Fluorescent Reporter Viruses Identify Non-Plaque-Forming Virions of Vaccinia Virus to Activate Viral Gene Expression

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Meinen Eltern

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I. INTRODUCTION

Over the last few decades the use of viruses as vaccine vectors has developed an increasingly important role in the field of vaccines in both veterinary and human medicine. The main advantages of viruses as a viral vector platform are, inter alia, the induction of high levels of antigen-specific humoral and cellular immune responses without the need of additional adjuvant application.

One of the most promising vectors is the Modified Vaccinia virus Ankara (MVA), a replication deficient and safety tested vaccinia virus (VACV) that is already licensed as a replacement smallpox vaccine in Europe. Furthermore, MVA represents an experimental non-replicating viral vector vaccine against various infections and cancer diseases with the objective to serve as a vaccine platform for rapid immunization against newly emerging pathogens.

A detailed understanding of MVA's biological properties is very important in terms of its potential as safe viral vector vaccine. Despite having known about the presence of a non-plaque forming particle subpopulation in purified vaccinia virus stock preparations for decades, the biological activities of these virions are still uncertain. In that context we wanted to put emphasis on these unknown particles in terms of their infectious potential and their ability to pass certain levels of the MVA molecular life cycle.

For this purpose we generated two indicator viruses that visualize the early and the late stage of the gene expression of MVA and MVA's ancestor virus Chorioallantois Vaccinia virus Ankara (CVA) via different fluorescent signals. After monitoring the dynamic life process of MVA's and CVA's cascade-like gene expression, we indeed found that the non-plaque forming particles are biologically active. More precisely, these particles are able to enter cells and switch on the first step of the virus molecular life cycle. About 10-20% of the single cell infections achieve the level of late gene expression. Thus, these data indicate that the non-plaque forming particle subpopulation might play an important role in

triggering the innate and adaptive immune response induced by vaccinia virus based vaccines.

II. LITERATURE REVIEW

1. Poxviruses

1.1. Taxonomy

Two subfamilies of poxviruses make up the family Poxviridae, Chordopoxvirinae infect vertebrates and the Entomopoxvirinae infect insects. The subfamily of *Chordopoxvirinae* is divided into nine genera, *Avipoxvirus, Cervidopoxvirus Capripoxvirus, Leporipxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus, Yatapoxvirus* and two unassigned species. Within the same genus the viruses show a similar host range and morphology along with a phylogenetic relation (MOSS, 2013b). The best known genus of the *Chordopoxvirinae* is the genus *Orthopoxvirus* because of its famous members variola virus, the causative agent of smallpox, and vaccina virus (VACV), the close relative used as vaccine to eradicate smallpox by the World Health Organization between 1958 and 1977 (FENNER et al., 1988). The cowpox virus seems to be the ancestral member of this genus, as it possesses all genes existent in other Orthopoxviruses, whereas some of these genes are absent in the other members (SHCHELKUNOV et al., 1998; MOSS, 2013b).

1.2. Virion morphology

The Poxviruses' (POXV) morphology is characterized by an oval to barrel-shaped architecture. The dimensions of the virions are around 250 nm in diameter and 360 nm in length. Thus POXVs belong to one of the largest well-studied animal virus families, which can even be visualized by light microscopy (figure 1). It is remarkable that POXVs have two infectious forms: the mature virion (MV), which contains a single outer membrane, and the extracellular enveloped virion (EV), which basically represents an MV but additionally possesses a further outer lipid membrane containing

unique EV-specific membrane proteins (PAYNE, 1978; ROPER et al., 1996; LOCKER et al., 2000; MOSS, 2006). Interestingly enough, MVs are the most frequent and basic infectious form, but an efficient cell-to-cell spread is induced by EVs (BLASCO & MOSS, 1992).

The biconcave core and the lateral bodies filling the space between the outer membrane and the core are the major characteristics of the virion's internal structure. The core encloses the s-shaped POXVs' genome - a linear double-stranded deoxyribonucleic acid (dsDNA) of 130-300 kilobase pairs (kb) (MOSS, 1996). The DNA strands are linked by single stranded hairpin loops with a high content of adenosine and thymine. Adjacent to the termini, there are inverted terminal repeats (ITRs) that include dozens of open reading frames (GARON et al., 1978; BAROUDY et al., 1982). Here, the localization of the genes exhibit a distinct pattern: Highly-conserved genes, essential for replication, are usually placed in the central region, whereas end terminal genes are often variable genes, which are not involved in replication but rather in host interactions (PERKUS et al., 1990; WERDEN et al., 2008). Further components within the core of the POXV particles are virus encoded enzymes and factors which are

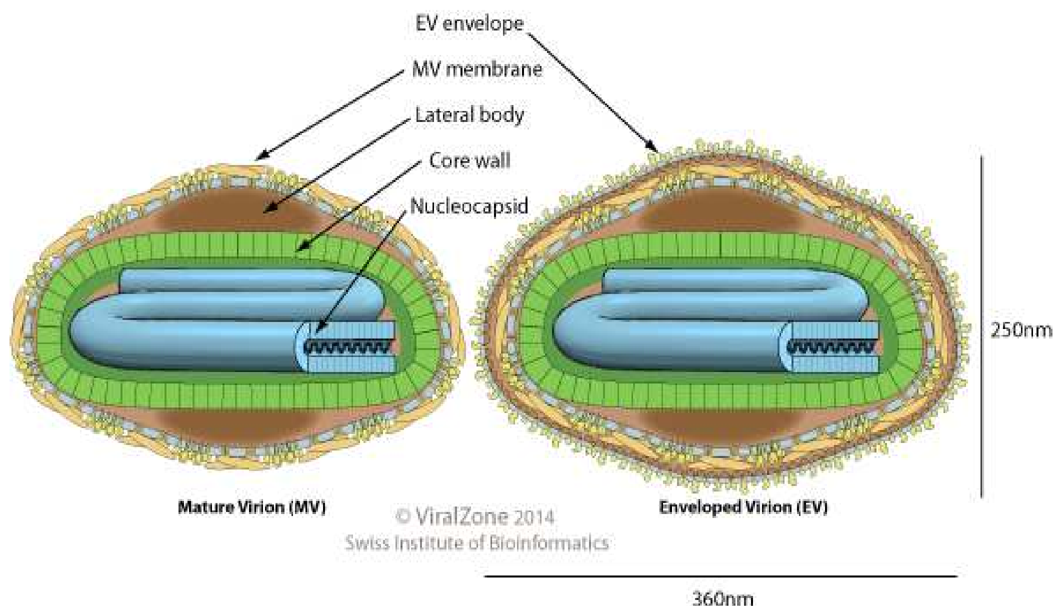

Figure 1: **Poxvirus morphology**

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

essential for the regulation of the early stage transcription, such as the multisubunit viral DNA dependent ribonucleic acid (RNA) polymerase and the mRNA capping enzymes (KATES & MCAUSLAN, 1967).

1.3. Poxviral diseases

The most well-known poxviral disease is the human smallpox caused by the variola virus, a member of the genus *Orthopoxvirus*. Smallpox, triggered by a solely human pathogen, was a dreaded illness which reached a mortality rate of up to 40%. The main transmission route was via airborne infectious respiratory droplets from variola infected patients. The virus first infected the mucosal surfaces of the nasopharyngeal tract before it moved on to the regional lymphatics and finally caused a viremia. The most common clinical type of variola infection was the ordinary smallpox: Here the first typical clinical symptoms were fever, backaches, headaches, vomiting and prostration after an incubation period of 7 to 17 days. This was followed by the appearance of a systemic rash whose pattern was centrifugal, mostly concentrated on the oral mucosa, face and extremities (FENNER et al., 1988; DAMON, 2013).

The earliest described cases of smallpox can be traced back to the fourth century AD (FENNER et al., 1988). In 1798, Edward Jenner was the first to publish the observation of a cross protection between cowpox and smallpox and thus he played a pioneering role in vaccine research (JENNER, 1798). Cowpox was later replaced by the VACV, which is closely related to the cowpox virus. The global vaccination program with VACV, commissioned by the World Health Organization (WHO) between 1958 and 1977, finally resulted in the eradication of smallpox in 1980 (FENNER et al., 1989).

Although naturally occurring variola virus is eradicated, humans are still faced with POXV diseases as several of the animal pathogenic POXVs have zoonotic potentials. In 2003, the U.S. was confronted with an outbreak of the monkeypox virus (MPXV) which resulted in 87 reported human infection cases (CDC, 2003; LIKOS et al., 2005). These first

human infections outside Africa were accidentally transmitted by prairie dogs that had had temporarily close contact to MPXV-infected rodents which were originally from Ghana (REYNOLDS et al., 2007). Due to the rather low virulence of the MPXV strain, there were fortunately no fatalities or human-to–human transmissions during this outbreak (REED et al., 2004). However, the existence of a natural reservoir of more virulent strains in rodents in central Africa makes MPXV infections a serious zoonotic POXV threat. This is especially true for immunocompromised patients and the unvaccinated population born after discontinuation of the mandatory vaccination against smallpox (TESH et al., 2004; XIAO et al., 2005; ESSBAUER et al., 2010).

Furthermore, POXVs are also pathogens to be feared in industrial livestock production, as clearly demonstrated by a recent outbreak of a highly-pathogenic fowlpox virus that killed all 10,000 breeding chickens in a poultry flock in 2009 (ZHAO et al., 2014). Another example is the camelpox virus which has a major economic impact on camel farming due to loss in terms of meat and milk production. It effects primarily young calves and pregnant female camels and is endemic, for example, in Middle Eastern, Asian and African camel-rearing countries (DURAFFOUR et al., 2011).

Due to natural reservoirs of POXVs and the fear of accidental or deliberate release of smallpox, coupled with a growing unvaccinated human population, POXVs still represent serious pathogens in the fields of human and veterinary medicine.

2. Life cycle of vaccinia virus as prototype poxvirus

2.1. Vaccinia virus entry

Vaccinia virus, the prototype of the genus Orthopoxvirus, played a key role in the early scientific research of viruses (FENNER et al., 1989). It was among the first viruses evaluated via electron microscopy (BORRIES et al., 1938). In addition, it was also among first viruses that could be

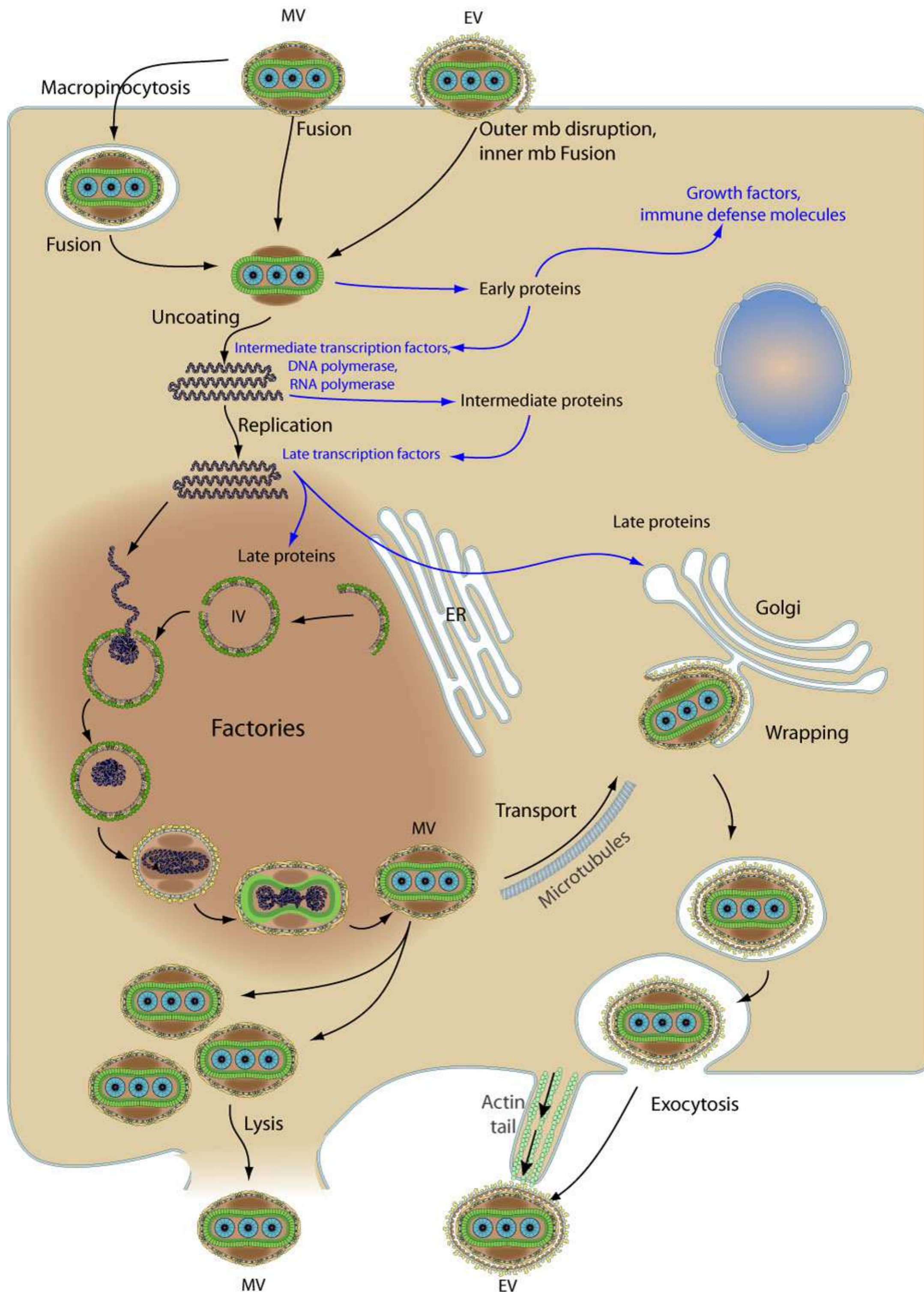

Figure 2: **Cellular cycle of poxvirus infection**
(Source: ViralZone; www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics; with permission)

amplified in cell cultures (PARKER & NYE, 1925; MAITLAND & MAITLAND, 1928) and were purified successfully to perform chemical

analysis of the virions (PARKER & RIVERS, 1935). As a model virus for the genus Orthopoxvirus and for the Poxviridae as well, the vast majority of information about POXVs life cycles and the interdependencies of virus-host interactions have been gained through studies using VACV (MOSS, 2013b).

The presence of two infectious forms of POXVs, EV and MV leads to the hypothesis of different entry pathways (figure 2). The attachment of the MVs is performed via four viral proteins binding glycosaminoglycans and laminin on the cell surface (CARTER et al., 2005; MOSS, 2012), followed by two feasible cellular entry mechanisms: Either MV's outer membrane immediately fuses with the plasma membrane or the virion is taken up more efficiently by an actin-dependent macropinocytosis (CARTER et al., 2005; MERCER & HELENIUS, 2008). The proteins involved in EV's attachment have not yet been described. An entry-fusion complex, which seems to consist of 12 proteins, is known to be involved in the virus-cell fusion (SENKEVICH et al., 2005; LALIBERTE et al., 2011; MOSS, 2012). As this complex is located in the MV membrane it was suspected that removal of the external EV membrane precedes EV cell fusion. This was indeed confirmed by electron micrographs of disrupted EV envelopes and the fusion of the inner membrane with the plasma membrane of the cell (LAW et al., 2006). Although many proteins involved in the virions entry are identified, the specific protein receptors have not yet been determined.

2.2. Cascade of gene expression

The POXV life cycle occurs entirely in the host cell cytoplasm without using the cellular replication system (MOSS, 1996). Hence, POXV virions hold a full transcription system for the regulation of the viral gene expression in order to initiate the first step of a cascade-like regulated transcription (MOSS, 2013b). This type of genetic programming is characterized by a selective expression of early, intermediate and late viral genes which is directed by transcription factors that are produced in each temporal stage to initiate the next level of gene expression (BALDICK &

MOSS, 1993; MCFADDEN, 2005).

Subsequent to the host cell entry of the VACV virions, early gene expression starts immediately with a detection of mRNA within 20 minutes (SANZ & MOSS, 1999). The early gene products initiate disruption of the virus core ("uncoating"), release of the genome into the cytoplasm and DNA replication (MCFADDEN, 2005). Cessation of early gene expression is regulated tightly due to the existence of specific nucleotide sequences serving as transcription termination signals. Early gene expression is immediately followed by DNA replication that occurs in specific cytoplasmic sites which are known as viral factories (YUEN & MOSS, 1987). These viral factories are enveloped by rough endoplasmic reticulum and can be identified by light and electron microscopic autoradiography (DOMI & BEAUD, 2000; SCHRAMM & LOCKER, 2005). Cessation of DNA replication coincides with the onset of intermediate stage transcription at about 2-3 hours after infection. Here, late stage transcription factors, DNA packaging and core associated proteins are encoded by the intermediate genes in order to initiate late gene transcription right after the termination of the intermediate stage (KECK et al., 1990; SANZ & MOSS, 1999). Both the intermediate and the late gene transcription are also called post-replicative gene expression, as inhibition of DNA replication revealed that the following transcription stages were blocked (ROSEL & MOSS, 1985; BROYLES, 2003). Regulation of the late gene transcription is not as stringent as the early gene expression since late transcription lacks specific termination signals. This in turn results in the synthesis of long read-through RNA transcripts and persistence of late gene transcription until the virus life cycle is finished (XIANG et al., 1998; MOSS, 2013b).

2.3. Assembly, exit and cell-to-cell spread

The earliest assembly stages that can be observed within the viral factories are crescent-shaped membrane structures with a honeycomb lattice on the surface. These membrane structures form the non-infectious

precursor virions of the MV - the so-called immature virions (IV) (GRIMLEY et al., 1970; WARD, 2005b). The IVs arise due to the absorption of a nucleoprotein mass, including the genome and the early transcription apparatus, while the membrane termini seal circularly (CONDIT et al., 2006). After that, the spherical IV is transformed into the MV which is associated with a disassembly of the lattice scaffold and internal restructuring (BROWN et al., 2006). The majority of MVs remain as the POXV's intracellular form within the infected host cell and can only be externalized by cell lysis (MOSS, 2013b). However, some MVs pass through a wrapping process involving two further membranes which are derived from the trans-Golgi network or endosomal cisterna (TOOZE et al., 1993; SCHMELZ et al., 1994). These membranes are essential for intracellular migration and EV generation. The wrapped-virion moves to the cellular periphery via microtubules followed by a fusion with the plasma membrane to release the EV (WARD & MOSS, 2001a; SMITH et al., 2002; WARD, 2005b). A smaller amount of the externalized EV is localized in the medium whereas most externalized EVs adhere to the cell surface. The adherent EVs are responsible for efficient cell-to-cell spread via actin containing microvilli (CUDMORE et al., 1995) whereas the liberated EVs are responsible for the wide range distribution and so contribute for the transmission within the host (PAYNE, 1980; VANDERPLASSCHEN et al., 1998). In contrast to this the MVs play a key role in the transmission between the host animals (MOSS, 2012).

3. Modified Vaccinia virus Ankara

3.1. Origin from Chorioallantois Vaccinia virus Ankara

The Modified Vaccinia virus Ankara is originated from a strain of VACV which was once utilized as a smallpox vaccine by the Turkish government (VACV Ankara). There, smallpox vaccine production was mediated by virus amplification in donkey-calf-donkey passages. When the virus was brought to Munich by Mayr and Herrlich, it was originally passaged on chorioallantoic membranes of embryonated chicken eggs and renamed to

Chorioallantois Vaccinia virus Ankara (CVA) (MAYR et al., 1975). In 1954/55, following two amplifications on bovine skin, CVA was also used as a smallpox vaccine in Munich. However, due to a rather frequent appearance of secondary lesions after primary vaccination the use of CVA based vaccines was discontinued (HERRLICH & MAYR, 1957; MAYR et al., 1975). In this context Mayr and coworkers analyzed the replicative capacity and virulence of CVA in several *in vitro* and *in vivo* infections, such as in chicken embryo fibroblasts, HeLa cells, rabbits, baby mice and embryonated chicken eggs (HERRLICH & MAYR, 1954; MAYR et al., 1955; MAYR & MUNZ, 1964). These studies determined a wide host tropism of CVA, compared it to other strains of VACV, and selected CVA for serial passaging experiments in several cell cultures in order to modify the host range of the virus (MAYR et al., 1975).

After 300 serial passages in chicken embryo fibroblasts (CEF) the biological properties of CVA had changed more obviously compared to the ones determined upon CVA passage in mammalian cells. After another 70 CEF passages, the CEF-modified CVA strain sustained a loss of the typical VACV properties, shown by altered growth capacity *in vitro* and a reduced virulence *in vivo* such as in rabbits, baby mice and chicken embryos (MAYR & MUNZ, 1964). Finally, at passage 516, the CVA-CEF descendant virus was named Modified Vaccinia virus Ankara (MVA) in order to clearly differentiate it from other attenuated VACV strains and to put emphasis on its altered biological properties (HOCHSTEIN-MINTZEL et al., 1972).

Further propagation of MVA through over 570 CEF passages resulted in an MVA strain that was confirmed to be avirulent even in immunosuppressed animals. These novel and promising properties were the impetus to test MVA as a priming vaccine prior to the administration of the conventional VACV vaccine in order to improve the safety of smallpox vaccination, especially for immunocompromised patients. In clinical field trails, 120,000 humans, including individuals at high risk for conventional smallpox vaccination, have been successfully vaccinated with MVA without the appearance of any significant adverse effects (STICKL et al., 1974; MAYR et al., 1978).

Eradication of smallpox and the end of mandatory vaccinations resulted in a loss of interest in improving and developing new and enhanced smallpox vaccines. However, in the recent decades the attention increased again due to fear of accidental or deliberate release of smallpox. Recent studies confirmed MVA's excellent safety profile, immunogenicity and efficacy against Orthopoxviruses in established animal models (DREXLER et al., 2003; KREMER et al., 2012b; VOLZ et al., 2014) so it was finally licensed as a standalone smallpox vaccine in the European Union and Canada (VOLLMAR et al., 2006; KENNEDY & GREENBERG, 2009).

3.2. In vivo attenuation and host cell tropism

MVA's attenuated biological properties are associated with a massive restriction of most known VACV virulence and immune evasion factors (MEYER et al., 1991; ANTOINE et al., 1998; MEISINGER-HENSCHEL et al., 2010). A loss of approximately 15 % of the parental viral genome, concerning six large genomic deletion sites and additionally many shorter deletions and point mutations, has been identified. The six major deletions, which were numbered with Roman numerals I to VI, are located in the more terminal regions of the genome, which encode more host range regulating genes rather than genes essential for viral replication (ALTENBURGER et al., 1989; MEYER et al., 1991). Interestingly enough, MVA's attenuated phenotype is not primarily attributed to the major deletions. A recent study showed that CVA mutant viruses containing MVA's six major deletion sites were only able to partly exhibit MVA's host range restrictions in different cell cultures and mice, suggesting that point mutations and smaller deletions provide significant contributions to govern MVA's attenuation (MEISINGER-HENSCHEL et al., 2010).

The comparison of the host range of MVA with the parental CVA strain in several cell lines identified three categories of cells: permissive, semi-permissive and non-permissive cells (DREXLER et al., 1998). The serial CEF passaging induced a high restriction of MVA replication on cells of avian origin (HOCHSTEIN-MINTZEL et al., 1972). Consequently, the

permissive category consists of primary CEF, but remarkably the Syrian hamster cell line BHK-21 belongs here as well. However, the BHK-21 cells are the only known mammalian cells that support an unimpaired replication and spread of MVA. The semipermissive category contains African green monkey cell lines such as BS-C-1 and CV-1, which enable limited spread of MVA. Nevertheless, most mammalian cells including human cells are classified into the non-permissive category. Here, MVA shows an inability to replicate productively, although it still remains infectious for the cells (CARROLL & MOSS, 1997). Despite this growth deficiency early, intermediate and late gene expression is not restricted. Due to a late block in the virus' assembly of viral particles MVA is not able to produce infectious progeny. Instead, only immature virions are formed in these non-permissive cells (SUTTER & MOSS, 1992).

Even though MVA's growth deficiency in different cell lines has been intensively evaluated, the genetic background is not yet completely clear. So far, Orthopoxviruses comprise five known major host range genes: C12L (SPI-1) (ALI et al., 1994), CP77 (GILLARD et al., 1985; HSIAO et al., 2006), C7L (PERKUS et al., 1990; OGUIURA et al., 1993), K1L (PERKUS et al., 1990) and E3L (CHANG et al., 1995; BEATTIE et al., 1996). As the CP77 is fragmented in all known VACV strains, this host range gene does not contribute to MVA attenuation. Apart from K1L and SPI-1 the other genes are still intact in the MVA genome (CARROLL & MOSS, 1997). In VACV and MVA the absence of K1L and C7L led to abortive replication in most mammalian cell lines (BACKES et al., 2010), while introducing K1L in MVA correlated only with multiplication in rabbit kidney derived RK-13 cells (SUTTER et al., 1994a; ZWILLING et al., 2010). The reconstruction of SPI-1 restored MVA propagation only in A549 (adenocarcinomic human alveolar basal epithelial cells) (WYATT et al., 1998). Furthermore, an MVA mutant lacking the host range gene E3L, which encodes the double stranded RNA-binding protein E3, showed a severe deficiency in viral DNA replication and late gene expression in human HeLa cells (LUDWIG et al., 2005).

Still, the POXVs cellular tropism results from the interactions of two factors: viral host range genes and, in addition, intracellular factors. These

cellular components have long been unknown, but a recent study using RNA interference screens revealed highly-conserved cellular proteins that may act as restriction factors for poxviral multiplication (SIVAN et al., 2015; VOLZ et al., 2015b). These data shed new light on the virus host interactions.

MVA's *in vitro* attenuation is also evident *in vivo*. It is mainly presented by the cessation of the typical VACV virulence character, which is particularly noticeable in humans, as demonstrated in the clinical human field trails mentioned above (STICKL et al., 1974; MAYR et al., 1978). Furthermore, MVA vaccination studies in several animal models such as dogs, piglets, rabbits, calves and macaques did not show the appearance of any significant adverse effects (MAYR et al., 1975). In addition, it has been reported that MVA was cleared within 48 hours after intraperitoneal infection of mice and that MVA was even found to be avirulent in newborn and irradiated mice (MAYR et al., 1978; MEYER et al., 1991; RAMIREZ et al., 2000). It is remarkable that MVA was even successfully tested in a preclinical safety study with immune-suppressed macaques. No clinical or pathological signs were found to be associated with the inoculation of high doses of MVA. In addition, infectious MVA could not be reisolated from these macaques (STITTELAAR et al., 2001). In contrast, generalized VACV infections could be observed in immunosuppressed (HIV positive) individuals, which were vaccinated with VACV vaccines (REDFIELD et al., 1987). Moreover, severe post vaccination complications could also be observed in VACV vaccinated infants during the eradication of smallpox (GURVICH, 1992). Thus, particularly with regard to immunocompromised or potentially immunocompromised individuals, such as infants and elderly, MVA seems to have an excellent safety profile to be used as a platform for recombinant human vaccines (STITTELAAR et al., 2001).

3.3. Development as vaccine platform

In 1983/84 recombinant VACV was the first virus that was established to express foreign antigens in order to induce immunity against other

pathogens (SMITH et al., 1983; MOSS et al., 1984; DRAPER & HEENEY, 2010). Since then, various viral vectors were generated as potential vaccine platforms, such as adenoviruses and alphaviruses (LUNDSTROM, 2005; CHOI & CHANG, 2013). An advantage to utilizing viral vectors as vaccine antigen carriers is their ability to infect cells. This allows for intracellular antigen production which results not only in a humoral immune response, but also in a strong cell-mediated immune response by activating antigen-specific CD8+ T cells (DRAPER & HEENEY, 2010). Furthermore, viral components trigger the adaptive immunity by inducing inflammatory reactions, which usually make the application of adjuvants unnecessary. In contrast to this, other vaccines, such as protein subunits or DNA vaccines, often need additional adjuvants to enhance immunogenicity (URA et al., 2014).

VACVs are ideally suited for the application as viral vector vaccine platform, due to the following biological properties: I. The large genome enables the insertion of large or even several foreign genes without affecting VACV infectivity; II. The life cycle occurs entirely in the cytoplasm outside the cellular nucleus, without any integration in the host genome; III. Depending on the selected promoter, high levels of foreign antigens can be produced in each phase of the life cycle for the induction of a strong adaptive immune response (MOSS, 1996). However, typical side effects of viral infections and even severe adverse reactions, which were observed during the smallpox eradication campaign, shifted the attention to the use of attenuated VACVs as vaccine platform (LANE et al., 1969; KENNEDY et al., 2009). Fortunately, it turned out that a reduced virulence is not always linked to an impaired immunogenicity. Indeed, it has been shown that MVA induced even a greater vaccination efficacy in mice and in non-human primates in comparison to replication competent VACV vector vaccines (SUTTER et al., 1994b; HIRSCH et al., 1996; RAMIREZ et al., 2000).

Sutter and Moss were the first who successfully evaluated the use of MVA as an expression vector. Furthermore, they determined that MVA expresses early genes as well as late genes in human cells, while the virion assembly is blocked (SUTTER & MOSS, 1992).This allows efficient

protein expression even under non-permissive conditions. Another attenuated VACV, the New York attenuated Vaccinia virus (NYVAC) generated by deleting specific regions in the genome of the VACV strain Copenhagen, synthesize only some of the late viral proteins in human cells (TARTAGLIA et al., 1992; PAOLETTI, 1996; NAJERA et al., 2006). In comparison to MVA this might constitute an adverse property in terms of high-level antigen production.

MVA, as a promising viral vaccine platform, is characterized by its proven safety and unimpaired gene expression in mammalian cells, but also by its high level of immunogenicity and protective capacity (SUTTER & MOSS, 1992; MOSS et al., 1996; STITTELAAR et al., 2001). The strong immunogenicity triggered by MVA is associated with the loss of several immune evasion factors. Unlike other VACVs, MVA can only produce a few of the receptor-like proteins that inhibit host cytokines, such as type I and type II interferon (BLANCHARD et al., 1998; WAIBLER et al., 2007). Unaffected secretion of the host cytokines initiates the expression of antiviral proteins which may contribute to the efficacy of a vaccine virus (BLANCHARD et al., 1998). Another MVA specific feature is the induction of an early immigration of CD4+ lymphocytes, monocytes and neutrophils to the site of infection via the cytokine CCL2 (LEHMANN et al., 2009).

MVA's proven immunogenicity, its safety profile as well as the established methods for large scale production provide excellent characteristics for the application as a viral vector vaccine (KREIJTZ et al., 2013). It is, thus, not surprising that over the last decade several MVA-based candidate vaccines have been widely tested in phase I and phase II clinical trials in the field of both veterinary and human medicine (KREIJTZ et al., 2013). In human medicine the ongoing research focuses particularly on the development of MVA-based viral vector vaccines against newly emerging infectious diseases, such as MERS, West Nile and Influenza, but also against infections that have presented major health challenges for years, such as AIDS, tuberculosis and malaria (GILBERT, 2013; KREIJTZ et al., 2013; VOLZ & SUTTER, 2013).

For instance, a recombinant MVA candidate vaccine against influenza A viruses of subtype H5N1 (MVA-HA-VN/04) has been proven to be highly

protective in preclinical mouse and macaque studies after the challenge with homologous and heterologous H5N1influenza viruses (KREIJTZ et al., 2007; KREIJTZ et al., 2009a; KREIJTZ et al., 2009b). These promising results allowed for a phase I/IIa clinical trial with 80 young volunteers. The vaccination resulted in high titers of H5N1 specific antibodies. Interestingly enough, a further booster vaccination after 12 months induced an increase of H5-specific antibodies despite the presence of MVA specific antibodies that emerged during the previous vaccination (KREIJTZ et al., 2014). Thus, this study confirmed that MVA has a great potential as a viral backbone for recombinant influenza vaccines.

Furthermore, a recombinant MVA candidate vaccine (MVA-MERS-S) against the newly emerged Middle East respiratory syndrome coronavirus (MERS-CoV) could be generated within a year after the new disease was first described in 2012 (ZAKI et al., 2012; SONG et al., 2013). Two years later in 2015, protective efficacy of MVA-MERS-S was confirmed in a preclinical mouse study after the challenge with MERS-Co-V (VOLZ et al., 2015a). This was followed by a collaborative study to demonstrate efficacy of vaccination in dromedary camels, which was published in 2016. The authors showed a highly significant reduction of viral load in the excretions of vaccinated and subsequently-challenged dromedaries, which are thought to mediate MERS-CoV transmission to humans (HAAGMANS et al., 2016). The brief time span between the first description of MERS-CoV and the availability of a recombinant MVA candidate vaccine against this previously unknown pathogen demonstrates the efficiency of MVA as a vaccine platform against emerging infectious diseases.

4. Engineering recombinant MVA and use of fluorescent proteins in scientific research

4.1. Engineering recombinant MVA

Generally, recombinant MVAs are generated by using the well-established

methodology of homologous DNA recombination in infected cells, which is a common occurrence during poxvirus replication (0.1%) (BALL, 1987; KREMER et al., 2012a). For recombinant engineering, the recombination event is directed by an MVA transfer plasmid. In order to ensure successful results, the MVA transfer plasmid has to contain certain components: the foreign gene sequence(s) of interest, a VACV promoter for the regulation of the foreign gene expression, a selection marker gene to facilitate isolation of recombinant MVA and two genomic MVA flanking sequences. These flanking sequences precede and follow the marker sequence as well as the gene of interest in order to enable homologous recombination to the selected MVA insertion site (KREMER et al., 2012a).

The strength of foreign gene expression is decisively influenced by the selected promoter. Several natural and synthetic virus-specific promoters that are assigned to the early, intermediate or late phase of gene expression are available (BROYLES, 2003; WYATT et al., 2008). However, there are also promoters that have activity in both early and late transcriptional stages, such as P7.5 (COCHRAN et al., 1985) and the modified promoter H5 (PmH5) (WYATT et al., 1996). It has been shown that the timing of gene expression induces different kinds of immune cells: Early VACV gene products seem to be preferentially targeted by CD8+ T cells, while intermediate and late gene products are predominantly recognized by CD4 T cells and specific antibodies (MOUTAFTSI et al., 2007; MOUTAFTSI et al., 2010; YANG et al., 2011). Therefore, tandem early and late promoters are commonly used to achieve high levels of foreign gene expression during the whole virus life cycle in order to induce a strong immune response (WENNIER et al., 2013).

A variety of different selection techniques to isolate recombinant MVAs are available. Early methodology relied on the use of VACV host range gene K1L whereby its acquisition in MVAs genome correlates with the ability to grow in rabbit kidney RK-13 cells (SUTTER et al., 1994a). Here, the VACV K1L gene is located next to the gene of interest within the transfer plasmid. Homologous recombination between the wild type MVA and the transfer plasmid results in a recombinant MVA expressing both the

recombinant antigen and K1L. This enables recombinant MVA to grow in RK-13 cells and ease the clonal isolation. Repetitive DNA sequences within the K1L expression cassette, allow K1L removal under nonselective growth permission, such as in MVA permissive Chicken embryo fibroblasts. This method of transient host range selection on RK-13 cells is well-established. However, the use of RK-13 cells may be restricted as recombinant MVAs generated in these cells have no record for clinical use (KREMER et al., 2012a).

Another more recent selection technique is based on the use of transiently expressed fluorescent markers, such as the green fluorescent protein (GFP). This technique has major advantages: I. The use of selecting agents and substrates is unnecessary; II. The recombinant MVA can be generated in CEF cells even without the addition of serum, III. The selection of recombinant MVA is easily possible via fluorescent microscopy. Similar to transient host range gene selection, the fluorescent reporter gene is located between repetitive DNA sequences, which allow for its removal in further rounds of plaque purification (KREMER et al., 2012a).

In order to verify successful isolation of recombinant MVA, proper quality controls including assays for genetic identity, replication deficiency and stable foreign gene expression are essential.

Generally, genetic stability can be analyzed by PCR monitoring. Foreign genes inserted in one of the characteristic six major deletions of the MVA genome are identified by using oligonucleotide primers that target the regions of the major deletions respectively (MEYER et al., 1991). Thus, the non-presence of wild type MVA and the correct insertion of foreign genes are easily proven. Stable expression of foreign genes can be evaluated on protein level via Western blot analysis. In some cases recombinant gene products interfere with the replication of recombinant MVA which could allow for generation of the wild type MVA. In this context serial cell culture passages at a low multiplicity of infection are used to confirm the stability of foreign gene expression. As replication deficient MVA can be handled under the biosafety level 1, it is also necessary to

test the replication capacity of recombinant MVA in comparison to the wild type MVA in permissive and non-permissive cells (KREMER et al., 2012a).

Taken all together the methods for generation and characterization of recombinant MVA are well-established and allow for rapid generation of recombinant MVA vaccines with the capability of large scale productions.

4.2. Fluorescent proteins

The discovery of the green fluorescent protein (GFP) (SHIMOMURA et al., 1962) revolutionized a large field of research related to processes in living cells and organisms. In the field of virology fluorescent proteins became unique instruments with a broad range of applications in many experimental settings, such as in studies of viral life cycle, in vivo tropism, pathogenesis and viral host cell interactions (WARD & MOSS, 2001a; DE VRIES et al., 2010; FUKUYAMA et al., 2015).

In 1962, Shimomura was the first to isolate GFP from the jellyfish Aequora victoria. He discovered the GFP along with the separate luminescent protein aequorin in the light producing organs of the jellyfish. Here, GFP absorbs the blue light (maximum 395 nm) produced by the protein aequorin and in turn exhibits the bright green fluorescence (maximum 509 nm) (SHIMOMURA et al., 1962). After 30 years, Douglas Prasher succeeded in sequencing and cloning of GFP, which allowed for large scale production of the fluorescent protein (PRASHER et al., 1992). The suitability of GFP as a marker to study gene expression and protein localization in living organisms was not reported until two years later (CHALFIE et al., 1994). Today a large redundant of GFP variants that are achieved through protein engineering are available. Due to mutations that affected the spectral characteristics of GFP, many variants with emission spectral profiles of blue, cyan and yellow emerged. One of the most-used wild type GFP variants is the enhanced GFP. It is an improved type in terms of a brighter and stronger green fluorescence in mammalian cells (ZHANG et al., 1996). The isolation of other GFP-like proteins from

nonbioluminescent Anthozoa species finally brought about fluorescent proteins emitting light in the red spectral region in 1999 (MATZ et al., 1999).

Regardless of their originating species, GFP-like proteins consist of 11 strands of beta sheet that form a barrel-like structure by surrounding an alpha helix. The chromophore, the part responsible for the light emission, is placed within the alpha helix (YANG et al., 1996). The fluorescent proteins derived from Aequora victoria have a naturally weak dimerization tendency. In contrast to this virtually all fluorescent proteins derived from Anthozoa species form stable tetrameric complexes, which bring about difficulties in experimental settings in cells (BAIRD et al., 2000; CAMPBELL et al., 2002). The oligomerization tendency often leads to formation of intracellular aggregations with the targeted proteins. In order to improve the usage of Anthozoa fluorescent proteins as general fluorescence tags, efforts were made to develop monomeric complexes (CAMPBELL et al., 2002). Protein engineering led to the generation of Anthozoa monomer derivatives with distinct fluorescences and altered wavelengths, such as mCherry (SHANER et al., 2004), mRaspberry (WANG et al., 2004b), mPlum (WANG et al., 2004b), and mGrape (SHANER et al., 2004). It turns out that mCherry is the most beneficial and commonly used of these red monomers (SHANER et al., 2005; DAY & DAVIDSON, 2009).

4.3. Use of fluorescent proteins in scientific research

Fluorescent proteins became important tools for numerous applications in the research of complex biological living systems. In the field of virology these fluorescent markers allow study of the biological properties of the viruses in cells, tissues and even entire organisms by using live imaging techniques (WARD & MOSS, 2001a; DE VRIES et al., 2010; FUKUYAMA et al., 2015).

Tracking fluorescence labeled viruses revealed new insights on their biodistribution in different animal models. Here, the expression of

fluorescent signals by recombinant viruses enables us to follow the course of infection and viral spread by using fluorescence macroscopic-imaging techniques. There have been many fluorescent tracking experiments with various viruses. For instance, the use of this method allowed the identification of lymphocytes as target cells for canine distemper virus (VON MESSLING et al., 2004). Also new findings about the cell tropism of measles infections in macaques could be determined (LEMON et al., 2011).

Furthermore, a widely used feature of fluorescent proteins is their ability to label proteins within cells and organisms in order to track the target proteins from expression through to degradation in real time. In virology this application is based on the generation of recombinant viruses which express fluorescent signals that are fused to the gene of interest. The site of fusion, which can be either at the protein's termini or inside the protein's chain, has to be well deliberated to prevent effects on the target protein's localization, targeting and function. Furthermore, the choice of a suitable fluorescent protein is also very important: it should not interfere with the characteristics of the target protein (CHUDAKOV et al., 2010) and it should produce a sufficient signal for reliable imaging in cells and tissues that have a strong auto fluorescence (RICH et al., 2014). Due to fluorescent fusion proteins it is possible to reveal information on a protein's movement and association with cellular components. For example, the use of GFP fusion proteins containing (I) MV-specific surface protein A27 or (II) the EV-specific B5-R membrane protein, demonstrated that VACV's intracellular EVs and MVs use microtubules for the intracellular transport (WARD & MOSS, 2001b; WARD, 2005a).

In addition to protein labeling, the gene regulatory network can be analyzed by using fluorescence imaging for monitoring the promoters' strength and activities as well (CHUDAKOV et al., 2010). The level and time of fluorescence expression concerning the regulation through a certain promoter can be determined, for example, via flow cytometry (DI PILATO et al., 2013). As fluorescent signals are typically stable for many hours, there are even destabilized fluorescent proteins available. Due to the fact that these proteins have a short time of maturation and

degradation, it is possible to precisely track the promoter's activation period in real time. (LI et al., 1998; CHUDAKOV et al., 2010).

Another unique application of GFP consists in its utilization as a model antigen in viral vector systems. Like other antigenic peptides, GFP-derived peptides are also presented by the major histcompatibility complexes. This in turn results in a GFP-specific CD8+ T cell immune response. Due to the availability of a GFP-epitope-specific peptide, it is easily possible to identify GFP-specific CD8+ T cells via ELISpot Assay (GAMBOTTO et al., 2000). GFP as a model antigen has been proven to be very useful in studying different antigen modifications on immunogenicity. This was nicely shown by a recent study that analyzed the GFP-specific CD8+ T cell response of different antigen localizations in the subcellular compartments (MARR et al., 2016).

So far, fluorescent proteins have been successfully established as excellent tools concerning analyzing virus host interactions, virus life cycles and the biodistribution as they enable the illustration of biological processes during viral infections in various *in vitro* and *in vivo* studies.

III. OBJECTIVES

In the view of the importance of MVA-based vector vaccines in clinical use as well as the need of a characterization of recombinant MVA candidate vaccines in terms of their purity, infectivity and recombinant gene expression this work describes the following:

(I) the generation of recombinant MVA and CVA expressing two fluorescent reporter proteins regulated by stage specific promoters

(II) characterization of both reporter viruses

- genetic analysis
- protein expression and replication analysis
- determination of the relation between the plaque-forming units and total virus particles in purified preparations of the reporter viruses

(III) characterization of vaccina virus infections with the reporter viruses

- monitoring the vaccinia virus life cycle
- evaluation of the relation between single cell infection and plaque formation in MVA permissive cells
- tracking single cell infection via live cell imaging

IV. RESULTS

The manuscript is presented in form accepted for publication (Lülf 2016 Virology, in press).

Non-plaque-forming virions of Modified Vaccinia virus Ankara express viral genes

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In cell culture infections with vaccinia virus the number of counted virus particles is substantially higher than the number of plaques obtained by titration. We found that standard vaccine preparations of recombinant Modified Vaccinia virus Ankara produce only about 20-30% plaque-forming virions in fully permissive cell cultures. To evaluate the biological activity of the non-plaque-forming particles, we generated recombinant viruses expressing fluorescent reporter proteins under transcriptional control of specific viral early and late promoters. Live cell imaging and automated counting by fluorescent microscopy indicated that virtually all virus particles can enter cells and switch on viral gene expression. Although most of the non-plaque-forming infections are arrested at the level of viral early gene expression, we detected activation of late viral transcription in 10-20% of single infected cells. Thus, non-plaque-forming particles are biologically active, and likely contribute to the immunogenicity of vaccinia virus vaccines.

Vaccinia virus (VACV) is the best-studied poxvirus and the prototype live virus vaccine used to eradicate human smallpox (for review see (MOSS, 2013b)). VACV was among the first animal viruses to be cultured and purified (STEINHARDT et al., 1913; PARKER & NYE, 1925; MAITLAND & MAITLAND, 1928; PARKER & RIVERS, 1935). Early studies with virus preparations from the skin of infected rabbits (STEINHARDT et al., 1913) or the chorioallantois membrane (CAM) of embryonated chicken eggs (DUMBELL et al., 1957) already provided evidence that more total particles (also called “elementary bodies”) are produced than infectious VACV virions. Indeed, a ratio of 10 total particles to 1 plaque-forming unit (PFU) was determined for purified preparations of VACV using a protocol of ultracentrifugation through sucrose-gradient similar to the one still used for producing vaccine preparations (JOKLIK, 1962). A single study using semi-purified (removal of cellular debris from virus harvest via low-speed centrifugation) MVA and comparing EM counted particles to tissue culture infectious dose 50 (TICD50) determined 2-3-fold more total particles than the TICD50 (GUGGENBERGER, 1989). This biological phenomenon is

true for many animal viruses, and even when disregarding morphologically or biochemically “incomplete” virions, 10 to 1 million virus particles are usually needed to initiate a single event of infection (ISAACS, 1957).

With a ratio of about 1:10 for CAM infections and tissue culture plaques, VACV has one of the highest ratios of virus infections to particles (JOKLIK, 1962; GALASSO & SHARP, 1963). Interestingly, early experiments suggested that the greater fraction of the non-plaque-forming VACV particles enter cells and are infectious in tissue culture (GALASSO & SHARP, 1964). Yet, it remained obscure whether these infections were able to initiate the virus life cycle, switching on gene expression and protein synthesis. Furthermore, the prevalence of non-plaque-forming particles in VACV vaccine preparations and their possible influence on immunogenicity or vaccine efficacy are largely unknown.

Today, more than three decades after eradicating smallpox as a natural disease, VACV is still needed as a reserve vaccine against smallpox due to the threat of bioterrorism (MOSS, 2011). Moreover, the virus is successfully used as a vector for developing new vaccines against other diseases (MACKETT et al., 1982; PANICALI & PAOLETTI, 1982). However, the VACV vaccines used during the smallpox eradication campaign would be currently considered to have an unsatisfactory safety profile, and safer strains of VACV such as Modified Vaccinia virus Ankara (MVA) have been established for vaccine development (for review see (MOSS, 2013a)).

MVA was obtained by extensive serial passaging of VACV strain Ankara in chicken embryo fibroblasts (CEF), resulting in DNA deletions and mutations at multiple sites of the viral genome (MAYR & MUNZ, 1964; MEYER et al., 1991; ANTOINE et al., 1998). MVA lost its ability to productively replicate in most mammalian cells, but maintained expression of all classes of viral and recombinant genes under conditions of non-permissive viral growth. Therefore MVA can serve as an exceptionally safe live viral vector vaccine (SUTTER & MOSS, 1992). Multiple recombinant MVAs are currently undergoing clinical testing for vaccination against various human infections including AIDS, tuberculosis, and malaria (for reviews see (GILBERT, 2013; GOMEZ et al., 2013)). Critically,

recent candidate MVA vaccines are being developed for experimental immunization of humans against emerging infections such as highly pathogenic avian influenza virus H5N1, the Middle East Respiratory Syndrome, West Nile fever, or Ebola hemorrhagic fever (KREIJTZ et al., 2014; VOLZ et al., 2015b; EWER et al., 2016; VOLZ et al., 2016).

As the clinical use of MVA vector vaccines expands more, vaccine preparations require non-clinical characterization for lot consistency, purity, infectivity, and recombinant gene expression. Our experience and that of others have indicated that the biological properties of the purified MVA particles in these preparations are generally unknown. More fundamental questions pertaining to the regulation of host-restriction and gene expression, as well as practical questions such as MVA vaccine immunogenicity also remain unclear.

Here, we studied recombinant MVA, and found over 70% non-plaque-forming virions in standard MVA vaccine preparations from avian cell lines. Specifically, we were able to monitor the ability of these virions to infect different host cells and activate viral gene expression using reporter viruses expressing fluorescent proteins under the control of early and late viral promoters. The resulting single cell infections synthesized early, and to a lesser extent late gene products. For comparison we also analyzed the particle properties and infection progression of recombinant virus preparations based on the fully replication competent parental VACV Ankara strain CVA152.

Non-plaque-forming virus particles in recombinant MVA vaccines

To characterize the particle content of standard MVA vaccine preparations we selected a sucrose-cushion purified preparation of an MVA candidate vaccine producing the Ebola virus glycoprotein (MVA-EBOV-GP) obtained from clonal isolation by plaque purification. First, to obtain standardized counts of total particles we used a virus counter device (ViroCyt Virus Counter 3100) known to be used for quality control monitoring by vaccine manufacturers. Virus counting is based on flow cytometry using two different fluorescent dyes to stain and co-localize proteins and nucleic

acids. For the MVA-EBOV-GP preparation we counted a total of 1.6 x10^10 virus particles (VP)/ml (Fig. 1A). In comparison, infectious virus particles quantified by plaque titration resulted in 4.8 x10^9 plaque-forming units (PFU)/ml, approximately 30% of the total virus particle count in this virus preparation.

The above data are in line with previous results from particle counts by electron microscopy, suggesting considerable fractions of non-plaque-forming particles are present in VACV preparations.

Monitoring the VACV life cycle with reporter viruses expressing fluorescent proteins

To investigate the biological properties of non-plaque forming VACV particles in more detail, we constructed the recombinant viruses MVA-GFP-mCherry and CVA-GFP-mCherry as reporter viruses (Fig. 1B). CVA-GFP-mCherry served to directly compare MVA to a fully replication-competent VACV, by using the Ankara strain CVA152 as a true ancestor virus of MVA. These recombinant viruses were designed to monitor the infection cycle by visualizing key steps in viral gene expression, detected through the synthesis of fluorescent marker proteins. Early GFP production should indicate virus entry and transcription of viral early genes; the detection of red fluorescent mCherry protein (late-mCherry) should signal the start of DNA synthesis and viral late gene expression.

The viruses were quality controlled by PCR analysis, confirming the stable integration of the GFP encoding sequences at the I8R/G1L intergenic site and mCherry sequences at deletion site III of both MVA and CVA152 genomes (Fig. 1B; Fig. S1; data not shown). Both recombinant viruses were genetically stable, and tested for growth, as well as production of recombinant GFP and mCherry proteins as detected by Western blot analysis (Fig. S2; data not shown). As expected, MVA-GFP-mCherry replicated efficiently in avian CEF and DF-1 cells but not in human HeLa or HaCat cells.

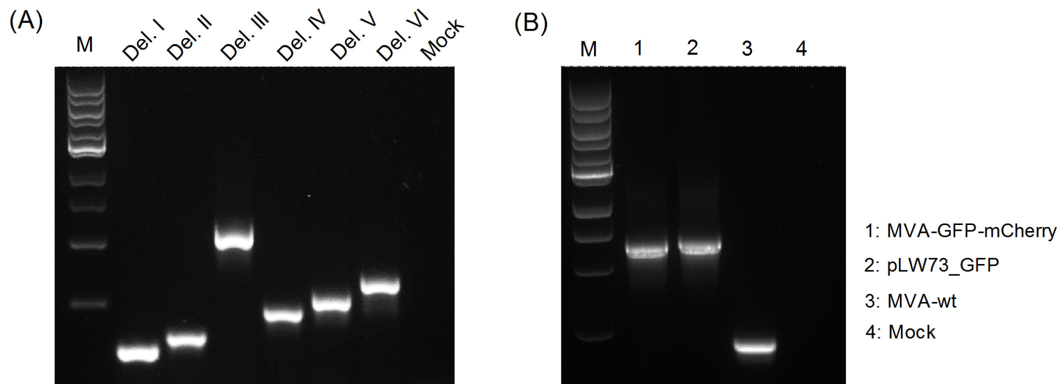

Fig. S1 Genetic integrity and genetic stability of MVA-GFP-mCherry. (A) PCR analysis of MVA-GFP-mCherry viral DNA using oligonucleotide primers flanking the six major deletions. (B) PCR analysis of MVA-GFP-mCherry viral DNA using oligonucleotide primers flanking the I8R/G1L insertion site.

To test the activity of our reporter viruses after tissue culture infection, we examined the production of the fluorescent proteins GFP and mCherry in chicken (CEF and DF-1) or human cells (HaCat and HeLa) by fluorescence microscopy (Fig. 1C). At 24 hours post infection (hpi), microscopy revealed foci in MVA-GFP-mCherry infected CEF and DF-1 cells; red fluorescent cells in the center were surrounded by green fluorescent cells, marking the early and late phases of the MVA infection. In contrast, in HeLa and HaCat cells we detected single green or red fluorescent cells, but no formation of plaques or foci with fluorescent cells.

Productive CVA-GFP-mCherry infections resulted in typical VACV plaques visible 24 hpi in all four cell cultures. Again red late-mCherry producing cells accumulated in the centers of large virus plaques, while green early-GFP synthesis was found around the periphery of the plaques. These results provided reasonable proof of principle for using these recombinant viruses to visualize various productive and abortive infection events in tissue culture.

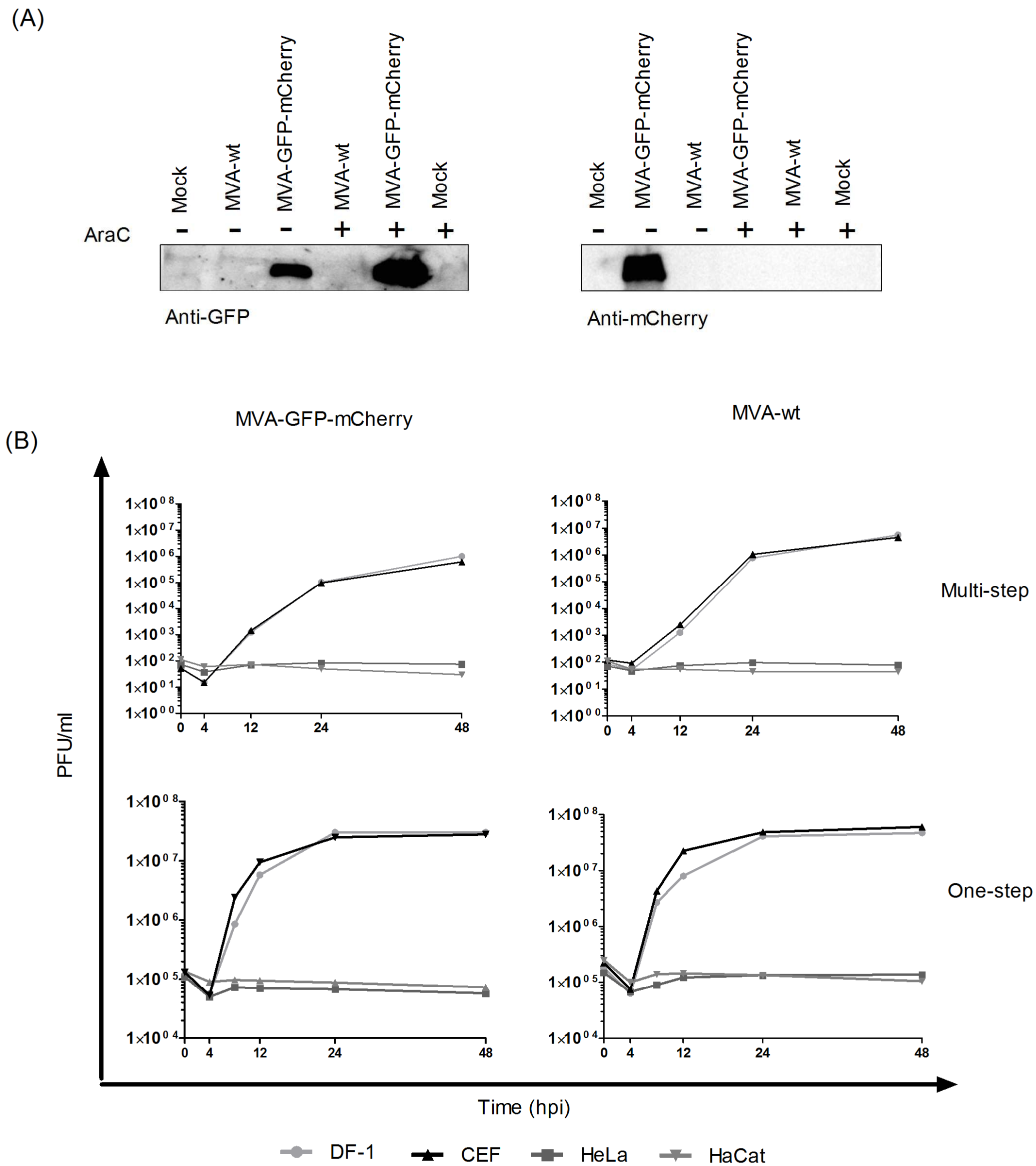

Fig. S2 Protein expression and replication analysis.

(A) Western blot analysis of total cell extracts from primary chicken embryo fibroblasts infected with MVA-GFP-mCherry or MVA wildtype at an m.o.i. of 10 without (-) or with (+) AraC, 24 hpi. (B) Cells were infected at an m.o.i. of 0.001 for multi-step growth analysis, and at an m.o.i. of 5 for one-step growth analysis. The cell cultures were harvested at the indicated time points and virus yields were determined by back titration and counting immunostained foci using a plaque assay. The average values of two independent experiments with double titrations are shown.

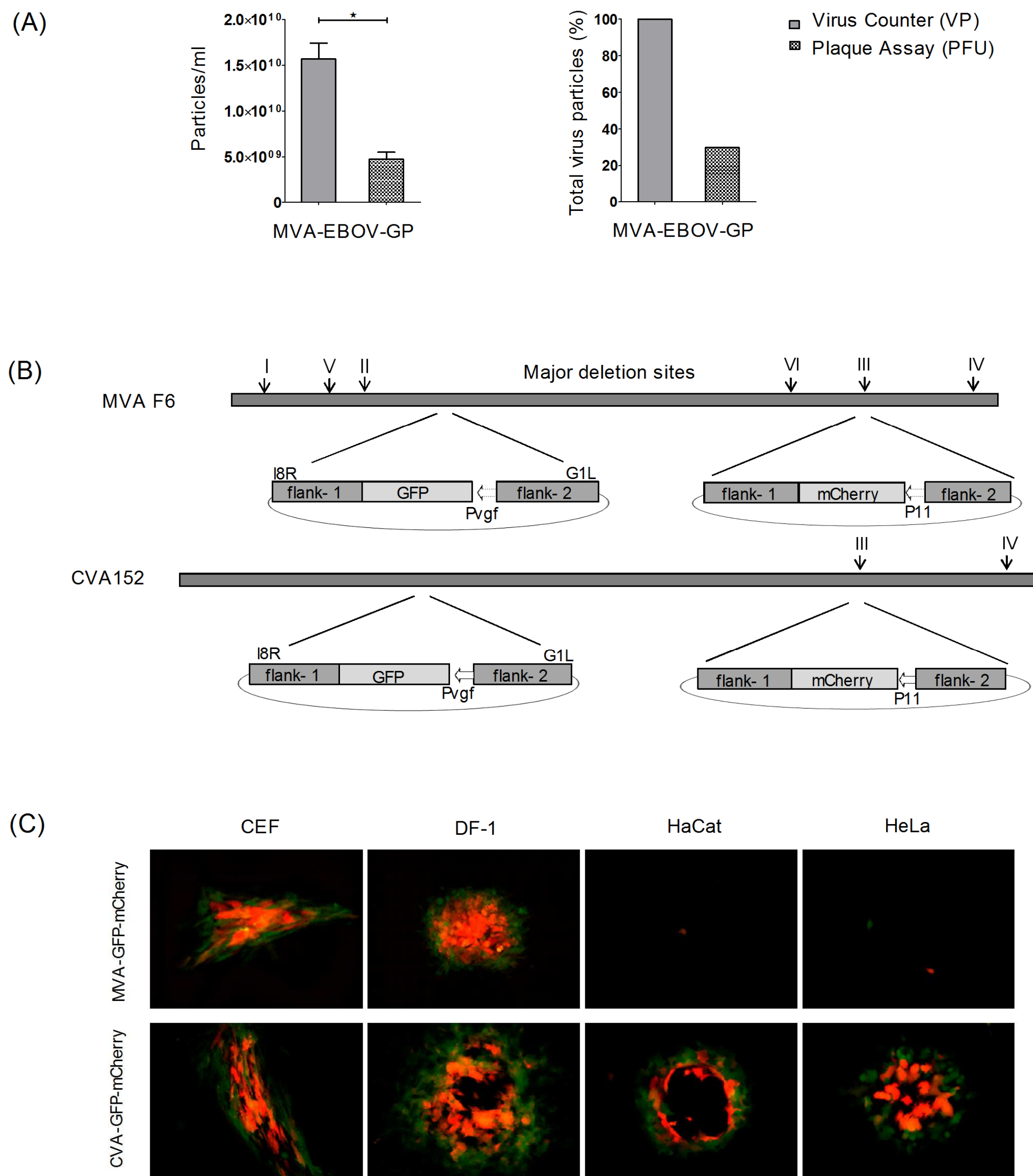

Fig. 1. Monitoring non-plaque-forming virus particles and gene expression with reporter fluorescent proteins. (A) Plaque forming units (PFU) and total virus particles (VP) in sucrose-cushion purified stock preparations of an MVA recombinant candidate (MVA-EBOV-GP) vaccine were quantified via plaque assays (PFU/ml) and virus Counter measurement (VirusCounter 3100-ViroCyt) (VP/ml). *P<0.05. (B) Schematic diagram of the MVA F6 and CVA152 genome indicating the I8R/G1L site, used to insert GFP regulated by the early vaccinia promoter Pvgf and the deletion III site used to insert mCherry under the transcriptional control of the late vaccinia promoter P11. (C) Fluorescence imaging of CEF, DF-1, HaCat and HeLa cells infected with MVA-GFP-mCherry and CVA-GFP-mCherry at an m.o.i of 0.005 at 24 hpi.

Infections with MVA-GFP-mCherry reveal numerous single cell infections without plaque formation

Again using standard sucrose-cushion purified preparations of MVA-GFP-mCherry and CVA-GFP-mCherry, we determined total virus particle numbers by particle counting, and measured infectivity by plaque titration (Fig. 2A). In agreement with our data above this analysis also revealed at least 3-fold more total particles than PFU. In detail, Virus Counter measurements recorded 1.5 x10^10 VP/ml for MVA-GFP-mCherry and 2.3x10^10 VP/ml for CVA-GFP-mCherry. In contrast, the plaque forming units as quantified by plaque titration amounted to 3.8x10^9 PFU/ml (~25% of the total particles) for MVA-GFP-mCherry and 3.5x10^9 PFU/ml (~15% of the total particles) for CVA-GFP-mCherry.

To monitor infection events in cell cultures permissive for productive virus replication, we analyzed the kinetics of GFP and mCherry expression upon MVA-GFP-mCherry infection in CEF and DF-1 cells (Fig. 2B). GFP and mCherry expression detected by fluorescence microscopy mostly revealed single green fluorescent cells at 12 hpi. At 14 hpi some of the single green cells shifted to red fluorescence, and adjacent cells started to show green fluorescence, forming foci of infected cells. MVA-GFP-mCherry formed more even and rounder plaques in DF-1 cells than the plaques in CEF, which were more irregularly shaped, corresponding to the well-known MVA plaque phenotypes in these cell cultures (Kremer et al., 2012). By 18 hpi we readily observed growing MVA-GFP-mCherry plaques in the cell monolayers, but the majority of the monitored single green fluorescent cells neither changed to red fluorescence nor developed into new virus plaques until 40 hpi.

The time point 24 hpi seemed suitable for determining the ratio of single infected cells to virus plaques in CEF and DF-1 after MVA-GFP-mCherry infection (Fig. 2C). Single fluorescent cells and virus plaques in 96-well cell monolayers were automatically quantified (Keyence Hybrid Cell Count Module, BZ-H3C). In both chicken cell cultures about 20-30% of all detected fluorescent events were identified as viral plaques. However, most of the infection events (>70%) were detected as single fluorescent cells. After imaging we fixed the infected cell cultures and performed a

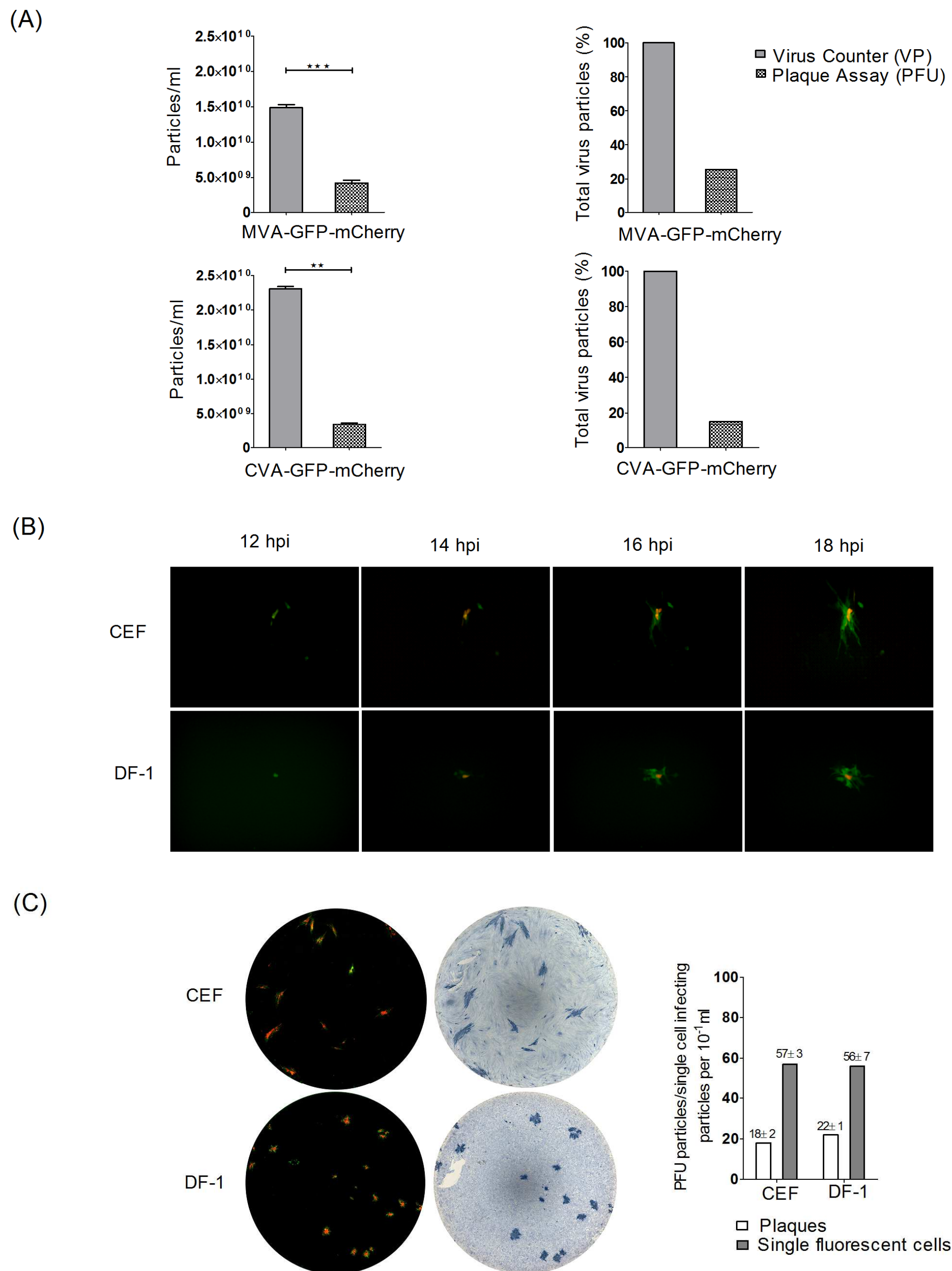

Fig. 2. Analysis of the MVA life cycle using the reporter virus. (A) Plaque forming units (PFU) and total virus particles (VP) in sucrose-cushion purified stock virus preparations of MVA-GFP-mCherry and CVA-GFP-mCherry were measured via plaque assays (PFU/ml) and virus Counter measurement (VirusCounter 3100-ViroCyt) (VP/ml). **P<0,01; ***P<0,001. (B) Time course of plaque development. CEF and DF-1 cells were infected with MVA-GFP-mCherry at an m.o.i. of 0.005. Infection events were monitored via fluorescence imaging in two hours increments. (C) CEF and DF-1cells grown in 96-well plates were infected with MVA-GFP-mCherry at an m.o.i. of 0.01. The wells were imaged via fluorescence microscopy at 24 hpi (left panel) and a VACV-specific immunostaining plaque assay was performed as a control (middle panel). Single infected fluorescent cells and plaques were quantified using Hybrid Cell Count Software (right panel).

standard VACV-specific immunostaining assay to confirm the data from plaque counting using the Hybrid Cell Count analysis assay. An equal number and very similar pattern of virus plaques were detected (Fig. 2C). These results indicate a surprisingly high number of single cell infection events, which closely correlated with the calculated number of non-plaque forming particles in the MVA-GFP-mCherry preparation.

VACV reporter viruses reveal viral early gene expression in most non-plaque forming infections

To more precisely follow up the outcome of MVA-GFP-Cherry infections in DF-1, CEF, and HeLa cells grown in 6-well plates, we selected 30 single GFP positive cells at 12 hpi. We automatically monitored the selected infected cells in the monolayers via live cell imaging (Keyence Time Lapse Module) over a time period of 24 hours (Fig. 3).

In DF-1 cells, 20% of the selected single infected green fluorescent cells resulted in the formation of plaques, 5% of the single cells proceeded to late gene expression as indicated by red fluorescence, and 75% of the infected cells remained green, i.e. in early gene expression. We could confirm these findings in CEF cells, where 16.9% of the selected cells formed plaques, 10.6% shifted to red fluorescence without plaque development, and 72.5% remained single green fluorescent cells. As expected, MVA-GFP-mCherry infection of human HeLa cells did not result in detectable virus plaques. However, the overall ratio of infected green cells arrested at the early stage of gene expression (83.9%) to infected red cells exhibiting late gene expression (16.1%) was similar to the proportion observed in the avian cell cultures (Fig. 3A).

Next, we analyzed infections with the replication-competent CVA-GFP-mCherry in DF-1 and HeLa cells using the same experimental setting (Fig. 3B). In DF-1 monolayers 10.1% of the selected single infected cells developed into typical plaques, 17.6% of the single cells displayed red fluorescence without formation of plaques, and 72.3% of the infected cells remained green. These results were comparable to those obtained with MVA-GFP-mCherry in DF-1 cell cultures. Interestingly, in HeLa cells replication-competent CVA-GFP-mCherry formed about three-fold more

plaques than seen in DF-1 cell infections. Overall, these data demonstrated that most tissue culture MVA or CVA infection events remain arrested at the level of viral early gene expression.

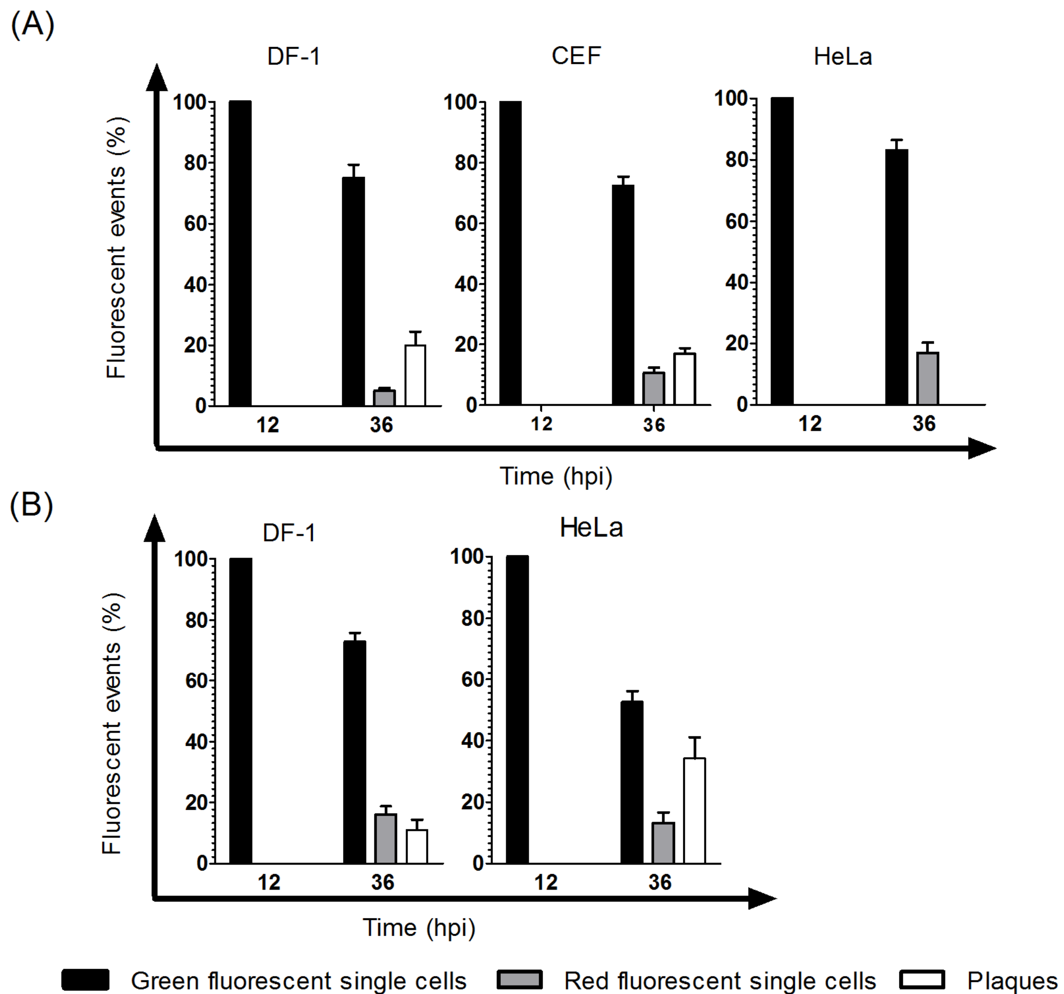

Fig. 3. Tracking single cell infection via live cell imaging.

Single green fluorescent DF-1, CEF and HeLa cells, infected with sucrose-cushion purified viral stock preparation of MVA-GFP-mCherry (A) or sucrose-cushion purified viral stock preparation of CVA-GFP-mCherry (B), were selected at 12 hpi and monitored for 24 hours in 30 minutes increments via Time Lapse Software. Each value represents the mean with SEM of at least three separate experiments.

Here, we tested the composition and biological activities of virus particles present in standard MVA vaccine preparations. For the first time we used new reporter viruses expressing the fluorescent markers GFP and mCherry under the control of an early and late viral promoter. Over the

past few years VACV biology has greatly benefited from advances in genetic engineering and fluorescent imaging techniques. Recombinant viruses with fluorescently tagged viral proteins have been elegantly used to elucidate the cell-to-cell spread and intracellular movement of VACV particles (FRISCHKNECHT et al., 1999; HOLLINSHEAD et al., 2001; WARD & MOSS, 2001a). In addition, fluorescent reporter viruses serve in screening experiments for antiviral drugs (DAL POZZO et al., 2008). Here, our reporter viruses successfully monitored VACV infection progression of both plaque-forming and non-plaque-forming virions and identified active transcription of viral early and late genes.

In infection research recombinant MVA viruses serve as candidate vaccines compatible with clinical use and industrial-scale production (for review see Volz 2016 Adv Virus Res, in press). The data quality required before initiating clinical trials include evaluation of the phenotypic properties of the vaccine virus preparations (European Medicines Agency. 2010. Guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines. EMA/CHMP/VWP/ 141697/2009).

Sucrose-gradient purified preparations of conventional VACV are known to comprise about 10-fold more total particles than infectious particles, e.g. as counted by electron microscopy and measured by plaque tests (JOKLIK, 1962). Also in this study, using an industry-standard virus counter and our regular protocol for MVA titration (KREMER et al., 2012a), we found a majority (>70%) of non-plaque-forming virions in standard MVA vaccine sucrose-cushion purified preparations from CEF or DF-1 cells, avian cell lines that are consistently used with MVA to amplify and purify normal titers from cell cultures.

Similar “noninfectious” particles have been found with many mammalian viruses, but the biological activities associated with these particles have largely been ignored (MARCUS et al., 2009; PIERSON & DIAMOND, 2012). Further characterizing these non-plaque-forming particles in MVA vector vaccine preparations seems relevant due to their potential role in inducing innate or adaptive immune responses (LOPEZ, 2014).

When testing sucrose-cushion purified preparations of MVA-GFP-mCherry

or CVA-GFP-mCherry in tissue culture infections we detected an unexpectedly high number of single fluorescent cells. Such monitoring of the infection events shows that non-plaque-forming particles also activate viral gene expression, although most of these single cell infections are restricted to synthesizing early gene products.

The de novo synthesis of early viral RNAs suggests that these non-plaque forming infections trigger innate immune signaling, as previously described for NFkB activation following VACV early transcription (MARTIN & SHISLER, 2009; WILLIS et al., 2011). Notably, our time lapse experiments allowed us to precisely follow the development of single viral plaques or the fate of single infected cells by observing the synthesis of GFP and mCherry (see video clips Fig. S3).

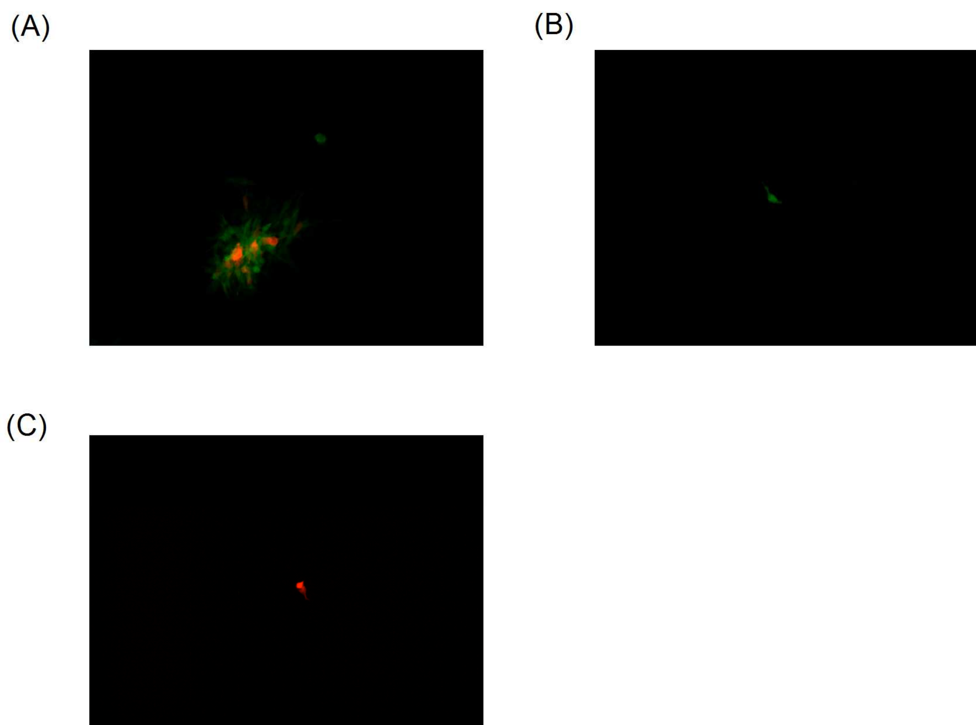

Fig. S3 Time Lapse videos depicting the fate of single green fluorescent DF-1 cells infected with MVA-GFP-mCherry: (A) Infection results in plaque formation with multiple cells proceeding from viral early (green fluorescence) to late (red fluorescence) gene expression (B) Single cell infection remains restricted to viral early gene expression as identified by green fluorescence (C) Single cell infection proceeds to viral late gene expression as identified by red fluorescence.

Regardless of the stage of viral gene expression (early or late) and the permissiveness for productive infection, many of the remaining single fluorescent cells underwent morphological changes, including phases of contraction with retractions of cell projections followed by cell rounding, membrane blebbing, and disintegration or detachment from the cell monolayer. Since the results from experiments with replication-competent CVA-GFP-mCherry confirmed the data seen for MVA-GFP-mCherry, we are confident about the general validity of these findings for infections with particles from different strains of VACV and possibly for infections with virions from other orthopoxviruses.

The mechanistic reason(s) for the substantial portion of abortive infection events still remain unclear. One possibility is that VACV preparations commonly contain fractions of “defective” virions unable to initiate a full cycle of viral gene expression and productive virus replication. We examined the virus particles in our stock preparations by electron microscopy and detected only mature brick-shaped virions with normal morphology (data not shown). Yet, this apparent absence of malformed particles does not formally exclude the possibility of mutations blocking later stages of infections. Alternatively, the biology of the host cell is essential for the replication of VACV and it is tempting to speculate that simply the condition of the infected host cell determines the outcome of an infection. The activation of host cell signaling pathways e.g. involving the MAPKs (mitogen-activated protein kinases) ERK (extracellular signal-regulated kinase) and JNK (Jun N-terminal kinase) has been shown to be critical for productive infection and VACV spread (DE MAGALHAES et al., 2001; ANDRADE et al., 2004; WANG et al., 2004a). Moreover, the activation of these host signaling pathways is specifically modulated in the favor of virus replication by various poxvirus regulatory proteins (DE MAGALHAES et al., 2001; WANG et al., 2006; SCHWENEKER et al., 2012; TORRES et al., 2016). These findings are used in the development of oncolytic poxviruses that better grow in tumor cells where such pathways are malignantly activated (BELL & MCFADDEN, 2014). Our observation of CVA-GFP-mCherry infection resulting in enhanced plaque formation in HeLa cells might be based on the preferential productive

replication of this virus in tumor cells (see Fig. 3B).

There are two major infectious forms of VACV particles: the mature virions (MV) and the extracellular enveloped virions (EV) (MOSS, 2013b). MVs are infectious virions surrounded by a lipoprotein membrane that accumulate within the cytoplasm of VACV infected cells (CONDIT et al., 2006). Since MV are the most abundant virion form present in vaccine preparations, the data obtained in our experiments might be essential for the quality control of MVA based vaccines. Both infectious forms, MV and EV, have distinct biological properties and activities: EV are responsible for efficient cell-to-cell spread and contribute to transmission within the host, while the MV play a key role in the transmission between the host animals (BLASCO & MOSS, 1992; CUDMORE et al., 1995; MOSS, 2012). Further experiments are needed to clarify on a possibly distinct role of EV infections on gene expression and plaque formation in different host cell substrates.

In conclusion, our experiments confirm that infection with non-plaque-forming particles of VACV results in gene expression. Despite the inability of these “defective” virus particles to induce plaque formation, these particles are still able to switch on early gene expression. Interestingly, already early work by Galasso and Sharp (1964) suggested that “non-replicating” VACV particles enter cells and exert biological activities. More recently, UV-inactivated recombinant VACV is used to express early genes as nonreplicative vector in tumor immunotherapy (ZAJAC et al., 2003). Thus, viral and recombinant early transcripts might serve to efficiently trigger innate immune responses and antigen production by recombinant vaccines similar to processes described for other vaccine-induced activations of immunity. In addition, these findings indicate that the choice of early or early-late promoters is an appropriate approach to most practically activate vaccine-induced immunity. Finally, our results further support the ability of MVA-based vaccines to stimulate a balanced interplay between innate and adaptive immunity as the key to robust protection against a selected pathogen.

Materials and methods

Cells

DF-1, HaCat and MA-104 Jena cells were cultured in VLE Dulbecco's MEM (Biochrom GmbH) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (SIGMA), 1% Penicillin-Streptomycin (SIGMA) and 2% Hepes-buffer (SIGMA). HeLa cells were maintained in Minimum Essential Medium Eagle (MEM) (SIGMA) containing 10% HI-FBS and antibiotics as described above. Primary chicken embryo fibroblasts (CEF) were prepared from 10-day-old chicken embryos (SPF eggs, VALO, Cuxhaven) and subsequently grown in MEM supplemented with 1% Penicillin- Streptomycin, 1% MEM non-essential amino acid solution (SIGMA) and 10% HI-FBS. All cells were maintained at 37°C in a 5% CO2 atmosphere.

Generation of recombinant viruses

Recombinant MVA-GFP-mCherry was generated by homologous recombination as described previously (KREMER et al., 2012a) MVA F6 (MEYER et al., 1991) served as the parental virus for generating MVA-GFP, used as the backbone virus for constructing MVA-GFP-mCherry. Vector plasmid pLW73 was used to direct the insertion of GFP, under the transcriptional control of vaccinia virus promoter Pvgf, into the G1L/I8R insertion site of the viral genome. Pvgf (5'-GTTTATATTACTGAATTAATAATATAAAATTCCCAATCTTGTCATAAA-3'; underlined A indicates transcriptional start site as described by Broyles et al., 1991.), a natural vaccinia virus promoter, controls the early expression of VACV ORF C11R mRNA that was recently reported to belong to the temporally first class of early genes (immediate-early class or E1.1 subclusters) (ASSARSSON et al., 2008; YANG et al., 2010). Plasmid pIIIgptex served as the vector for inserting mCherry, placed under the vaccinia virus promoter P11, into Deletion III. Recombinant MVA was obtained by clonal isolation in plaque purifications using five to eight passages in CEF following the plasmid transfections. Finally, recombinant viruses were amplified in CEF. Screening for expression of the fluorescent proteins was used to identify and isolate recombinant viruses. Quality

control experiments were performed using standard methodology (KREMER et al., 2012a).

The genetic identity and genetic stability of the reporter virus were assessed via PCR analysis of genomic viral DNA. The replicative capacity of the recombinant virus was confirmed by one-step and multiple-step growth experiments in CEF, DF-1, HeLa and HaCat cells. CVA-GFP-mCherry was constructed in a similar manner to MVA-GFP-mCherry, but the fully replication-competent virus CVA152 (WAIBLER et al., 2009) was used as the parental virus and the propagation was carried out in MA-104 cells. CVA152 is a direct ancestor virus of MVA and its genome sequence is identical to MVA at the insertion sites Deletion III and I8R/G1L. Sucrose-cushion purified virus stocks of MVA-GFP-mCherry and CVA-GFP-mCherry were generated by ultracentrifugation through a 36% sucrose cushion, followed by resuspension in 10 mM Tris buffer pH 9 using standard procedures as described in (KREMER et al., 2012a) and (JOKLIK, 1962).

Quantification of virus

Plaque assay

Viral plaque forming units (PFU) were determined via standard plaque assays, as described in Kremer and coworkers (KREMER et al., 2012a). Briefly, confluent CEF cell monolayers in 6-well plates were infected with serial 10-fold dilutions of the viruses. After 2 h of incubation at 37°C, the cells were washed once with phosphate buffered saline (PBS) and incubated with fresh medium containing 2% HI-FBS at 37°C for two days, followed by a vaccinia specific immunostaining. Here, the infected cells were fixed in acetone-methanol (1:1) for 5 min, washed with PBS and incubated for 60 min with a polyclonal rabbit anti-vaccinia virus antibody. After an additional washing step (PBS), the cells were incubated with peroxidase-conjugated AffiniPure goat anti-rabbit secondary antibody for 60 min. Plaques were visualized using TrueBlue peroxidase substrate solution (KPL), counted and calculated as described previously (KREMER et al., 2012a). All virus titrations were performed in duplicate and repeated at least three times.

Total virus particle counts

Total virus particles were estimated using ViroCyt VirusCounter 3100 following the manufacturer's instructions (ViroCyt, Bloomfield, CO). Briefly, the sucrose-cushion purified virus samples were diluted 1:200 and 1:400 in ViroCyt sample dilution buffer. Subsequently, 300 µl of each sample were incubated with 150 µl Combo Dye solution, a combination of two fluorescent dyes specific for proteins and nucleic acids (STOFFEL et al., 2005). After 30 min of light-protected incubation at room temperature, the virus particles were quantified in the virus counter. Simultaneous detection of both fluorescent signals using the virus counter's dual channel flow cytometer was measured as one virus particle. Inter-sample washes, followed by a cleanliness control run were performed between each sample analysis, and medium from uninfected CEFs was used as a negative control. Total virus particle concentration was recorded as virus particles per ml (VP/ml) and determined for at least three times per virus sample.

Fluorescent microscopy

Fluorescent images were obtained using an inverted fluorescence microscope (Keyence BZ-X700) with a x20 Plan Fluor NA 0.45 Ph1 objective and a z-section setting. Emission of GFP filter (excitation wavelength 470/40 nm, absorption wavelength 525/50 nm) and TexasRed filter (excitation wavelength 560/40 nm, absorption wavelength 630/75 nm) were used for detecting green and red fluorescence. Raw imaging data were processed and analyzed with BZ-X Analyzer Software.

Hybrid cell count

The ratio of single infected fluorescent cells to plaque-forming fluorescent units was assessed during MVA-GFP-mCherry infection in permissive cells by culturing CEF and DF-1 cells in 96-well plates. Each well was infected at an m.o.i. of 0.01 and received 100 µl of inoculum. After 30 min incubation at 4°C (cold start), cells were washed with PBS three times and incubated at 37°C with 200 µl fresh medium containing 2% HI-FBS. 24 h after infection multiple fluorescent and bright-field images of the infected wells were acquired using a 20x objective, and then stitched together via

the Keyence Merge function. Afterwards a vaccinia specific immunostaining of the captured wells was performed, as described above (Plaque assay). Bright-field images of these wells served as controls. The total single fluorescent cell count was quantified using single extraction function of the Hybrid Cell Count Software (Keyence Hybrid Cell Count Module, BZ-H3C) and total foci were counted manually.

Live cell imaging

For live cell imaging, DF-1, CEF and HeLa cells cultured in 6-well plates, were infected with a sucrose-cushion purified viral stock preparation of MVA-GFP-mCherry or a sucrose-cushion purified viral stock preparation of CVA-GFP-mCherry at an m.o.i. of 0.005 via cold start as described above. After three washing steps with PBS, 2 ml fresh medium containing 2% FBS was added and the cells were incubated at 37°C. At 12 hpi about 30 single green fluorescent cells were selected for each separate experiment. The development of the single infected cells was observed in 30 minutes increments for a further 24 hours using Keyence Time Lapse Module, BZ-H3XT. Time lapse analysis was performed at 37°C with 5% CO_2 concentration using an incubation chamber (Tokai Hit, Incubation Systems for Microscopes) to allow 24 h recording. All time lapse experiments with recombinant MVA and CVA viruses were performed at least three times.

Western blot analysis

Chicken embryo fibroblasts were infected at an m.o.i. of 10 with MVA-GFP-mCherry and CVA-GFP-mCherry in the absence or presence of cytosine arabinoside (AraC, 1 mg/ml). MVA wild type or mock-infected cells served as controls. Total cell extracts were prepared at 24hpi. After separating by 10% SDS-PAGE proteins were analyzed via Western Blots using anti-GFP rabbit fraction antibody (life technologies, 1:250) and anti-mCherry rat fraction antibody (kindly provided by Elisabeth Kremmer, Helmholtz Zentrum München, 1:10 dilution) as primary antibody. For detection of GFP and mCherry via MicroChemisystems (biostep), anti-rabbit HRP-conjugated antibody (Sigma, 1:5000 dilution) and goat anti-rat HRP-conjugated antibody (BioLegends, 1:2000) were used as secondary antibodies.

Statistical analyses

The differences in virus particle titres were assessed for statistical significance with GraphPad Prism version 5 software (GraphPad software, San Diego, USA); using the Mann-Whitney U test and P-values less than 0.05 were considered to be statistically significant.

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V. DISCUSSION

The heterogeneity of MVA and VACV populations in terms of the particle's infectivity has been known for many years. The plaque-forming particles constitute a minority in purified MVA and VACV viral stock preparations. However, the majority of the particles form a subpopulation whose effect for the biology of infections is still uncertain. The objective of the current study was to provide new insights in the biological activities of this unknown particle subset. Our newly constructed reporter viruses, MVA-GFP-mCherry and CVA-GFP-mCherry, enabled us to analyze the complexity of MVA and CVA infections in life-time. The expression of GFP and mCherry, controlled by specific early and late promoters, allowed for detection of productive and abortive infections as well as for the specific level of gene expression. Our *in vitro* data showed that a minority of the viral particles replicated productively. The fraction of non-plaque forming (NPF) particles was likely represented by infections that were unable to pass beyond the stage of early gene expression. As these abortive infections constituted a surprisingly high proportion of the total infections, we assume that nearly all virions of MVA and CVA have the capability to activate at least the stage of early gene expression.

Population heterogeneity of other viruses

The presence of NPF-particles has been observed for decades in the population of several RNA and DNA viruses (WILDY & WATSON, 1962; RAINBOW & MAK, 1970). Depending on the specific biology of the viruses, the ratio of particles to infectious units varies from 1 to 1000 (Table1). Regarding the high values of particle-to-PFU ratio, the question arises whether these NPF-particles have certain viral functions that may affect the course of viral infections.

The first biologically active but "non-productive" form of virus particles was

Virus	Particle-to-PFU ratio
Polyomaviridae	
Polyomavirus	38-50
Simian virus 40	100-200
Adenoviridae	
Adenovirus type 12	320[1]
Poxviridae	
Vaccinia virus	6-7[2]
MVA	4-5[2]
Orthomyxoviridae	
Influenza virus	20-50
Picornaviridae	
Poliovirus	30-1.000
Reoviridae	
Reovirus	10
Alphaviridae	
Semliki Forest virus	1-2
Herpesviridae	
Herpes simplex virus	50-200

Table 1: **Particle-to-PFU ratio of different viruses**
(Principles of Virology, Flint et al., Third Edition, ASM Press; modified)
[1] (Lülf et al., 2016), [2] (GREEN et al., 1967)

detected by Henle and Henle in the 1940s, who described the now widely known defective interfering (DI) particles of influenza virus populations

(HENLE & HENLE, 1943). This fraction of particles constitutes a subpopulation with special properties: they contain smaller units of the genome, their replication depends on the presence of a functional standard virus, they interfere with the propagation of the “helper viruses” during coinfection and the amount of DI particles increase during passages at high multiplicity of infection (HUANG & BALTIMORE, 1970). Over the years, DI particles were detected *in vitro* in many other virus families, especially in RNA viruses such as vesicular stomatitis virus (HACKETT et al., 1967), sendai virus (KINGSBURY et al., 1970) and polio virus (COLE et al., 1971). While it has long been known that VACV preparations exhibit a significant difference between total virus particles and NPF-particles, such DI particles have never been found in POXVs (ISAACS, 1957; GALASSO & SHARP, 1963).

In addition to DI particles, a further subpopulation of NPF-particles can be detected in Influenza A virus - the semi-infectious (SI) particles (BROOKE, 2014). This fraction is spread-incompetent and non-interfering, but has infectious potential. It can be isolated *in vitro* as well as *in vivo*. The SI particles are able to infect cells and express restricted numbers of essential viral proteins. Depending on which proteins are synthesized, these particles activate different innate signaling pathways. Thus, SI particles can be assigned to distinct, detected subpopulations: noninfectious cell killing particles (niCKP) (NGUNJIRI et al., 2008; BROOKE, 2014), IFN-inducing particles and IFN-induction suppressing particles (MARCUS et al., 2005; NGUNJIRI et al., 2012; BROOKE, 2014). Among the NPF-virions in CVA and MVA preparations, we also determined differences in their ability to achieve the levels of gene expression (either early gene expression or until late gene expression). It is also very likely that the activation of early or early and late stages in the VACV molecular life cycle initiate different innate signaling pathways.

Among the DNA viruses, NPF-particles capable of a range of biological activities can also be detected. For human adenoviruses it is known that such particles have lower density compared to functional infectious virions due to deletions of their viral genome. For this reason, the subpopulations can be easily analyzed by separating them through centrifugation in a

density gradient. Thus, some viral functions like cytolysis and tumor (T) - antigen production could also be attributed to the fraction of NPF-virions of adenovirus Type 12. (RAINBOW & MAK, 1970; MAK, 1971). This goes in line with studies of defective virus particles of Simian Virus 40, which are able to induce virus-specific T antigens (UCHIDA et al., 1968). Studies of the NPF-particles with limited biological functions, or rather DI-particles of various DNA and RNA viruses, indicated that their genomes, when compared with genomes of standard plaque-forming-particles, differ in terms of their size. The morphology and structural proteins of the different particle subpopulations appear identical. (PATTNAIK & WERTZ, 1991; DIMMOCK & EASTON, 2014; FRENSING, 2015) In the field of VACVs studies analyzing the biological activities of NPF-particles are very limited. In the 1960s Galasso and Sharp had a very early suspicion that all NPF-particles are at least capable of infecting cells (GALASSO & SHARP, 1964). This approach had not yet been further pursued until very recently. However, the current study has confirmed the presumption of Galasso and Sharp. Indeed, the results indicate that essentially all NPF-particles are capable of reaching the stage of early gene expression. This may have several implications for the future of infection research, especially with regard to MVA as a vaccine platform.

Detailed studies will be required to understand the mechanisms that are crucial to execute the observed abortive infection or rather the origin of the NPF-particles. One interesting aspect could be the evaluation of mutations within the NPF-particles' genome. Depending on the particular proteins that can be expressed, the NPF-particles might have the potential to rise beyond the stage of early gene expression. However, the abortive events might also result from the interactions with the respective host. On a cellular level the specific activation status of the cell might restrict VACV life cycle to the early stage. In addition this might also be induced by the activation of specific cell signaling pathways that are activated by VACV proteins, such as the well-studied vaccinia virus growth factor (VGF) or the regulatory VACV protein O1 targeting the ERK signaling pathway (BULLER et al., 1988; SCHWENEKER et al., 2012).

Impact of the NPF-virions on the biology of infections

Although the NPF-particle subpopulation of different mammalian viruses was previously thought to have only restricted viral functions *in vitro*, there is growing evidence that this fraction contributes to the pathogenesis of infections *in vivo*. The induction of the innate immune response appears to be especially affected by these NPF-particles (FRENSING, 2015). During viral infection, virus-associated molecules such as double-stranded RNA (dsRNA) are recognized through the host's pattern-recognition receptors. These induce a signaling cascade which specifically activates among other things the expression of type I interferons via different transcription factors such as NFkB (KAWAI & AKIRA, 2006). This in turn initiates the expression of antiviral proteins that interfere with viral replication.

Especially, the DI genomes of some viruses seem to induce the innate immune response. For instance, the copy-back DI genomes of the Sendai virus, which have complementary ends, are able to form long stretches of dsRNA. Strahle et al. state that this presumably explains the strong interferon (IFN) beta activation through the Sendai virus (STRAHLE et al., 2006). Furthermore, it has been shown that Sendai virus and Influenza A virus DI genomes are naturally generated in lungs of infected mice and also contribute to an IFN induction *in vivo* (TAPIA et al., 2013).

However, the NPF-particles of MVA and CVA preparations described in the current study have different biological properties in comparison to DI particles. Virtually all of these NPF-particles are able to enter cells and switch on the early stage of gene expression. It seems very likely that this has an impact on the course of infections. There are several studies that provide insight on the early phase of the MVA life cycle triggering cellular signaling pathways.

Regarding the cellular basis of the adaptive immune response, it has been shown that recombinant genes expressed under the control of early VACV promoters induce greater cytotoxic CD8+ T cell responses compared to late promoters (COUPAR et al., 1986; ZHOU et al., 1991; BRONTE et al., 1997). Generally, the induction of a strong T-cell response has become increasingly important in vaccine development as several studies

confirmed that T-cell responses play a critical role for the immunity against infectious diseases (PANTALEO & KOUP, 2004; KREMER et al., 2012b; ALTENBURG et al., 2014). Moreover, it has been reported that the VACV gene expression arrest at the early phase of the viral molecular life cycle in human and murine dendritic cells proposing to express target antigens under the control of early promoters (BRODER et al., 1994; BRONTE et al., 1997; SUBKLEWE et al., 1999; KASTENMULLER et al., 2006). In addition, these dendritic cells are essential for the cross-presentation of viral antigens (SIGAL et al., 1999; ACKERMAN et al., 2003; LARSSON et al., 2004), which is relevant for protection against viruses that do not directly infect antigen-presenting cells. Here, exogenous antigens, avoiding the endogenous processing pathway, are taken up via phagocytosis and displayed to cytotoxic CD8+ T cells via major histocompatibility complex class I molecules (CHAPLIN, 2010). The use of early promoters has also been reported to be crucial for the reactivation of memory T-cells. Kastenmüller et al. demonstrated that the number of specific CD8+ T cells during boost vaccination was highly affected by the timing of antigen expression. They suggest that the competitions of CD8+ T cell activities are responsible for this outcome (KASTENMULLER et al., 2007).

The early phase of MVA’s life cycle influences not only the adaptive immunity but has also impact on the innate immunity. In general, dsRNA plays an important role in the activation of the innate immune response against viral infections (JACOBS & LANGLAND, 1996; HALLER et al., 2006). For VACV, the majority of complementary RNA arises during the intermediate and late phase of VACV replication while little complementary RNA is synthesized in early stages (COLBY et al., 1971; BOONE et al., 1979). This might indicate that the majority of the appropriate signaling pathways are activated in the late stage of infection. However, a recent study demonstrated that an early event during MVA replication is capable of activating NFkB transcription factors, which is based on a dsRNA dependent protein kinase (PKR)-mediated process (HAYDEN et al., 2006; LYNCH et al., 2009). As dsRNA is supposed to be one of the major stimuli

of the PKR, the authors presume that the induction of PKR might be caused by the small quantities of dsRNA produced at the early phase during the MVA life cycle (GARCIA et al., 2006; LYNCH et al., 2009). A further study reported that MVA proteins, synthesized in the early phase, possess NFkB activation function (MARTIN & SHISLER, 2009). Another study found that MVA is able to activate human dendritic cells via an NFkB dependent process (DRILLIEN et al., 2004). In contrast to this, it has been shown that both the replication-competent VACV strain Western Reserve and a cowpox virus prevent NFkB activation during infections of human embryonic kidney cells, as they encode proteins that inhibit the related signaling pathways (LYNCH et al., 2009).

Generally, Orthopoxviruses developed a multitude of strategies to evade the host cell immune response. VACV genes encode accessory proteins that interfere with host cytokines, such as TNF, in order to inhibit the activation of transcription factors (HU et al., 1994; SMITH et al., 1996; PICKUP, 2007). An important distinction is that it has been shown that MVA lacks several of these immune evasion factors, as most of the corresponding genes are fragmented (ANTOINE et al., 1998; BLANCHARD et al., 1998).

Another indication that the early phase of MVA's life cycle contributes significantly to triggering the innate immune response can be seen in the early induction of interferon alpha during MVA infection. Waibler et al. demonstrated that the secretion of IFN alpha in MVA infected bone marrow derived plasmacytoid dendritic cells is fully independent of viral replication as well as intermediate and late gene expression since an inhibition of viral replication did not result in a decrease of IFN-alpha levels (WAIBLER et al., 2007). In line with these results, it was reported that interleukin (IL) -1beta and IL-8, both pro-inflammatory cytokines, as well as IFN-beta production were not affected in THP-1 cells which were infected with UV- treated MVA (DELALOYE et al., 2009). Dai et al. postulate that viral DNA, released upon MVA entry, triggers the type I IFN production in murine conventional dendritic cells and, furthermore, Delaloye et al. assume that the MVA envelope or a core protein is one of the responsible triggers for the IL-8 production (DELALOYE et al., 2009;

DAI et al., 2014). These findings indicate that MVA infection induces host cell cytokine production even before the viral early protein synthesis. Moreover, MVA is able to induce significant type I interferon responses upon *in vivo* infection of mice whereas a systemic release of type I IFN cannot be detected following the infection with conventional replication-competent VACV. Interestingly enough, the same study showed that two MVA ancestor viruses, CVA-152 and CVA-386 which were isolated after the 152nd and 386th CEF passage, do not induce IFN alpha while CVA 386 induces IFN beta. Waibler et al. relate this to the consecutive loss of immune evasion factors during MVA attenuation (WAIBLER et al., 2009).

Taken together, these studies demonstrate that MVA activates the innate immune response very early during the viral life cycle. Thus, the ability of MVA's NPF-particles to enter cells and to activate the early gene expression may be an important factor in triggering cellular signaling pathways. Indeed, it seems to be very likely that the biological properties of the NPF-particles contribute to MVA's strong intrinsic immunogenicity. The viral function of the NPF-particles, as a stimulus for the innate immune response, may enhance the efficacy of MVA-based vaccines. Hence, the NPF-particles might act as a natural adjuvant. Furthermore, the work of others reporting efficient antigen-specific cytotoxic CD8+ T cell immune responses of viral antigens expressed during the early phase of the VACV molecular life cycle, indicate the relevance of the early gene expression for the stimulation of the adaptive immunity (COUPAR et al., 1986; ZHOU et al., 1991; BRONTE et al., 1997; KASTENMULLER et al., 2007). These findings and our results clearly emphasize the use of early or early-late promoters for recombinant antigens to influence an efficient and optimal innate and adaptive immune response.

MVA-GFP-mCherry – novel tool for basic research

The construction of MVA-GFP-mCherry constitutes a novel approach for tracking viral functions. Quality controls including determination of genetic stability and replication capacity confirmed the suitability of MVA-GFP-mCherry for its use as a reporter virus. Various issues concerning the

virus life cycle, such as virus replication, host range effects and the impact of host factors on the viral replication, can be pursued by using this reporter virus. This was nicely shown by the current study. Here, MVA-GFP-mCherry provided simultaneous monitoring of the early and late gene expression during MVA infections, which enabled the visualization of the well-known cascade-like gene expression of POXVs in real time (MOSS et al., 1991). Another useful aspect of the reporter virus is the capability to differentiate between productive and abortive infections in various cell cultures *in vitro* and also to investigate the infection of target cells/tissues *in vivo*. Especially with regard to the characterization of the particle population it is very helpful to gain insight into the general replicative potential of single particles. MVA-GFP-mCherry even enables analyzing the particles viral function in more detail since the fluorescent reporter proteins indicate which level of the viral life cycle is achieved. The certain stage of arrest indicated that the virus probably lacks the competence to express essential proteins for the next step of gene expression.

For several decades, changes in the course of gene expression had to be determined via Western or Northern blotting as well as metabolic labeling analysis. These well-established traditional methods can provide information regarding changes in each stage of the viral expression cascade. However, they are not able to attribute the particular steps of gene expression to single cell infection events. Thus, abortive infections remain undetected. In contrast to this the reporter virus allows tracking of individual infection events as well as the determination of the point of inhibition due to the usage of stage specific promoters. Another benefit of the application of MVA-GFP-mCherry is the time-saving aspect. Due to direct and simultaneous microscopic observation, analysis of the viral life cycle using the reporter virus is much faster in comparison to traditional indirect methodologies.

There are various applications for which the reporter virus can be used as a tool for basic research. For instance, MVA-GFP-mCherry mutant viruses lacking certain genes can be generated to easily analyze the impact of specific viral proteins on the viral life cycle. In addition, reconstruction of defective genes in the reporter virus genome can be applied to study the

function of individual VACV genes and their contribution to the viral replication in non-permissive cells. The reporter virus constitutes an innovative tool, especially for the analysis of cellular host factors and their involvement in the virus-host interactions. For example, monitoring the effect of cellular gene knockdowns on the viral expression cascade helps to further the knowledge of the function of these host factors.

All in all, the newly constructed reporter virus has been proven to be a powerful tool in studying the progression of viral spread at the level of individual particle infections. The virus shed new light on the largely ignored role of NPF-particles during MVA infections and it might further help to gain a better understanding of the basic mechanisms by which MVA's cell tropism and host range are controlled.

Future perspectives

The initial experiments with MVA-GFP-mCherry confirmed its ability to be used as a reporter virus in *in vitro* studies. However, *in vivo* imaging of fluorescent reporter viruses has also been established in various animal models for several viruses, such as canine distemper virus (VON MESSLING et al., 2004) and measles (LEMON et al., 2011). Thus, MVA-GFP-mCherry might not only be useful for *in vitro* characterizations but also for *in vivo* studies. This strategy might be beneficial for addressing issues, particularly those related to biosafety and environmental safety, in terms of development and marketing authorization of recombinant MVA products. In particular, the evaluation of the biodistribution and clearance of genetically modified viruses in vaccinated individuals is an important aspect in the European guidelines of live recombinant viral vectored vaccines (European Medicines Agency. 2010. Guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines. EMA/CHMP/VWP/ 141697/2009). Biodistribution in patients and virus shedding from patients are major safety parameters for considering the potential risk of vector dissemination into the environment (BALDO et al., 2013). In addition to the type of viral vector, the application route is also an essential factor that contributes to the distribution within the body. So far

there are only a few biodistribution and shedding studies of MVA-based vector vaccines available (RAMIREZ et al., 2000; STITTELAAR et al., 2001; GOMEZ et al., 2007). In this context, the reporter virus MVA-GFP-mCherry might facilitate the study of viral distribution in *in vivo* models. Macroscopic fluorescence imaging may indicate the organ-specific distribution of MVA while microscopic imaging can provide information about MVA's specific cell tropism. Thus, with the help of MVA-GFP-mCherry the viral distribution might be tracked from different sites of application to dispersion and clearance within the patients. One particular aspect that would be very interesting to explore with the help of this reporter virus is the fate of MVA in the chicken model – the ancient host of MVA.

VI. SUMMARY

Fluorescent Reporter Viruses Identify Non-Plaque-Formig Virions of Vaccinia Virus to Activate Viral Gene Expression

The use of viruses as vaccine backbones has become more and more important for innovative vaccine development over the last decades. The particular ability to induce both the humoral as well as the cellular immune response has been considered to be a tremendous benefit of viral vector platforms. Thus, viral vaccine vectors are deemed to be an attractive alternative to traditional vaccine strategies. In this context, the Modified Vaccinia virus Ankara (MVA) has been proven to be a very promising viral vaccine platform due to its capability of inducing an extraordinarily strong immune response along with its excellent safety profile. Concerning the clinical testing of recombinant MVA vaccine candidates, knowledge about the phenotypic and biological properties of the virus preparation is essential.

Interestingly virus preparations of vaccinia virus (VACV) and also MVA seem to comprise substantially more total particles than infectious particles as seen *in vitro* in cell culture. Here, the majority of the viral particles constitute a subpopulation without the ability to form plaques in permissive cells. However, there is not much known about the biological activity as well as the biological functionality of these particles.

With regard to the unknown biological activities of the non-plaque (NPF) forming particles, the objective of this study was to investigate their effect on the biology of infections. For this purpose we generated a recombinant MVA and parental Chorioallantois Vaccinia virus Ankara (CVA) reporter viruses which express fluorescent proteins under the transcriptional control of early and late specific promoters. These reporter viruses allowed us to elegantly follow single infections in infected cell culture – in other words, the potential of individual virions to activate viral gene expression. Hence, we were able to differentiate between productive and abortive infections in MVA permissive cells and beyond that to identify the level of gene expression. Our *in vitro* studies in several cell lines indicated that

nearly all virions are infectious and are able to at least switch on the early gene expression. These novel findings shed new light on this “indiscernible” subpopulation of NPF-particles.

VII. ZUSAMMENFASSUNG

Fluoreszierende Reporterviren identifizieren nicht-Plaque-bildende Virionen mit biologischer Aktivität in Vaccinia virus Präparationen

Die Verwendung von Viren als Impstoffvektoren hat in den letzten Jahrzehnten im Bereich der Impfstoffentwicklung zunehmend an Bedeutung gewonnen. Vor allem die Fähigkeit sowohl die humorale als auch die zelluläre Immunantwort zu induzieren, wird als enormer Vorteil der viralen Impfstoffplattformen angesehen. Somit stellen virale Impfstoffvektoren eine attraktive Alternative zu den traditionellen Impfstrategien dar. In diesem Zusammenhang hat sich das modifizierte Vaccinia virus Ankara (MVA) als sehr viel versprechende Impfstoffplattform erwiesen. Dies ist besonders auf die starke Aktivierung der angeborenen Immunantwort sowie das ausgezeichnete Sicherheitsprofil zurückzuführen. Im Hinblick auf den klinischen Einsatz von rekombinanten MVA Impfstoff-Kandidaten sind fundierte Kenntnisse über die phänotypischen und biologischen Eigenschaften von gereinigten Viruspräparationen essentiell.

Die Mehrheit der Viruspartikel in aufgereinigten MVA- und Vaccinia virus (VACV)-Präparationen ist einer Fraktion zugehörig, die nicht in der Lage ist in permissiven Zellen Plaques zu bilden. Dieses Phänomen ist schon seit Jahrzehnten bekannt, jedoch ist die biologische Aktivität und dessen Auswirkung auf die Infektion bislang noch nicht untersucht worden.

Angesichts der Frage ob diese nicht-Plaque-bildenden (NPF) Partikel auch die Immunogenität oder Wirksamkeit eines MVA-Impfstoffes beeinflussen könnten, wurde die biologische Aktivität dieser Partikel im Rahmen der vorliegenden Arbeit untersucht. Hierzu wurden rekombinante Reporterviren entwickelt. Die Gensequenzen des grün fluoreszierenden Proteins (GFP) und eines rot fluoreszierenden Proteins (mCherry) wurden in das MVA Genom sowie in das Chorioallantois Vaccinia virus Ankara (CVA) Genom, dem Ursprungsvirus von MVA, integriert. GFP und mCherry wurden dabei unter der Transkriptionskontrolle eines VACV-spezifischen frühen sowie eines späten Promotors produziert. Dies

ermöglichte uns einzelne Infektionsereignisse in infizierten Zellkulturen genau zu verfolgen, sodass wir zwischen produktiven und abortiven Infektionen differenzieren und die erreichte Stufe der viralen Genexpression bestimmen konnten. Unsere *in vitro* Studien in verschiedenen Zellen deuten darauf hin, dass nahezu alle MVA sowie CVA Virionen in der Lage sind Zellen zu infizieren und die Synthese viraler Genprodukte zu starten. Diese Erkenntnisse geben einen neuen Einblick auf die bisher „unsichtbare“ Subpopulation der NPF-Partikel und legen deren Beitrag zur Wirksamkeit von MVA-Impfstoffpräparationen nahe.

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IX. ABBREVIATION

AraC	Cytosine arabinoside
CAM	Chorioallantois membrane
CEF	Chicken embryo fibroblasts
CVA	Chorioallantois Vaccinia virus Ankara
DI	Defective interfering
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
ERK	Extracellular signal-regulated kinase
EV	Enveloped virion
GFP	Green fluorescent protein
HI-FBS	Heat inactivated fetal bovine serum
HIV	Human immunodeficiency virus
hpi	Hours post infection
IFN	Interferon
IL	Interleukin
INF	Interferon
ITRs	Inverted terminal repeats
IV	Immature virion
JNK	Jun N-terminal kinase
kb	Kilobase
kDa	Kilodalton
MAPKs	Mitogen-activated protein kinases
MEM	Minimum Essential Medium Eagle

MERS	Middle East respiratory syndrome
MERS-CoV	Middle East respiratory syndrome coronavirus
MOI	Multiplicity of infection
MPXV	Monkeypox virus
MV	Mature virion
MVA	Modified Vaccinia virus Ankara
niCKp	Noninfectious cell killing particle
NPF	Non-plaque-forming
NYVAC	New York attenuated vaccinia virus
ORF	Origin of replication
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
PKR	Double-stranded RNA-dependent protein kinase
PmH5	Modified promoter H5
POXV	Poxvirus
RNA	Ribonucleic acid
SI	Semi infectious
TICD50	Tissue culture infectious dose 50
TNF	Tumor necrosis factor alpha
VACV	Vaccinia virus
VP	Virus particles
WHO	World Health Organization

X. DANKSAGUNG

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