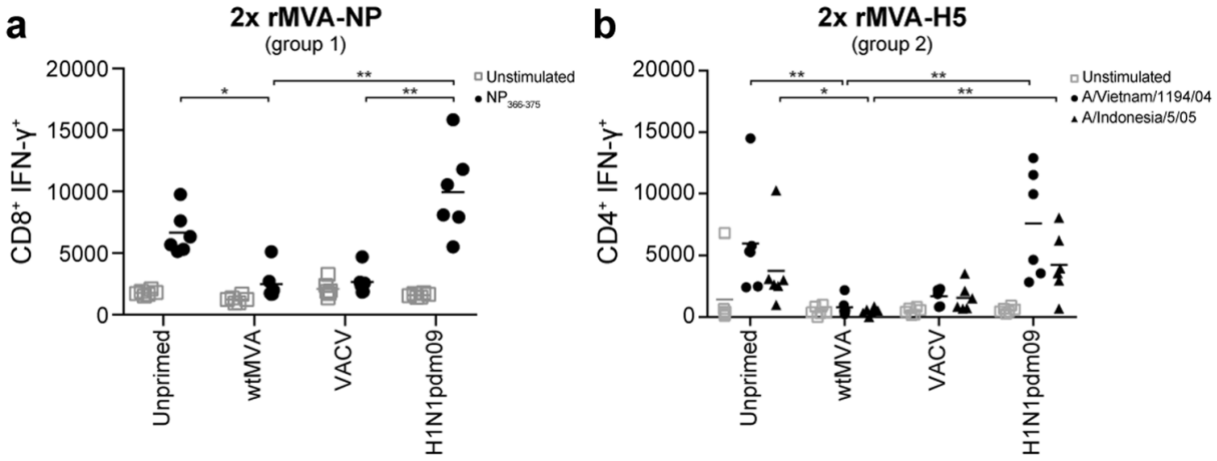


Figure 19: Pre-existing vaccinia virus-specific significantly reduces antigen-specific T cell responses in mice*. Measured with flow-cytometry after intracellular cytokine staining (ICS) of splenocytes, unprimed mice had no prior wild type MVA (wtMVA) or vaccinia virus (VACV) immunization, wtMVA primed mice received two shots of wtMVA at eight and four weeks prior to rMVA vaccination, VACV primed mice received one shot of VACV four weeks prior to rMVA vaccination, CD8+ IFN-γ-producing cells in (a), CD4+ IFN-γ-producing cells in (b) are shown, published in: (Altenburg et al., 2018).*

As displayed in **Figure 20**, Bejon et al observed a negative correlation between anti-vector T cell responses and T cell responses against the antigenic insert (r= -0.33, p= 0.03) in their clinical phase 1 malaria vaccine trial in using a two different recombinant poxvirus vectors (FP9 and MVA), both encoding for the malaria-antigen ME-TRAP (Bejon et al., 2006).

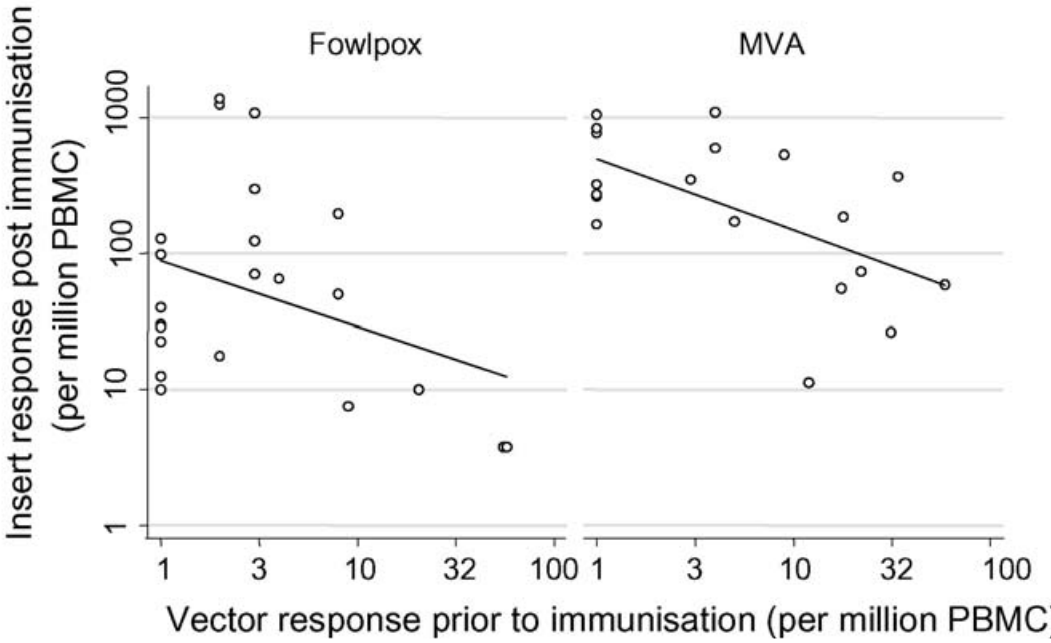


Figure 20: Negative correlation of pre-vaccination anti-vector immunity and insert-specific response (1 week) post vaccination in humans. *Measured in IFN-γ ELISpot, left panel shows insert-specific response after FP9 ME-TRAP vaccination, right panel indicates insert specific response after FP9 ME-TRAP vaccination followed by MVA ME-TRAP vaccination three weeks later, published in: (Bejon et al., 2006).*

The discrepancies between the observations reported by Bejon et al. and the findings here could be related to methodological factors. Bejon et. al used the Western Reserve strain of vaccinia virus to stimulate cells in the ELISpot assay at a MOI of 3. Bejon et al. recruited only male study participants, whereas the ten study participants of the MVA-MERS-S-cohort were all female. Further Bejon et al. measured vaccine-induced antigen-specific responses as early as one week after immunization. Here, MERS-S-specific immune responses peaked 14 – 28 days after vaccination.

By contrast, a more recent study from La Rosa et al. made similar observations as in the MVA-MERS-S study. They evaluated a recombinant MVA vaccine expressing CMV antigens in study participants with and without prior smallpox vaccination. They could not observe differences between study subjects who previously received smallpox vaccination suggesting that repeated administration of MVA might not interfere greatly with vaccine immunogenicity (La Rosa et al., 2017).

In this study, anti-MERS-S1 antibody responses were successfully boosted after second and third immunizations, despite the presence of vector-immunity. There was no correlation between anti-vaccinia virus and anti-MERS-S1 IgG responses.

Our results coincide with the observations of Kreijtz et al. in their MVA-based H5N1 influenza vaccine trial showing that anti-vector immunity did not prevent boosting of influenza-specific immune responses. They hypothesized that the entry of MVA into the cells, following expression and presentation of the encoded antigen, could not be hampered by pre-existing MVA vector-specific antibodies (Kreijtz et al., 2014).

4.5 Conclusions

Repeated vaccination with the vaccine candidate MVA-MERS-S induced MVA vector-specific cellular and humoral immune responses. Even in the face of vector-immunity, MERS-S-specific cellular and humoral immune responses were boosted after repeated immunizations with MVA-MERS-S.
Further, there was a positive correlation between MVA vector-specific and MERS-S-specific T cell responses. Anti-vaccinia virus IgG and anti-MERS-S1 IgG responses did not show a correlation. Based on these data, there is no evidence for a negative influence of vaccine-induced MVA vector-specific immunity on the immunogenicity of the antigenic insert MERS-S. However, further studies are required to determine the exact impact of vector-immunity against MVA.

4.6 Limitations of this study

This study has some limitations. First, in this first-in-human vaccine trial, the number of study participants was low and only ten study participants received three immunizations. Further, the study cohort was homogenous with all ten study participants being young, white women. Based on these data one cannot extrapolate to the general population.

4.7 Further directions

An extended time interval between prime-boost immunizations could be included. A study from Palgen et al. indicated that innate and secondary humoral immune responses are improved by increasing the time between MVA immunizations to two months (Palgen et al., 2020).

Sample collection of later time points post-vaccination i.e. within the scope of an observational study would allow assertions on the longevity of vaccine-induced responses to the antigenic insert and to the MVA vector.

In further studies, polyfunctionality and proliferation of T cells could be evaluated via flow-cytometry in order to gain a more detailed understanding of T cell-mediated immunity to MVA.

Lastly, for a deeper understanding of the effect of pre-existing immunity on vaccine efficacy, subsequent study protocols could include study participants with prior smallpox or recombinant MVA vaccination, respectively.

5. Summary

Middle East Respiratory Syndrome (MERS) is caused by MERS coronavirus (MERS-CoV) associated with a high case-fatality rate of up to 35%. With no specific treatment available and considering the high epidemic potential of MERS-CoV infection, fast and efficient development of a protective vaccine is of great interest.

The vaccine candidate MVA-MERS-S was proven safe and immunogenic in small and large animal models as well as in a recent first-in-human phase 1 vaccine trial conducted in this working group. MVA-MERS-S is a viral vector vaccine utilizing the attenuated poxvirus Modified Vaccinia virus Ankara (MVA) which expresses the MERS-S-protein. While MVA has increasingly been used as viral vaccine vector, the influence of anti-vector immunity on the formation of antigen-specific immunity remains poorly understood.

The aim of this work was the evaluation of vector-immunity within the scope of a phase 1 trial with the vaccine candidate MVA-MERS-S. Cellular and humoral immune responses to the MVA vector were assessed at different time points post vaccination. To measure MVA vector-specific T cell responses an interferon-γ (IFN-γ) ELISpot assay was established. Anti-vaccinia virus IgG was detected using an indirect immunofluorescence test (IFT). Lastly, MVA vector-specific immune responses were correlated to MERS-S-specific immune responses.

All ten study participants of the MVA-MERS-S study had detectable anti-vector T cell responses and IgG antibodies 14 days after the second vaccination. MVA vector-specific and MERS-S-specific T cell responses measured 14 – 28 days after the third immunization showed strong positive correlation ($r= 0.7333$, $p= 0.0202$).

Repeated vaccination with the vaccine candidate MVA-MERS-S induced MVA vector-specific cellular and humoral immune responses as presumed. Nevertheless, MERS-S-specific cellular and humoral immune responses were boosted after repeated immunizations with MVA-MERS-S even in the face of vector-immunity. There is no evidence for a negative influence of vaccine-induced MVA vector-specific immunity on the immunogenicity of the antigenic insert MERS-S. Further studies are required to determine the exact impact of MVA vector-specific immunity on vaccine immunogenicity.

A detailed understanding of the development of vector immunity and its effect on immune responses to the antigenic insert may help to optimize future vector vaccine strategies.

6. Zusammenfassung

Das Middle East Respiratory Syndrome (MERS) wird durch das MERS-Coronavirus (MERS-CoV) verursacht und ist assoziiert mit einem hohen Fall-Verstobenen-Anteil von bis zu 35 %. Da aktuell keine spezifische Therapiemöglichkeiten verfügbar sind und MERS-CoV ein hohes epidemisches Potenzial besitzt, ist eine schnelle und effiziente Impfstoffentwicklung von großem Interesse.

Der Vakzinkandidat MVA-MERS-S war sicher und immunogen in kleinen und großen Tiermodellen, sowie in einer anschließenden first-in-human Phase 1 Impfstoffstudie, die in dieser Arbeitsgruppe durchgeführt wurde.

MVA-MERS-S ist ein viraler Vakzinvektor, der das attenuierte modifizierte Vacciniavirus Ankara (MVA) benutzt, welches das MERS-S-Protein exprimiert. Während MVA zunehmend als viraler Vakzinvektor verwendet wird, bleibt der Einfluss von gegen den Vektor gerichteten Immunantworten auf die Bildung antigen-spezifischer Immunantworten wenig verstanden.

Das Ziel dieser Arbeit war die Evaluation von Vektorimmunität im Rahmen einer Phase 1 Studie mit dem Vakzinkandidaten MVA-MERS-S. Zelluläre und humorale Immunantworten gegen den MVA-Vektor wurden zu unterschiedlichen Zeitpunkten nach Impfung untersucht. Um MVA vektorspezifische T-Zellantworten zu messen wurde ein Interferon-*γ* (IFN-*γ*) ELISpot-Assay etabliert. Anti-Vacciniavirus Immunglobulin G (IgG) wurde mittels eines indirekten Immunfluoreszenztests (IFT) detektiert. Abschließend wurden die vektorspezifischen Immunantworten mit den gegen das MERS-S-Antigen gerichteten Immunantworten korreliert.

Alle zehn Studienteilnehmer*innen hatten detektierbare gegen den Vektor gerichtete humorale und zelluläre Immunantworten 14 Tage nach der zweiten Impfung. MVA vektorspezifische und MERS-S-spezifische T- Zellantworten zeigten eine positive Korrelation 14 – 28 Tage nach der dritten Impfung ($r= 0.7333$, $p= 0.0202$). MERS-S-spezifische Immunantworten konnten trotz Vektorimmunität nach mehrmaliger MVA-MERS-S-Impfung geboostet werden.

In dieser Studie gibt es keinen Anhalt für einen negativen Einfluss von Impfstoff-induzierter Vektorimmunität auf die Immunogenität von MERS-S. Es sind noch weiterführende Studien notwendig, um die genaue Auswirkung von Vektorimmunität festzustellen. Ein detailliertes Wissen über die Bildung von Vektorimmunität und deren Effekt auf antigenspezifische Immunantworten kann helfen zukünftige Vektorimpfstoff-Strategien und deren Wirksamkeit zu verbessern.

7. Abbreviations

aa ..... *Amino acid*
ARDS ..... *Acute respiratory distress syndrome*
BCG ..... *Bacille de Calmette et Guérin*
COVID-19 ..... *Coronavirus Disease 2019*
CMV ..... *Cytomegalovirus*
CPN ..... *Vaccinia Virus Copenhagen*
CTL ..... *Cytotoxic T lymphocyte*
CVA ..... *Chorioallantois Vaccinia virus Ankara*
DMSO ..... *Dimethylsulfoxid*
DNA ..... *Deoxyribonucleic acid*
DPP4 ..... *Dipeptidyl-peptidase 4*
EDTA ..... *Ethylenediaminetetraacetic acid*
ELISA ..... *Enzyme-linked Immunosorbent Assay*
ELISpot ..... *Enzyme-linked ImmunoSpot*
EMA ..... *Europe Medicines Agency*
EMC ..... *Erasmus Medical Center*
FBS ..... *Fetal Bovine Serum*
FDA ..... *Food and Drug Administration*
FITC ..... *Fluorescein isothiocyanate*
HCoV ..... *Human coronavirus*
HLA ..... *Human leucocyte antigen*
HRP ..... *Horseradish peroxidase*
ICS ..... *Intracellular cytokine staining*
ICTV ..... *International Committee on Taxonomy of Viruses*
IFN-γ ..... *Interferon-γ*
IFT ..... *Immunofluorescence test*
Ig ..... *Immunoglobulin*
IQR ..... *Interquartile range*
IVS ..... *In vitro Stimulation*
LED ..... *Light-emitting diode*
MERS ..... *Middle East Respiratory Syndrome*
MERS-CoV ..... *Middle East Respiratory Syndrome Coronavirus*

MOI ..... *Multiplicity of infection*
MU ..... *Multi Use*
MVA ..... *Modified Vaccinia virus Ankara*
NYCBOH ..... *New York City Board of Health*
OLP ..... *Overlapping peptide*
ORF ..... *Open reading frame*
PBMC ..... *Peripheral Blood Mononuclear Cells*
PBS ..... *Phosphate Buffered Saline*
PFU ..... *Plaque-forming unit*
PHA ..... *Phytohemagglutinin*
PHEIC ..... *Public Health Emergency of International Concern*
PUVA ..... *Psoralen and ultraviolet A*
PvE ..... *Postvaccinal Encephalitis*
RBD ..... *Receptor binding domain*
RNA ..... *Ribonucleic acid*
SARS ..... *Severe Acute Respiratory Syndrome*
SARS-CoV ..... *Severe Acute Respiratory Syndrome Coronavirus*
SFC ..... *Spot forming cell*
TMB ..... *3,3',5,5'-Tetramethylbenzidine*
UKE ..... *University Medical Center Hamburg-Eppendorf*
VSV ..... *Vesicular stomatitis virus*
WHO ..... *World Health Organization*
wtMVA ..... *Wild type MVA*

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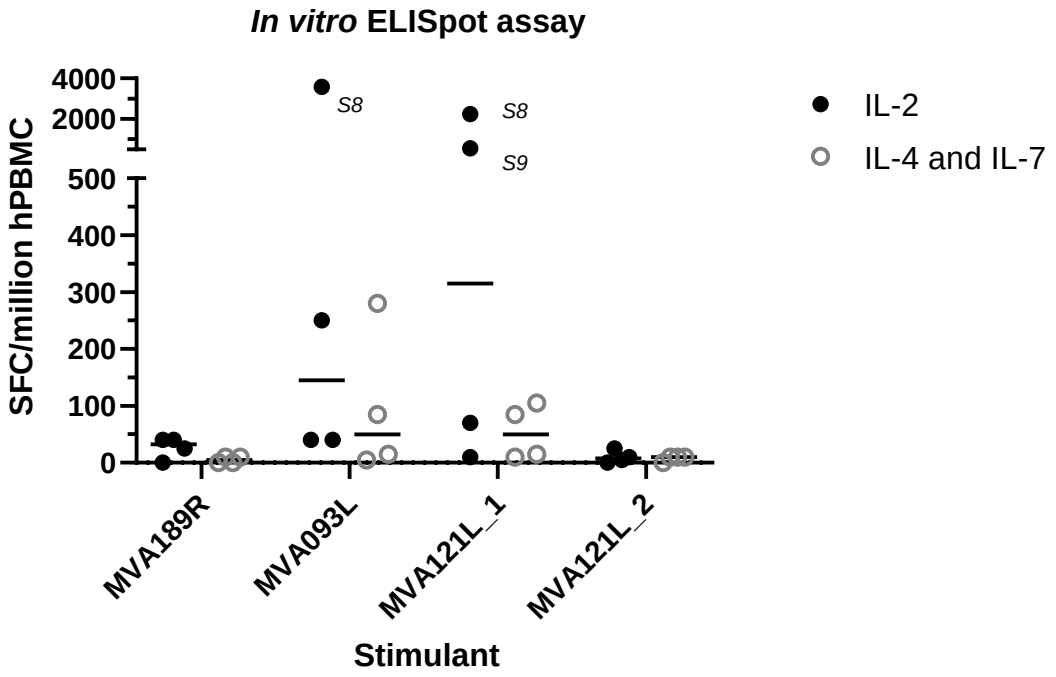
9. Appendix



Appendix 1: ELISpot results of Smallpox vaccine cohort, in vitro assay. *after in vitro stimulation with OLP and IL2 (black dots) or IL4 +IL7 (grey circles), samples from Smallpox vaccine cohort (n=4), tested with OLP, dots indicate mean per sample, bars indicate median per group.*

Establishment of an interferon γ ELISpot assay to evaluate vector-immunity within the scope of a homologous prime boost immunization with the vaccine candidate MVA-MERS-S

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Background: **The WHO Blueprint priority disease Middle East Respiratory Syndrome (MERS)** is caused by MERS coronavirus (MERS-CoV). Infection can lead to **severe pneumonia, multi-organ failure and death**[1]. With **no specific treatment** available and considering the **high epidemic potential** of MERS-CoV infection, fast and efficient development of a protective vaccine is of great interest[1].
The vaccine candidate **MVA-MERS-S** was proven safe and immunogenic in small and large animal models[2,3] as well as in a subsequent **first-in-human Phase I vaccine trial conducted in our working group**. MVA-MERS-S is a viral vector vaccine utilizing the **attenuated and replication-deficient poxvirus Modified Vaccinia virus Ankara (MVA).** While MVA has increasingly been used as viral vaccine vector, the influence of anti-vector immunity on formation of antigen-specific immunity remains poorly understood. **Vector-immunity is seen as possible drawback of viral vector vaccines.** Therefore, a deeper understanding of vector-immunity may help to optimize vector vaccine strategies and vaccine efficacy in the future.
Aim: In order to evaluate vector-immunity in MVA-MERS-S vaccinated individuals, we aim to establish an **ELISpot assay to measure the MVA vector-specific T-cell mediated release of interferon γ (IFN-γ).**
Methods: We recruited 10 healthy adults with prior smallpox vaccination in their childhood as test cohort for our assays. Whole blood samples were collected and peripheral blood mononuclear cells (PBMCs) were isolated and cryopreserved. We **identified immunogenic MVA overlapping peptide pools (MVA093L, MVA189R and MVA121L)** via literature review[4] and established an MVA vector-specific ***ex vivo* and *in vitro* ELISpot** protocol. For *in vitro* ELISpot assays cryopreserved PBMCs were cultured with MVA peptide pools in the presence of interleukin-2 (IL-2) for 10 days. Subsequently, the ELISpot assay was performed. We recently started to assess anti-MVA IFN-γ T cell responses in our MVA-MERS-S cohort.
Results: In our smallpox vaccine cohort we could detect **MVA093L-specific T cell responses in 1 out of 10 adults *ex vivo*** (49 years after smallpox vaccination). Further, **in 2 out of 3 adults we could detect MVA121L-specific T cell responses after *in vitro* stimulation** with over 60-fold rise compared to *ex vivo* results. We were able to detect **MVA vector-specific immunity in the MVA-MERS-S cohort after repeated immunizations**. So far, induction and quantity of MVA vector-specific T cell responses seem **dose-dependent.** Even in the presence of vector-immunity, **T cell responses to the MERS-S antigen were boosted.**

1. Background

A) Middle East Respiratory Syndrome

https://www.irishmirror.ie/news/world-news/kiss-death-deliver-farmers-still-318822

https://commons.wikimedia.org/wiki/File:MERS-Cov_map.png

Facts on MERS (MERS Situation update September 2019, WHO)[1]
2468 cases
851 deaths (CFR: 34,4%)
27 countries, 84% of cases in Saudi Arabia
3 cases in Germany
Zoonosis, Reservoir: dromedary camels
human-to-human transmission possible

B) MVA-MERS-S Phase I vaccine trial

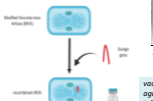
Spike (S)-protein

Study design
1.10⁸ PFU
1.10⁸ PFU
1.10⁸ PFU

- First-in-human trial
- 23 adults
- 2 dose groups
- homologous prime-boost vaccination: D0, D28
- Subgroup (n=10) received 2nd boost 12 months +/- 3months after 1st immunization
- Low adverse events

→ ***Vaccine is safe and immunogenic*** *(Koch et al., in preparation).*

C) Modified Vaccinia virus Ankara as viral vaccine vector*



A. Fathi, unpublished

vaccine trials against influenza[2], MERS, HIV[4], malaria[3], also oncolytic cancer vaccines[5].

**To date no recombinant MVA vaccine has been licensed!*

D) Vector-immunity

What is vector-immunity?

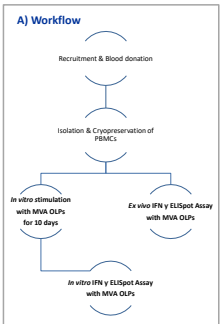
The immunization generates immune responses against the inserted antigen and also against the vector. This may reduce vaccine efficacy[5].

Why is it relevant?

1. Worldwide vaccination campaigns that led to smallpox eradication in 1979
   - A high proportion of the population already has "seen" Vaccinia virus.
   - Persistence of antibodies up to 75 years, T cell response half-life of 8-15 years[6]
2. Multiple immunizations with recombinant MVA in homologous prime-boost regimens

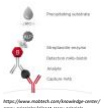
2. Methods

A) Workflow



B) The ELISpot Assay *(=Enzyme Linked ImmunoSpot)*

1. Membrane coated with capture antibodies ,e.g. to capture IFN γ
2. Stimulation of cells with antigen, e.g. MVA → Cytokine secretion
3. Capture antibodies bind cytokines
4. Detection antibodies bind cytokines (different binding site)
5. Enzymatic precepitation of substrate, which is linked to detection antibodies → visible spots
6. Spot count



https://www.mabtech.com/knowledge-center/assay-principles/elispot-assay-principle

3a. Results: Smallpox vaccine cohort

Fig. 1: *Ex vivo* ELISpot
Detectable T cell responses to MVA093L pool in 1 out of 10 participants (49 years after smallpox vaccination)

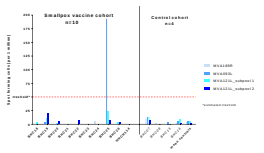


Fig. 1: PBMCs were stimulated with MVA overlapping peptide pools (OLPs) [1µg/ml] for 16h; Control cohort: healthy adults with no prior smallpox or MVA vaccination

Fig. 2: *In vitro* ELISpot
Detectable T cell responses to MVA121L_subpool 1 in 2 out of 3 participants

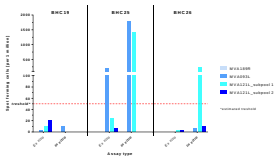
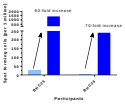


Fig. 2: PBMCs were cultured with MVA OLPs [0,5µg/ml] and IL-2 [100IU/ml] for 10 days, subsequently ELISpot assay was performed as described in Fig. 1

Fig. 3: Characteristics of participants

Participant	Age	Sex	No. of smallpox vaccinations	Time after 1st immunization [years]	Time after 2nd immunization [years]	Verification
BHC18	63	male	3	62	52	remembered
BHC19	51	female	1	51		Vaccination certificate
BHC20	58	male	2	57	47	remembered
BHC21	61	female	2	60	50	remembered
BHC22	50	female	1	49		Vaccination certificate
BHC23	62	female	2	61	50	Vaccination certificate
BHC24	47	female	1	45		Vaccination certificate
BHC25	51	female	2	49	39	Vaccination certificate
BHC26	55	male	2	54		Vaccination certificate
HHCH114	49	female	1	47		scar
Mean	*54,7*		*1,7*	*53,5*	*47,5*	
SD	*5,6*		*0,6*	*5,9*	*4,2*	

Fig. 4: 60- respectively 70-fold augmented MVA121L-specific T cell responses after *in vitro* stimulation



3b. Results (preliminary): MVA-MERS-S cohort

Fig. 5: *Ex vivo* ELISpot
Dose-dependent induction of T cell responses against MVA vector after repeated immunizations

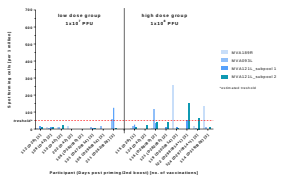


Fig. 6: *Ex vivo* ELISpot
Dose-dependent induction of T cell responses against MERS-S antigen after repeated immunizations

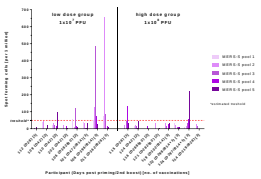


Fig. 7: Trend to higher T cell responses against MERS-S antigen in low dose group, vice versa in high dose group

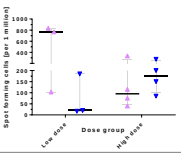


Fig. 7: MERS-S and MVA vector-specific T cell responses of all tested peptide pools after 3 immunizations in low dose (n=3) and high dose (n=4) group, lines indicate median

4. Conclusion and next steps

- The ELISpot assay we established seems poxvirus-specific and promising to detect also MVA vector-specific T cell responses.
- Although persistence of T cell responses after smallpox vaccination has been described as limited (half-life 8-15 years)[8], we found 1 out of 10 assay responder ex *vivo* and 2 out of 3 assay responder *in vitro* in our Smallpox vaccine cohort.
- With *in vitro* stimulation, we could augment poxvirus-specific T cell responses 60- respectively 70-fold.
- We were able to detect MVA vector-specific immunity in the MVA-MERS-S vaccinated cohort.
- So far, the induction of MVA vector-specific T cell responses seems dose-dependent.
- Repeated immunizations with MVA-MERS-S seem to boost MERS-S specific T cell responses regardless of vector-immunity.

***Next steps*:**
→ *Repeat the ELISpot assay with wild type MVA and compare sensitivity.*
→ *Resume the ex vivo ELISpot assays in our MVA-MERS-S cohort analyzing up to 5 different time points per individual (D0, D28, D42, D180, Boost D28).*
→ *Correlate results to MERS-S-specific ELISpot assays.*

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Appendix 2: Poster from DZIF/DGI Joint Annual meeting 2019, Bad Nauheim, Germany.

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11. Curriculum Vitae

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

12. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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