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Vaccine generation against gamma herpesvirus infection in a murine surrogate model

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I. List of abbreviations

AIDS	Murine herpesvirus 68
APC	Antigen presenting cells
APS	Amonium per sulphate
BAC	Bacterial artificial chromosome
BMDC	Bone marrow derived dendritic cells
BFA	Brefeldin A
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
CVA	Chorioallantois vaccinia virus Ankara
CEF	Chicken embryo fibroblast
CD	Cluster of differentiation
CPE	Cytopathic effect
DC	Dendritic cells
dpi	Days post infection
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
EMA	Ethidium monoazide
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HSV	Herpes simplex virus
HA	Hemagglutinin
ICS	Intracellular cytokine staining
IFN	Interferon
i.n.	Intra nasal
i.p.	Intra peritoneal
KSHV	Kaposi's sarcoma herpesvirus
IL	Interleukin
MHV68	Murine herpesvirus 68

MHC	Major histocompatibility complex
MOI	Molecules of infection
MLN	Mediastinal lymph nodes
ORF	Open reading frame
PCR	Polymerase chain reaction
PTLD	Post transplant lymphoproliferative disorder
PRR	Pattern recognition receptor
TCID50	Tissue culture infectious dose 50
TAP	Transporter associated
TCGF	T cell growth factor
TNF	Tumor necrosis factor
VZV	Varicella zoster virus

II. Summary

Gamma herpesviruses are known to establish a lifelong persistent infection in association with a number of malignancies in the immunosuppressed host. A mouse model of gamma herpesvirus (MHV68) has been utilized to elucidate the immune response of gamma herpesvirus infection, having genetic similarities with human lymphocrypto- (e.g EBV) and rhadinoviruses (e.g KSHV). During infection, gamma herpesviruses undergo a lytic infection cycle for 7 to 12 days and establish latent infection after 13 to 42 days. Moreover, the latent virus may reactivate due to immune suppression in transplant patients likewise in AIDS patients. Innate immunity is activated by, but fails to control the viral infection. Generally, adaptive T-cells play an important role in initially controlling the acute infection and subsequently maintaining the viral infection in a latent state. However, the nature of the T-cell response and specific viral epitopes eliciting protective immune responses are poorly understood. During infection, gamma herpesviruses undergo a lytic and latent phase of infection and express distinct antigens in each phase. We have used recombinant vaccines to analyze the adaptive immune response upon MHV68 infection, in particular CD4+ and CD8+ T-cells involved in the lytic, latent and reactivation phase, respectively. Particularly, recombinant modified vaccinia virus Ankara (rec-MVA) are widely used for antigen delivery in clinical research for its high level of gene expression and immunogenicity. Therefore, we have generated rec-MVA vaccines encoding MHC class I and II-restricted epitopes of MHV68 by using MVA-BAC homologous recombination technology. We have established antigen-specific cytotoxic T cell lines against MHV68 antigens, which specifically recognized exo and endogenously presented peptide ligands. We have found strong immune responses induced by rec-MVA vaccines for MHC-class I/II-restricted epitopes of MHV68 in immunized mice during the acute and memory phase after single or prime/boost vaccinations. In addition, the protective capacity of prophylactic rec-MVA vaccination against a challenge with MHV68 has beenshown in the acute and latent phase of infection. Briefly, all of this information will augment the advancement of vaccine development against the herpes virus infection.

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1. Introduction

1.1. Herpesvirus

The human herpesviruses are enveloped viruses, containing 100-200nm large double stranded DNA genome, packaged in an icosahedral capsid surrounded by viral tegument proteins (Spear and Longnecker 2003, Modrow S 2010). They are considered as a most complex human viruses because of having large genome including 70-200 predicted open reading frames and encode higher number of proteins (Stern-Ginossar, Weisburd *et al*. 2012). The virus envelop is composed of a variety of glycoproteins which serve as receptors to interact with the host cell surface and facilitate the internalization of virus (Krummenacher, Carfi *et al*. 2013). The virion is released in the cytosol of the host cell and viral DNA transfers to the nucleus for viral replication. Most common features of herpesviruses are asymptomatic infections, establishment of latent viral infection and reactivation of virus replication upon immune suppression, leading to a new episode of disease. Herpesviruses are classified into *alpha* herpesviruses (e.g varicella zoster virus [VZV] and herpes simplex virus [HSV]-1 and 2) which target the neurons for long term latency, *beta* herpesviruses (e.g cytomegalovirus) which persist latency in myeloid lineage cells and *gamma* herpesviruses (e.g Epsteinbarr virus [EBV] and Kaposi's sarcoma virus [KSHV]) which mainly infect B-lymphocytes to undergo latent infection (Grinde 2013).

The human gamma herpesviruses like EBV and KSHV are known to cause persistent infections and establish life-long latency. In immune-competent individuals, these viruses remain in quiescent state and in immune-compromised individuals they are associated with several malignancies and lymphoproliferative disorders. Therefore understanding immune control of these viruses is major health issue. However, these human gamma herpesviruses have a narrow host-range tropism; they are able to infect only humans or some non-human primates under special conditions. Therefore, disease progression and molecular pathogenesis of human herpesviruses are critical to understand. To overcome this problem, a mouse model of murine gamma herpesvirus 68 (MHV68) has been utilized to elucidate the pathogenesis of and immune response during gamma herpesvirus infections (Kim, Flano *et al*. 2002). In this project, we were mainly interested in utilizing MHV68 to establish a model for vaccine-based immunoprophylactic or -therapeutic approaches supporting an advanced strategy against the human gamma herpesvirus like EBV and KSHV infections.

1.2. Clinical significance of human gamma herpesvirus

Human gamma herpesviruses are identified as a lymphocryptoviruses e.g Epstein Barr virus (EBV) and a rhadinoviruses e.g Kaposi's sarcoma-associated herpesvirus (KSHV) (Lacoste, Lavergne *et al*. 2010). These herpesviruses causing lifelong infections, which are widespread and most adults carry multiple herpesviruses (Fields, Knipe *et al*. 2013). EBV is an exclusive human pathogen that was first recognized as an oncogenic herpesvirus, which is able to infect or to cause tumours in both lymphocytes and epithelial cells (Ali, Al-Shraim *et al*. 2015). KSHV is also known to induce lymphomas by infecting and immortalizing the B cells leading to several malignancies (Chen, Hsu *et al*. 2014). Previously it was reported that worldwide 95% of population is seropositive to EBV (Fruehling, Swart *et al*. 1998) and 35% seropositive for KSHV upon high regional differences (Whitby, Luppi *et al*. 1998).

Clinically primary infection of EBV and KSHV are mostly silent in immune-competent individuals and establish asymptomatic lifelong persistency followed by an acute infection. However, it can cause severe malignancies in the infected individuals with compromised immunity like in AIDS or transplant patients. On the basis of expression profiles of viral genes, the infection cycle of herpesviruses is divided into lytic, latent and reactivation phase. During the lytic infection, expression cascade of viral genes are strongly active and ensures the virus DNA replication and its packaging into new viral progeny. While latency is a silent viral state characterized by the restricted gene expression that help the virus to escape the immune system and allowing for a long-term persistent state (Thakker and Verma 2016). In case of EBV latent infection, the major latency protein is EBNA-1 (EBV nuclear antigen-1), likewise LANA-1 (latency-associated nuclear antigen-1) during KSHV infection, and these latency-associated proteins are responsible for their maintenance of genome during latency, proliferation and immune evasion. However, upon immune suppression there is a switch from latent to lytic infection that is called reactivation phase. This switch is a tightly regulated process initiated by the expression of the EBV BRLF-1/RTA gene (Hong, Holley-Guthrie *et al*. 1997) or KSHV ORF50/RTA lytic gene products (Purushothaman, Uppal *et al*. 2015), which are necessary and sufficient to induce lytic replication of the virus. The process of limiting antigen expression from lytic to latent infection is most important long–term T cell evasion mechanism (Feng, Moses *et al*. 2013).

Both of these viruses are associated with tumourogenesis upon reactivation in immune compromised patients, such as Hodgkin's lymphoma, Burkitt's lymphoma, post-transplant lympho proliferative disorder (PTLD), nasopharyngeal carcinoma mainly caused by EBV

infection (Rezk and Weiss 2007) and primary effusion lymphoma, Kaposi sarcoma, HIV co-infection with endemic Kaposi Sarcoma, plasmablastic variant of multicentric Castleman's disease caused by KSHV (Thakker and Verma 2016). Overall these viruses are associated with severe clinical disease manifestations and are able to cause endemic carcinomas in AIDS patients which explain the clinical importance of these viruses and a reason to study or understand the disease progression to generate therapeutic tools for them.

1.3. MHV68 virus as a murine model

The non-primate murine gamma herpesvirus 68 (MHV68; also known as MuHV4 or γHV68) has extensive biological similarities and genetic homology with human gamma herpesviruses. MHV68 is a natural rodent pathogen that was originally isolated from free living rodents, *Apodemus falvicollis* and *Clethronomys glareolus* (Blaskovic, Stancekova *et al*. 1980). Later studies indicated that MHV68 is endemic in *Apodemus sylvaticus*, most likely the natural host species (Blasdell, McCracken *et al*. 2003). MHV68 belongs to a sub division of gamma herpesvirus, the genus *Rhadinovirus* genetically closely related to the human gamma herpesvirus EBV and KSHV (Efstathiou, Ho *et al*. 1990, Virgin, Latreille *et al*. 1997). MHV68 is a double stranded DNA virus of ~118kbp genome size with at least 80 open reading frames (ORFs), of which 80% of the genes are homologous to EBV (Efstathiou, Ho *et al*. 1990), 90% homologous to KSHV and few host cellular genes (Fig.1.1) (Virgin, Latreille *et al*. 1997). Likewise, as EBV and KSHV, MHV68 has the same infection cycle comprising lytic and latent phases.

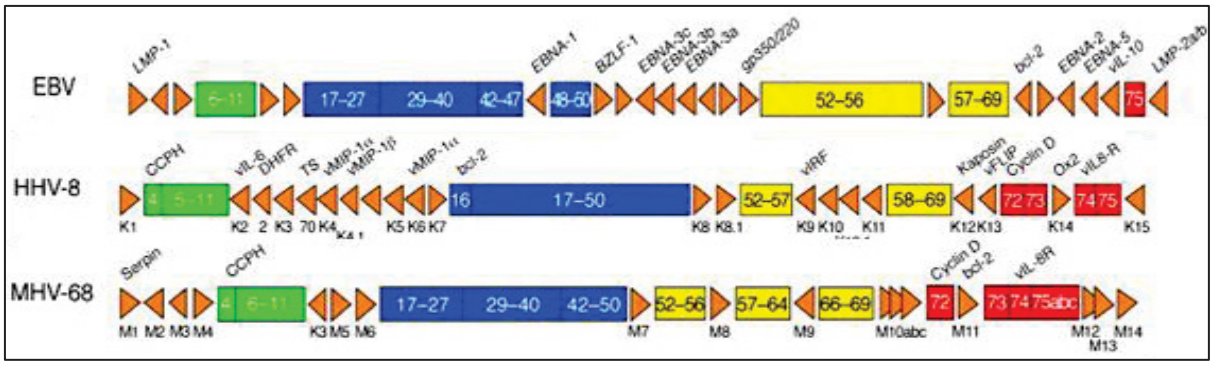


Fig. 1.1 Genetic organization of gamma herpesviruses (Efstathiou, Ho *et al*. 1990)

1.4. Pathogenesis of MHV68

There is no convincing data about the natural route of infection as the history of MHV68 infection in free rodents is unknown. Previously, most groups have been utilizing the

intranasal (i.n.) route of virus inoculation in order to establish reproducible stages of infection. After i.n. infection, the lytic phase is established during the acute infection of alveolar epithelial and mononuclear cells in the lung (Sunil-Chandra, Efstathiou *et al*. 1992, Stewart, Usherwood *et al*. 1998)[a]. The virus is then spread to the spleen and mediastinal lymph nodes (MLN) where they infect B cells, macrophages and dendritic cells to establish a latent chronic infection (Sunil-Chandra, Efstathiou *et al*. 1992, Flano, Husain *et al*. 2000, Nash, Dutia *et al*. 2001)[b]. During the lytic phase, the viral genome must be linearized and replication takes place by viral DNA polymerase, reflecting a viral takeover of the host cell (Wilson and Mohr 2012). The acute infection of MHV68 is eliminated within 10-12 days from the lungs mainly by macrophages and CD8+ T cells (Ehtisham, Sunil-Chandra *et al*. 1993, Weck, Barkon *et al*. 1996), but still persists in lung (Stewart, Usherwood *et al*. 1998).

In order to establish latency, the viral genome becomes circularized to get an episomal DNA form, is packed into histone chaperons and replication takes place by cellular DNA polymerase (Wilson and Mohr 2012). The latent infection in the spleen has a peak at 14-21 days post infection which starts to decline within 1 to 2 weeks. The latent virus preceeds the chronic infection which is associated with splenomegaly caused by the increased number of B and T cells (Sunil-Chandra, Efstathiou *et al*. 1992, Usherwood, Stewart *et al*. 1996). During latency, the virus is dependent on host's epigenetic mechanisms to silence viral genes and to down regulate antigen expression which allows switching into an immunologically silent and persistent mode of infection. Upon immune suppression, MHV68 reactivates from latency allowing replication and transmission to neighbouring host cells. Currently, MHV68 inbred strains of mice exclusively serve as animal models to evaluate viral and host determinants of gamma herpesvirus pathogenesis *in vitro* and *in vivo* (Nash and Sunil-Chandra 1994, Virgin, Latreille *et al*. 1997, Hu and Usherwood 2014).

1.5. Selected MHV68 genes for the generation of recombinant MVA

The T cell response against MHV68 is directed towards a broad repertoire of epitopes. In acute infection, the virus is cleared by 10 to 12 days post infection, and then follows latent infection (Sunil-Chandra, Efstathiou *et al*. 1992). Previous studies have shown two kinetic patterns of epitope-specific CD8+ T cells responses, following acute and latent MHV68 infection (Freeman, Lanzer *et al*. 2010). In C57BL/6 mice, $ORF6_{487}$ and $ORF61_{524}$ are well characterized epitopes encoded by the MHV68 ORF6 and ORF61 antigens, respectively. The CD8+ T cell responses to these epitopes follows distinct kinetics, where $ORF6_{487}$ specific cells are dominant in early infection and $ORF61_{524}$ specific cells continue to expand through

early latency and persist at higher levels during latent infection (Stevenson, Belz *et al*. 1999). This distinct kinetics of the T cell response correlate with the presentation of the $ORF6_{487}$ epitope during lytic infection and $ORF61_{524}$ epitope presentation during both, lytic and latent phase (Liu, Flano *et al*. 1999, Obar, Fuse *et al*. 2006).

Additionally, MHV68 ORF8 encoding glycoprotein gB is highly conserved between herpesviruses. It is not essential for virus structure, but for penetration into the host cell (Stewart, Janjua *et al*. 1994). The subdominant $ORF8_{604}$ epitope also induces intermediate levels of CD8+ T cell responses in C57BL/6 mice (Gredmark-Russ, Cheung *et al*. 2008). Recently, MHC class-II restricted epitopes have also been identified to be presented by ORF6, ORF61 and ORF8 antigens characterizing the CD4+ T cell response during latency (Freeman, Burkum *et al*. 2014).

For CD4+ T cell responses, the best characterized antigen is ORF51 (or M7) that encodes for glycoprotein 150 (gp150). MHV68 gp150 is a homologue of gp350/220 from EBV and gp35/37 from KSHV (Virgin, Latreille *et al*. 1997). gp150 is also known as a neutralizing determinant of MHV68 (Gillet, May *et al*. 2007) and strongly induces CD4+ T cell responses specific for the $ORF51_{67}$ epitope (Liu, Usherwood *et al*. 1999) and the recently published $ORF51_{111}$ epitope (Freeman, Burkum *et al*. 2014).

Moreover, MHV68 M2 is a latency-associated antigen and also plays an important role during reactivation from latency (Macrae, Usherwood *et al*. 2003, Herskowitz, Jacoby *et al*. 2005). M2 is able to manipulate B cell signalling and differentiation in order to establish long term latency in memory B cells (Simas, Marques *et al*. 2004). M2 utilizes another immune modulator strategy by inducing IL-10 expression in primary B cells (Siegel, Herskowitz *et al*. 2008). In Balb/c mice, the $M2_{91}/k^d$ epitope is known for stimulating CD8+ T cell responses (Usherwood, Roy *et al*. 2000), while in C57BL/6 mice $M2_{124}$ I-Ab epitope has been identified to induce CD4+ T responses (Freeman, Burkum *et al*. 2014).

Lastly, the MHV68 M3 gene is expressed as an early lytic gene in macrophages and dendritic cells. Similar to the KSHV K3 gene, M3 inhibits the antigen presenting machinery by downregulating MHC-I molecules and also by degrading TAP (Boname, de Lima *et al*. 2004). M3 limits CTL migration into the site of latency in infected mice, providing bystander protection for latently infected cells (Stevenson 2004). The $M3_{150}/k^d$ epitope has been identified to induce CD8+ T cell responses (Obar, Donovan *et al*. 2004), while in C57BL/6 mice no epitope has been reported yet. We have also performed epitope screening for the M3 antigen in C57BL/6 mice as part of an additional project (data not shown).

Given that information, we have selected ORF6, ORF61, ORF8, ORF51, M2 and M3 antigens from MHV68 to be expressed by recombinant MVA vaccines to induce antigen-specific T cell responses. Each of these MHV68 antigens is known for encoding epitopes specific for CD8+ or CD4+ T cells which have been generated and used as read out in this project (Table.1).

Table 1 MHV68-specific CD8+ and CD4+ T cell epitopes. IE-immediate early, E-early, L-late

Gene	Function	Amino acid positions	Epitope restriction	Response	Gene expression
ORF6	ss DNA binding protein	487-495	D^b	E-L	E
		593-607	$I\text{-}A^b$		
ORF61	Ribonucleotide reductase, large subunit	524-531	K^b	E-L	L
		343-357	$I\text{-}A^b$		
		691-705	$I\text{-}A^b$		
ORF8	Glycoprotein B (gpB)	604-612	K^b	L	L
		285-299	$I\text{-}A^b$		
		326-340	$I\text{-}A^b$		
		482-496	$I\text{-}A^b$		
ORF51	Glycoprotein 150 (gp150)	67-83	$I\text{-}A^b$	E-L	L
		111-125			
M2	Immune regulation/ latency	91-99	K^d	E-L	E-L
		124-138	$I\text{-}A^b$		
M3	MHC-I inhibitor	150-158	K^d	L	IE

1.6. Role of T cell immunity and viral evasion of immune response

1.6.1. CD8+ T cell immunity

CD8 T cells play a major role in controlling the acute lytic infection mainly during the primary MHV68 infection (Ehtisham, Sunil-Chandra *et al*. 1993, Weck, Barkon *et al*. 1996, Stevenson, Cardin *et al*. 1999). Increased numbers of CD8+ T cells and their priming result a decreased infectious virus titre in lungs after intra-nasal infection (Ehtisham, Sunil-Chandra *et al*. 1993, Stevenson, Belz *et al*. 1999). However the long term latent viral load seems very

little influenced by the primary lytic replication after mucosal infection (Coleman, Lima *et al*. 2003).

In previous studies, intact antiviral CD8+ T cell responses has been reported in MHC class II-deficient I-Ab-/- mice which lack CD4+ T cells, however, reducing the infectious virus titre in lungs with delayed kinetics (Cardin, Brooks *et al*. 1996, Stevenson, Belz *et al*. 1998). Additionally, in absence of CD4 T cells, these CD8+ T cells were able to proliferate efficiently in response to a challenge with their cognate epitopes presented after post exposure vaccination with recombinant vaccinia viruses. However, the expanded CD8+ T cell population were not sufficient to prevent from lytic and chronic MHV68 infection (Belz, Stevenson *et al*. 2000).

The viral infection can shut off the host cellular gene expression which facilitates viral replication and a slowdown of the immune activation. KSHV ORF37 gene encoded SOX, shutoffs host gene expression and induces host mRNA degradation by hyperadenylation of the transcripts, thereby retaining the host mRNAs in the nucleus (Lee and Glaunsinger 2009, Kumar and Glaunsinger 2010). Likewise, EBV BGLF5 and MHV68 muSOX homologues to KSHV SOX, can promote mRNA degradation and MHC-I gene expression shutoff resulting in the impairment of endogenous antigen recognition by virusspecific CD8+ T cells (Rowe, Glaunsinger *et al*. 2007, Zuo, Thomas *et al*. 2008, Buisson, Geoui *et al*. 2009, Covarrubias, Richner *et al*. 2009, Bagneris, Briggs *et al*. 2011).

In latently infected lymphoid tissues, these herpesviruses utilize different mechanisms to evade CD8+ T cell responses. The MHV68-encoded K3 (mK3) protein is homologous to KSHV-encoded K3 and K5, having ubiquitin ligase activity. mK3 is predominantly present in ER membranes where it binds to MHC class-I molecules and catalyses their ubiquitination leading to rapid proteasome-dependent degradation (Boname and Stevenson 2001). mK3 is also responsible for the degradation of TAP and tapsin (Boname, de Lima *et al*. 2004, Boname, May *et al*. 2005). The reduced MHC-I expression induced by mK3 is sufficient to block antigen presentation (Stevenson, Efstathiou *et al*. 2000). Likewise two EBV lytic cycle proteins, BILF1 (down regulates the expression of MHC-1 molecules (Zuo, Currin *et al*. 2009)) and BNLF2a (inhibits TAP mediated import of antigenic peptides into the ER by suppressing the binding of both ATP and peptide to TAP molecule (Hislop, Ressing *et al*. 2007).

In case of MHV68, the chemokine binding protein M3 is efficiently involved in the evasion of CD8+ T cells *in vivo* (Rice, de Lima *et al*. 2002) and also contributes to the establishment of latent viral load (Bridgeman, Stevenson *et al*. 2001, van Berkel, Levine *et al*. 2002).

MHV68 M3, a lytic gene transcript in infected lymphoid tissue, protects latently infected B cells by inhibiting the migration of CD8 T cells into the sites where latency-associated antigens are expressed (Stevenson 2004). Furthermore, the KSHV LANA (latency-associated nuclear antigen) homologous to mLANA encoded by MHV68 ORF73 and EBV EBNA1 proteins are involved in establishment and maintenance of viral latency. These proteins play an important role in inhibiting their own proteasomal degradation and retarding their own translation, leading to the impaired antigen processing by proteasome and MHC-I restricted presentation (Levitskaya, Coram *et al*. 1995, Levitskaya, Sharipo *et al*. 1997, Fowler, Marques *et al*. 2003, Bennett, May *et al*. 2005, Kwun, da Silva *et al*. 2007).

1.6.2. CD4+ T cell immunity

Cytotoxic CD4+ T cells play an important role to control gamma herpesvirus infections, because there is a down regulation of MHC class-I molecules leading to the impaired CD8+ T cell recognition by viral immune evasion mechanisms (Stevenson, Efstathiou *et al*. 2000) and, because the major latency reservoir is within MHC class-II positive B cells (Blackman, Flano *et al*. 2000).

In addition, CD4+ T cells play an important role for virus-specific antibody production and also serve as effectors cells to induce and maintain the cytotoxic CD8+ T cell response. CD4+ T cell-depleted immune competent mice have shown very little efficacy for antibody responses and died within 4 months after infection (Stevenson, Cardin *et al*. 1999). While in B cell-deficient mice, absence of antibody production leads to the lethal viral recrudescence. During acute infection in lungs, CD4+ T cells were able to control MHV68 infection in B-cell-deficient as well as CD8+ T-cell-depleted mice showing their role as effectors cells. However, mice are severely susceptible to infection, if both CD4+ and CD8+ T cells were depleted (Stewart, Usherwood *et al*. 1998, Christensen, Cardin *et al*. 1999). CD4+ T cell effector functions can also be mediated by IFNγ production or by cytotoxicity. In IFNγ-/- and IFNγ receptor -/- mice, MHV68 causes chronic infections indicating the role of IFNγ in limiting the lytic infection or suppression of reactivation from latent infection (Dutia, Clarke *et al*. 1997, Weck, Dal Canto *et al*. 1997).

The finding that CD4+ T cells lyse latently MHV68-infected B cells, also suggests that CD4+ T cells can directly control latency rather than just serving via helper T cell functions (Stuller and Flano 2009). Apparently CD4+ T cells are continuously stimulated by infected B cells and dendritic cells (Freeman, Burkum *et al*. 2011), but gamma herpesviruses also counteract CD4+ T cell activation. During KSHV infection, the vIRF3 protein was shown to inhibit class

II trans activator (CIITA), which usually promotes the expression of MHC-II molecules at the transcriptional level (Ting and Trowsdale 2002). Consequently, latently infected primary effusion lymphoma cells are unable to be recognized by LANA-1-specific MHC-II-restricted CD4+ T cells (Schmidt, Wies *et al*. 2011). While in EBV infected B cells MHC-II-dependent antigen presentation is restricted by BZLF-1 mediating down regulation of invariant chain CD74 post-transcriptionally (Zuo, Thomas *et al*. 2011). Moreover, higher levels of IL-10 expression have be shown during EBV (Burdin, Peronne *et al*. 1993), KSHV (Jones, Aoki *et al*. 1999)(76) and MHV68 (Peacock and Bost 2001) infection, which inhibits antigen-specific T cell proliferation by down-regulating MHC-II in B cells (Go, Castle *et al*. 1990) and IFNγ-induced MHC-II expression in monocytes (de Waal Malefyt, Haanen *et al*. 1991). Besides the strong role of CD4 + T cells, there is still room to define further key antigenic targets for CD4+ T cell activation and control.

1.7. MVA as viral vector vaccine

Modified vaccinia virus Ankara (MVA) is an attenuated strain of vaccinia virus (VACV). MVA has been generated from parental Chorioallantois vaccinia virus Ankara (CVA) by more than 570 continuous passages in chicken embryo fibroblasts (CEF) (Carroll and Moss 1997, Blanchard, Alcami *et al*. 1998). The serial passaging resulted in large genomic deletions, truncations and gene fragmentations. Therefore, a significant reduction from total 229 to about 193 open reading frames resulted in MVA. MVA has lost ~15% of its parental genome suffering six major deletions resulting in replication deficiency in most mammalian cells (Antoine, Scheiflinger *et al*. 1998, Meisinger-Henschel, Schmidt *et al*. 2007, Gomez, Najera *et al*. 2008, Baur, Brinkmann *et al*. 2010).

The six deletion sites offer the advantage to integrate foreign DNA for the usage as vector system to express recombinant antigens and also to serve as antigen delivery system for vaccination. Likewise, MVA has a capacity for packaging large recombinant DNA and can express large or multiple antigens in one viral vector (Staib, Lowel *et al*. 2003, Prieur, Gilbert *et al*. 2004, Robinson, Sharma *et al*. 2007). Furthermore, MVA has lack of persistence or integration of viral DNA into the host genome as the poxvirus encoded genes are transcribed in the cytoplasm of infected cells (Stickl, Hochstein-Mintzel *et al*. 1974). MVA posses high immunogenicity targeting both innate and adaptive immunity (Waibler, Anzaghe *et al*. 2007, Lehmann, Kastenmuller *et al*. 2009, Frenz, Waibler *et al*. 2010) and also know to have safe clinical records (Moss 1996).

Additionally, MVA is also known to encode virulence factors that can target the innate signal pathways like IFNs and TLRs (Delaloye, Roger *et al*. 2009, Price, Torres-Dominguez *et al*. 2013). These innate responses can also trigger strong adaptive immune responses e.g. TLR signalling pathways are crucial for CD8+ T cell memory development upon vaccinia virus infection (Amiset, Fend *et al*. 2012). Previously, MVA has been utilized as an excellent tool for vaccination. In case of smallpox virus infection, it has been proved safe and strongly effective (McCurdy, Larkin *et al*. 2004, Parrino, McCurdy *et al*. 2007, Greenberg, Overton *et al*. 2013, Greenberg, Hurley *et al*. 2015). By using advanced techniques for genetic modifications, insertion of a specific target gene into the MVA genome that encodes for an antigen, has been done successfully (Drexler, Staib *et al*. 2004). MVA expressing recombinant antigens have been extensively utilized as a vaccine in clinical trials, for example recombinant MVA vaccines against malaria (McConkey, Reece *et al*. 2003, Moorthy, Pinder *et al*. 2003, Moorthy, Imoukhuede *et al*. 2004), cervical cancer (Albarran, de la Garza *et al*. 2007), retrovirus infections (Chamcha, Kannanganat *et al*. 2016), influenza (Altenburg, Kreijtz *et al*. 2014) and prostate cancer (Amato and Stepankiw 2012). Briefly, MVA has been characterized as one of the most promising and immunogenic live viral vectors for the development of vaccines.

1.8. Generation of recombinant MVA vaccines by *en passant* homologous recombineering of BAC

The generation of recombinant MVA vaccines for target gene expression is carried out by homologous recombination between the viral genome and the transfer vector plasmid (Staib, Drexler *et al*. 2004). The deletion sites within the MVA genome are usually used for insertion of target antigens (Sutter *et al*. 1992). Previously, several methods have been decribed to generate recombinant MVA (rec-MVA), like LacZ or gpt-based selection or screening systems (Scheiflinger, Dorner *et al*. 1998), essential gene rescue for D4R-defective MVA (Ricci, Schafer *et al*. 2011) and host range selection (Galindo, Lorenzo *et al*. 2001). In this project, we have generated recombinant MVA vaccines expressing MHV68 antigens encoding epitopes which are MHC-I/II restricted by using the MVA-BAC homologous DNA recombination techonology (Cottingham, Andersen *et al*. 2008, Cottingham and Gilbert 2010, Cottingham and Carroll 2013).

The rapid generation of BAC-based rec-MVA has been utilizing the *en passant* recombineering of bacterial artificial chromosome (BAC) (Tischer, von Einem *et al*. 2006,

Tischer, Kaufer *et al*. 2007, Tischer, Smith *et al*. 2010). BAC replicons have high capacity (upto 300kbp) for cloning large DNA and RNA virus genomes. The backbone of BAC vectors contain a minimal fertility factor replicon (mini-F) which facilitates the maintenance and consistent replication of the BAC construct in *E*.coli. A modification of the BAC system is the integration of the well established red recombination system that is derived from bacteriophage λ and integrated in the genomic DNA of *E*.coli. Here, the BAC system utilizes double-stranded DNA (dsDNA) ends as substrate for recombination and consists of three components (Zagursky and Hays 1983, Murphy 1998, Zhang, Buchholz *et al*. 1998). The first component is the Gam protein which protects from degradation of dsDNA free ends by blocking the endogenous RecB/C/D in *E*.coli (Sakaki, Karu *et al*. 1973). A second component is alpha or RecE, a 5'-3'exonuclease generating single-stranded 3' DNA overhangs in the presence of Gam (Kushner, Nagaishi *et al*. 1974, Kovall and Matthews 1997). The third component is the single-stranded binding protein βeta or RecT that can bind to and also protect ssDNA from degradation (Hall, Kane *et al*. 1993, Wu, Xing *et al*. 2006). Additionaly, βeta helps to anneal ssDNA to the complementary sequences of the lagging DNA strand from the replicating viral DNA (Ellis, Yu *et al*. 2001, Tischer, Smith *et al*. 2010).

The major advantage of the red recombination system is that only short 50bp of homologous sequences are required for recombination (Tischer and Kaufer 2012). For positive selection of recombinant virus clones, a marker gene has been included in the transfer cassette. In order to remove the marker gene from recombinant viruses, a 18bp *I-Sce*I homing endonuclease site is present adjacent to the marker gene. Subsequently, the marker gene is removed by *en passant* recombination of the homologous sequences flanked to the adjacent sites of the marker gene. In this system, the genome manipulation is carried out in GS1783 *E*.coli cells, which harbor the BAC-virus genome. These GS1783 *E*.coli cells employ their red recombinase gene and *I-Sce*I endonuclease gene inserted into their genome, under the control of a heat inducible promoter and a L-arabinose substrate inducible promoter, respectively. Following BAC rescue, the complete BAC expression cassette is removed by inter molecular homologous recombination from the recombinant viruses (self- excising BAC). The BAC rescue is carried out in the presence of a helper virus which provides early transcription products for the replication of recombinant virus in permissive cell lines (Cottingham, Andersen *et al*. 2008, Cottingham and Gilbert 2010, Cottingham and Carroll 2013).

1.9. Adaptive Immunity

After infection, the innate immune response is activated leading to call the adaptive immune response to act together in eliminating viral or bacterial pathogens by macrophages, NK cells and granulocytes. Unlike innate immunity, adaptive immunity is highly antigen-specific for the pathogens which trigger them. Adaptive immune responses have two broad classes, one is *antibody mediated response* and the other is *cell mediated immune response,* carried out by lymphocytes mainly B and T cells. In the antibody response, B cells are activated to produce antibodies (immunoglobulins), which circulate in blood or body fluids to neutralize the pathogen. In case of adaptive immune responses, T cells start to proliferate and differentiate into effector cells to kill the virus or virus-infected cells. This activity continues for several weeks and then the host immune system is silenced again due to the elimination of the pathogen and the subsequent loss of activated effector T cells by apoptosis. At the same time, T cells also differentiate into memory T cells which reactivate in case of a relapse of infection or second encounter of pathogens (Bruce Alberts 2002). In this project, we have mainly focused on adaptive immune responses in the context of enhancing host immunity against herpesvirus infections.

1.10. Antigen presenting cells (APCs)

APCs are professionally specialized to internalize and present antigens to T cells, either by phagocytosis like in macrophages and dendritic cells (DCs) or by receptor mediated endocytosis like in B cells. These APCs are derived from bone marrow precursors (hematopoietic stem cells (HSCs)), which further differentiate and develop into myeloid or lymphoid precursors. Myeloid precursors generate DCs, macrophages and monocytes, while lymphoid precursors give rise to T and B cells. Upon internalization, APCs process antigens into peptide fragments and then present these peptides on their surface through membrane bound MHC-I or MHC-II molecules. T cells recognize and interact with peptide-MHC I/II molecule complexes on the surface of APCs and are activated in the context of co-stimulatory signals (den Haan, Arens *et al*. 2014). Among all APCs, DCs have a broad range of antigen presentation and are essential to activate (prime) naïve T cells. DCs have membrane-bound pattern recognition receptors (PRRs) to identify pathogens leading to cell damage or signals to destroy pathogens. DCs are involved in presentation of peptides from proteins synthesized within the infected DC itself so-called “direct presentation” or present antigens acquired from other infected cells so-called “cross presentation” (Mann and Li 2014).

1.10.1. Direct antigen presentation

The activation of T cells is mainly dependant on the presentation of antigen by MHC molecules. MHC class-I molecules present the peptide to the CD8+ T cells, while peptide-loaded MHC class-II molecules interact with CD4+ T cells. Once the cell is infected, the viral antigens pass through proteasome-mediated degradation to convert into peptide fragments in the cytosol. These peptides are transported into the lumen of the endoplasmic reticulum (ER) by TAP (transporter associated with antigen processing), trimmed by aminopeptidases and then loaded at a length of 8 to 10 amino acids on MHC-I complexes. MHC class-I molecules are heterodimers, consisting of a heavy α-chain (single transmembrane polypeptide chain) and a light chain β_2 microglobulin. MHC class-I molecules are stabilized by ERp57, calreticulin and tapasin to allow a functional peptide loading complex (PLC) without peptide. Furthermore, TAP interacts with tapasin to get access to MHC class-I molecules and assembles the peptides into the MHC class-I peptide binding grooves. MHC-peptide complex molecules are then released from the PLC, exit the ER and reach the cell surface via the secretory pathway of the Golgi apparatus (Neefjes, Jongsma *et al*. 2011)
In contrast to endogenous antigen presentation, exogenous antigens are internalized by endocytosis, degraded inside endosomes and deliver antigenic peptides to the MHC class-II loading compartment (MIIC). MHC class-II molecules are protected from premature peptide loading inside the ER lumen by the invariant chain (li). MHC class-II is also a heterodimer, consisting of α and β chains which form a groove allowing the binding of peptides with a size of 15-24 amino acids. By passing through the MIIC/CIIV compartment, the li-invariant chain is degraded from MHC-II-li complex, and finally antigenic peptides are loaded on free MHC class-II complexes and may be presented to CD4+ T cells on the surface of APCs (Busch, Rinderknecht *et al*. 2005).

1.10.2. Antigen cross-presentation

The ability of antigen presenting cells which are not directly infected, to take up antigens exogenously, and process and present them to cytotoxic T lymphocytes (CTLs) via MHC class-I molecules is called as antigen cross-presentation. Among all APCs, DCs are the only professional cross-presenters *in vivo* while macrophages and B cells are also known to do this job *in vitro* (Hopkins and Connolly 2012). By following endocytotic pathways, DCs can cross-present cell-associated as well as soluble antigens (Zhao, Mifsud *et al*. 2012). After endocytosis, exogenous antigens are degraded by proteasomes in an yet unidentified

compartment e.g. phagosomes (Joffre, Segura *et al*. 2012). These degraded antigenic peptides or phagosomes are transported to the cytosol via specialized compartments in the ER. Furthermore, the degraded peptides follow the traditional MHC class-I presentation pathway to be presented to CD8+ T cells.

In context of rec-MVA vaccines, MVA are highly efficient to infect DCs *in vivo* leading to the strong induction of adaptive immune response (Becker, Norder *et al*. 2014). The infected DCs are able to express high levels of viral antigens, producing considerable amount of IFNα and then become apoptotic. The apoptotic DCs are encountered and phagocytosed by un-infected DCs and serve as antigen reservoir for cross-presentation. Cross presentation of antigens upon rec-MVA vaccination leads to an enhanced virus-specific adaptive immune response and makes these vaccines even more efficient (Pascutti, Rodriguez *et al*. 2011, Guzman, Cubillos-Zapata *et al*. 2012, Iborra, Izquierdo *et al*. 2012).

1.11. Aim of thesis

In this study, we have focused on gamma herpesviruses which are known to establish life long persistent infections in their hosts. Murine gamma herpesvirus (MHV68) serves as an animal model which shares genetic similarities and basic aspects of human gamma herpesvirus infections. During infection, MHV68 undergoes a lytic infection phase in the lungs and further establishes a latent infection in the spleen. Moreover, the latent virus may reactivate due to immune suppression in immune-compromised patients. We aimed to to study the immune response of gamma herpesvirus infection upon vaccination using MHV68 as a model. We mainly focused on adaptive immune responses to MHV68 infection, in particular CD4+ and CD8+ T cells, involved in controlling the lytic and/or latent phase. CD8+ T-cells help to limit MHV68 lytic replication but are not sufficient to prevent from the latent phase of infection. CD4+ T-cells have also important roles either directly as effector cells or as helpers of B cells for the antibody response. In addition, they control viral lytic replication indirectly by producing IFN-γ. Therefore, we aimed to identify and subsequently used multiple antigens which are expressed in the lytic and latent stages of infection and encode CD4+ and CD8+ T cell epitopes as targets for immunotherapy. Selected MHV68 antigens harboring T cell epitopes, should be incorporated in recombinant MVA vectors. Recombinant modified vaccinia virus Ankara (rec-MVA) vectors are widely used for antigen delivery in clinical research for their safety and immunogenicity. We aimed to generate recombinant MVA vaccines expressing MHV68 antigens, encoding epitopes which are MHC class I and II restricted, by using the MVA-BAC homologous DNA recombination technology. Finally, rec-

MVAs should be tested for recombinant protein expression, subjected to growth kinetics analysis and also for genomic sequencing to evaluate the functionality and stability of each construct.

Furthermore, we aimed to characterize the rec-MVAs for their immunogenicity *in vitro* and *ex vivo*. We generated epitope-specific CD8+/ CD4+ T cell lines and used them for exo- and endogenous epitope recognition analysis *in vitro*. By performing *ex vivo* immune analyes, we intended to analyse the antigen-specific CD8+/ CD4+ T cell response during the acute and memory phase after immunization of mice with rec-MVA vaccines. Since MVA has been known to induce stong responses upon prime-boost vaccination, we also tested this strategy for rec-MVA vaccination. Last but not least, we aimed to analysed these vaccines for experimental prophylactic vaccination approaches in MHV68 mouse challenge experiments. The results will allow to calculate the efficiency of rec-MVA vaccination and to further develop vaccination strategies for prophylactic or therapeutic purposes against herpesvirus infections.

2. Materials

2.1. Chemicals

Acetic Acid	Merck
Agarose	Biozym
Ampicilin	Roth
Bacto-Agar	Fluka
β-mercaptoethanol	Roth
Bromophenol blue	Merck
Brefeldin A	Sigma
Calcium chloride	Roth
Chloramphenicol	Sigma
Dimethylsulfoxide (DMSO)	Roth/ Sigma
DNA-Size Standards Hyperladder I	Bioline
Deoxinucleotides triphosphate (dNTPs)	Qiagen
Dithiothreitol (DTT)	Merck
DNase	Roche
DreamTaq Master Mix (2x)	ThermoFisher
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco
Endonucleases (restriction enzymes)	New England Biolabs
Enzyme buffers (10x)	New England Biolabs
Ethanol absolute	Merck
Ethylenediaminetetraacetic acid (EDTA)	Merck
Fetal calf serum (FCS)	PAN, Biotech
Fugene	Roche
Glycerol	Roth
Hydrochloric acid (32%)	Roth
High fidelity DNA polymerase	M0530L, Biolabs
Isopropanol	Merck
Kanamycin	Sigma
LB-Broth (Lennox)	Roth
Lumi-light Western Blotting Substrate	Roche
Magnesium chloride	Roth
Methanol	Sigma
Nonfat-dried milk	Sufocin
Paraformaldehyde	Merck
Pharm-Lyse (lysing buffer, BMDC preparation)	BD
Phosphate buffered saline (PBS, 1x)	Gibco
Penicillin/Streptomycin (P/S)	Gibco
Phenol-chlorophorm	Roth
Ponceau S	Roth
Proteinase K	AGS GmbH

Roswell Park Memorial Institute medium (RPMI)	Gibco
Sodium acetate (NaAc)	Meck
Sodium azide (NaN_3)	Merck
Sodium chloride (HCl)	Sigma
Sodium dodecyl sulfate (SDS)	Roth
Sodium hydroxide NaOH)	Merck
Sucrose	VWR
T4-DNA-Ligase	New England Biolabs
T4-DNA-Ligase buffer (10x)	New England Biolabs
TEMED	Roth
Tris-Base	Roth
Tris-borate EDTA buffer (TBE, 10x)	Sigma
Triton X-100	Sigma
Trypan blue stain (0.4%)	Gibco
Trypsin-EDTA (0.05%)	Gibco
TurboFect	ThermoFisher
Tween 20	Merck

2.2. Equipments

Agarose gel electrophoresis chamber	Neolab
Blotting-Equipment	Neolab
Centrifuges	5810R, Eppendorf Megafuge 16R, Heraeus
Ultra Centrifuged	Thermo Scientific Sorvall WX80
Electrophoresis chambers	Neolab
Electrotransformator	Bio-Rad
Flow cytometry equipment	FACSCanto II™, BD
Freezer (-80°C)	Herafreeze, Thermo Scientific
	Forma 88000 Series, Thermo Scientific
Freezer (-20°C)	GNP 4166 Premium NoFrost, IG 1166 Premium, Liebherr
Fridge (+ 4°C)	LKUv 1610 MediLine, Liebherr FKUv 1610 Premium, Liebherr KB 4260 Premium, Liebherr KUR18421, AntiBacteria, Bosch
Heating plate	MR Hei-Tec, Neolab
Ice machine	AF124, Scotman
Incubators	Heracell 150i/ 240i, ThermoScientific
Micropipettes	Pipetman P2-1000, Gilson
Microscope	CKX41, Olympus LSM 780, Zeiss
Microwave	Gn3431MA
Micro Pistill	9.314 496, Lab logistic group

Multi-channel pipette	Pipetman P200N, Gilson
Nitrogen Tank	Biosafe MD, Cryotherm
pH-meter	PB-11, Satorius
Power-Supplies	MS 3AP, MS Major Science
Sonification machine	Sonopuls HD200/UW200,Bandeline
Thermoblock	ThermoStat plus, Eppendorf
Thermomixer	Thermomixer Comfort, Eppendorf
Thermocycler	T1 und T3000, Biometra
UV-Transilluminator	UVsolo TS, Biometra
Vortexer	Vortex Genie 2, Scientific Industries VV3, VWR
Waterbath	type 1083, GFL
Weighing machine	AEJ 220-4M, KERN & Sohn GmbH Ew 4200-2nm, KERN & Sohn GmbH
Weighing shaker	St 5 cat, Neolab
Western blot chamber	Neolab
Working sterile bench	Bio Safety class II, Labogene

2.3. Solutions and buffers

Cell culture medium	RPMI/ DMEM 10 % FBS 1 % P/S

Freezing-medium	90 % FCS 10% DMSO

LB (Luria-Bertani)-medium	1% (w/v) Bacto-Trypton 5.5% (w/v) Bacto-Yeast-Extract 1% (w/v) NaCl

LB-Agar	1.5% (w/v) Agar in LB-medium

Immunization buffer (2x)	4ml Tris (20mM) 56ml NaCl (280mM) 140ml Aquadest pH 7.4

Solutions for Western Blot:

10x Lämmli-running buffer	250 mM Tris-base 2M glycine

Ponceau	0.1% Ponceau S 5% Acetic acid

5x sample loading buffer	250 mM Tris pH 6,8 10% SDS 7.5% Glycine 12.5% β-Mercaptoethanol 0.5% Bromphenol blue

Separating gel (12%)	10% Acrylamide 43 mM Tris/HCl, pH 8.8 0.1% SDS (w/v) H_2O TEMED APS

Stacking gel (12%)	5% Acrylamide (v/v) 65 mM Tris/HCl, pH 6.8 0.1% (w/v) SDS 14% (w/v) sucrose H_2O TEMED 0.13% (w/v) APS
10x TBST	0.1 M Tris/HCl pH 8.0 1.5 M NaCl 0.5% (v/v) Tween 20

10x Transfer buffer	58 g Tris-base 29 g Glycine

1x Transfer buffer	100 ml 10x Transfer buffer 200 ml Methanol 700 ml dd H_2O

Tyr-lysis buffer	50 mM Tris-HCl pH 8 150 mM NaCl 0.02% NaN_3 1% Triton X-100

2.4. Kits

BD Cytofix/Cytoperm Kit	BD Pharmingen
Plasmid Midiprep Kit	Qiagen
PCR-Master-Mix	Roche
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR purification Kit	Qiagen
DNA-Isolation Kit	Qiagen

2.5. Plasmids

Name	**Provider**
pH5_dVI_MVA plasmid	I.Drexler
pIRES-EGFP	Albert Zimmerman

2.6. Bacteria

Name	**Description**	**Source**
E.coli DHB10	Electro-competent	Gibco BRL
E.coli GS1783	BAC clone: pMVAF BAC self excising	Gift from Matthew G.Cottingham

2.7. Oligonucleotides

List of oligonucleotides applied for PCR and sequencing analysis (from Eurofins)

Name	**Sequence 5' → 3'**
F.P-FLI_dVI (P1)	CCACGCACGTATCTAAAG
R.P-FLII_dVI (P4)	GGCGTCCATATGAGTAAC
Forward-Promoter-Primer	CTCCTTCATTACAGAAACGGC
Reverse-Promoter-Primer (P3)	GATGCTATACTCGTTTATATTAG
F.P-FLI_dVI _Seq-1	GCTACTTCAATGCGAGGAAA
F.P-FLI_dVI _Seq-2	GTCACATCACTAATCGGTTC
R.P-FLII_dVI _Seq-1	GGTAAGAATCACGATGGTACTCC
F.P-FLII_dVI _Seq-1	TCGTGATATACCTGTGATCCG
F.P-FLII_dVI _Seq-2	GGGGCTCCTAATGGAGATTT
R.P_MVA-BAC_Seq-1	GGTTGGAAAATCACGCTGAAAC
RFV_F.P	AAAGATGCGTACATTGGACCC
RFV_R.P	GTTCGAGACTAGAAAAGCGCC
GFP_F.P	TTGTACAGCTCGTCCATGCCGAG
GFP_R.P	GCAAGGCCGGATCTGGGAATTC
M2_BamHI_R.P	GGGGGGATCCCACCATGGCCCCAACACCCCCACAAG
M2_AflII_HA_F.P	GGGGCTTAAGTTAAGCGTAATCTGGAACATCGTATGG GTACTCCTCGCCCCACTCCACAAAACC
M3-BamHI_R.P	GGGGGGATCCCACCATGGCCTTCCTATCCACATCTG
M3_AflII_HA_F.P	GGGGCTTAAGTCAAGCGTAATCTGGAACATCGTATGG GTAATGATCCCCAAAATACTCCAGC
M3-F.P_Seq-1	CTGTGGAAGAGCTGGGTCAAG
ORF61_BamHI_R.P	GGGGGGATCCCACCATGGCGACCCAAACCATGGAC
ORF61_AflII_HA_F.P	GGGGCTTAAGTTAAGCGTAATCTGGAACATCGTATGG GTATTGACAGTGCAGACAAGAAGC
ORF61-F.P_Seq-1	GCTGCTAGGTTTTATGACACC
ORF61-F.P_Seq-2	GGAGTGTGCATACCAGAGAT
ORF61-F.P_Seq-3	CCTTTGCTATGATGGGGTTG
ORF8_SwaI_F.P	GGGGATTTAAATCACCATGAGAGTCGCCCACCTAACC

ORF8_AflII_HA_R.P	GGGGCTTAAGCTAAGCGTAATCTGGAACATCGTATGG GTAGAGGGAAGTTGATTCATCTTCC
ORF8-F.P_Seq-1	GCCCTACATCTTCAAAATCAGG
ORF8-F.P_Seq-2	GCCACCAGACAAACTGCCACG
ORF8-F.P_Seq-3	TGGAAAGCCTGTCGCTGCCA
ORF6_BamHI_F.P	GGGGGGATCCCACCATGGAGACAACCAATGTCTG

2.8. Antibodies

List of antibodies used for flow cytometry analysis

Specificity	Clone	Isotype	Conjugate	Manufacture
CD8e	500A2	hamster IgG2	PB	BD Pharmingen
CD8a	53-6.7	rat	PB (eFluor® 450)	eBioscience
CD4	GK1.5	rat IgG2b	PB	eBioscience
CD62L	MEL-14		PE-Cy7	eBioscience
IFNγ	XMG1.2	rat	APC	eBioscience
TNFα	-	rat	FITC	BD Pharmingen
IL2	JES6-5H4	-	PE	eBioscience
antiHA	-	Rabbit	-	Sigma
anti Rabbit	-	IgG	Peroxidase	Dianova

2.9. Cell lines

List of used cell lines

Cell line	Characteristics	Reference
BHK21	Baby hamster kidney cells	ATCC CCL-10
HeLa	Human cervical carcinoma	ATCC CCL-2
DC2.4	Immortalized murine dendritic cells	Georg Häcker
BMDC	Bone marrow dendritic cells from	C57/BL6 mice
DF-1	Immortalized chicken embryo fibroblasts	
EL4	Mouse ascites lymphoma lymphoblast	ATCC Nr. TIB 39
CEFs	adhesive primary cells, chicken embryo	Generated from SPF eggs

	fibroblast	(VALO BioMedia)
CD8-βgal$_{499}$	Murine CD8+ T cell line	Generated in this project
CD8-D13$_{118}$	Murine CD8+ T cell line	Generated in this project
CD8-ORF6$_{487}$	Murine CD8+ T cell line	Generated in this project
CD4-ORF6$_{593}$	Murine CD4+ T cell line	Generated in this project
CD8-ORF61$_{524}$	Murine CD8+ T cell line	Generated in this project
CD4-ORF61$_{343}$	Murine CD4+ T cell line	Generated in this project
CD4-ORF61$_{691}$	Murine CD4+ T cell line	Generated in this project
CD4-ORF51$_{111}$	Murine CD4+ T cell line	Generated in this project
CD4-M2$_{124}$	Murine CD4+ T cell line	Generated in this project
CD4-B$_{546}$	Murine CD4+ T cell line	Provided by I.Drexler
CD4-Ova$_{265}$	Murine CD4+ T cell line	Provided by I.Drexler

2.10. Viruses

Name	**Source**
MHV68 Strain g2.4 (WUMS) NCBI Accession: NC_001826	Gifted from Heiko Adler
MVA-F6 (Modified virus Ankara wild type)	Provided by I.Drexler
rec-P7.5-Ova-MVA	Provided by I.Drexler
rec-P7.5-lacZ-MVA	Provided by I.Drexler
rec-ORF6-MVA	Generated in this project
rec-ORF61-MVA	Generated in this project
rec-ORF8-MVA	Generated in this project
rec-ORF51-MVA	Generated in this project
rec-M2-MVA	Generated in this project
rec-M3-MVA	Generated in this project

2.11. Peptides

Peptides	**MHC class restriction**	**Amino acid sequence**
ORF6 $_{487–495}$	H2- D^b	AGPHNDMEI
ORF61 $_{524–531}$	H2- K^b	TSINFVKI

$ORF51_{111-125}$	$I\text{-}A^b$	PTEADPKAAPSAGHV
$M2_{124-1381}$	$I\text{-}A^b$	NSEPVYIQPISTRSL
$ORF6_{593-607}$	$I\text{-}A^b$	CNTNWLPPCPIIHNL
$ORF61_{343-357}$	$I\text{-}A^b$	EQGAYEAVVPIKSVM
$ORF61_{691-705}$	$I\text{-}A^b$	LFLNEDYASSASNIK
$OVA_{265-280}$	$I\text{-}A^b$	TEWTSSNVMEERKIKV
$B5_{46-60}$	$I\text{-}A^b$	FTCDQGYHSSDPNAV
βgal_{96-103}	H2- K^b	DAPIYTNV
$B8_{20}$	H2- K^b	TSYKFESV

2.12. Mice

C57BL/6 mice (MHC restrictions →H2- K^b/ H2- D^b/ $I\text{-}A^b$)

Reference: http://jaxmice.jax.org/strain/000664.html

3. Methods

3.1. Molecular Biological Methods

3.1.1. Generation of electrocompetent *E. Coli* cells (DH10B cells)

An inoculum from a fresh overnight E. Coli DH10B culture in 50ml was added to 500ml LB medium. Cells were grown at 37˚C with vigorous shaking to get an OD_{600} nm of 0.4-0.6, indicating exponential growth. The following steps were carried out on ice to achieve high transformation efficiency. Cells were harvested by centrifugation at 5000rpm for 15min at 4˚C (Centrifuge Avanti J-25, rotor JA-10). Pellet was resuspended in 500ml of ice-cold sterile distilled water. Cells were washed 3 times followed by centrifugation. The cells were resuspended in a final volume of 2ml ice-cold sterile 10% glycerol (~(2-5)x10^{10} cell/ml). For storage, 50µl of electrocompetent cells were aliquoted in each tube and quickly placed on dry ice and stored at -80˚C.

3.1.2. Generation of elctrocompetent GS1783 *E. coli* cells

GS1783 (2.5µl) cells were inoculated in 100ml pre-warmed LB medium suplemented with 30µg/ml chloramphenicol. Cells were grown with vigorous shaking at 30-32˚C to an OD_{562} nm of 0.5-0.7. The cell suspension was transferred immediately to a pre-warmed water bath at 42˚C with shaking at 220rpm for 15min. Furthermore, cells were placed on ice-water mix for 20min. Cells were harvested by centrifugation at 6300rpm (Beckman coulter JA10) for 5min at 4˚C. The pellet was resuspended in 5ml medium containing 10% ice-cold glycerol and again centrifuged at 9800rpm for 1min. Cell pellet was washed 3 times in 5ml of 10% glycerol. The cells were resuspended in a final volume of 900µl of 10% ice-cold glycerol. For storage, 50µl of electrocompetent cells were aliquoted in each tube and stored at -80˚C.

3.1.3. Transformation

Electrocompetent *E. coli* cells were used for uptake of DNA-ligated products (plasmid and insert gene DNA) by electroporation. 50µl of electrocompetent cells in each tube were thawed on ice. An appropriate amount of DNA was added to the bacterial cells. This mixture was transferred to the cuvette (0.2 cm gene pulser, Bio-Rad) and pulsed at 2 kV, 200 Ω, 25 µF (For GS1783 *E. coli*, 1.5 kV, 200 Ω, 25 µF was used). Electropration was followed by addition of 900µl of LB medium (without antibiotic) to the pulsed sample immediately and incubated at 37˚C for 1h, in a water bath with continuous shaking (for GS1783 cells, 30-32˚C)

to express the antibiotic resistant gene product. 10% of bacterial suspension was distributed directly on the LB agar plate containing antibiotics. The remaining 90% of cell suspension was centrifuged at 5000rpm for 5min, resuspended in 100-200µl of fresh LB medium and distributed on another LB agar plate containing antibiotic. The agar plates were incubated at 37˚C for overnight or desired time period (for GS1783 cells, 30-32˚C).

3.1.4. DNA Isolation from virus and bacteria

3.1.4.1. Isolation of MHV68 viral DNA

BHK cells (80-90% confluent) were infected by using MOI 10 of MHV68 virus. After 24h, cells were harvested by scraping and centrifugation at 10000rpm (5810R, Eppendorf) for 1min. DNA was extracted from the cell pellet by using DNA extraction protocol from Qiagen Kit (QIAamp DNA Mini Kit, cat# 51304).

3.1.4.2. Isolation of recombinant MVA DNA

The monolayer of DF-1 cells (immortalized chicken embryo fibroblasts) was infected by using MOI 10 of virus and incubated for 24h at 37˚C. After infection, medium was removed and infected cells were harvested in 400µl aqua dest and subsequently transferred into a 1.5ml tube. Afterwards 50µl of 10X TEN buffer (pH 7.4) were added to cells, followed by 3 cycles of freez and thaw. Cell suspension was centrifuged at 2500 rpm for 5min at RT to remove cell debris. Supernatant was transferred to a clean tube. 50µl of proteinase K and 23µl of SDS (20%) were added to the supernatant and incubated for 1-4h at 56˚C. Equal volume of phenol and choloroform (1:1) was added, mixed and centrifuged at highest speed for 5min at RT. The supernatant was transferred to a new tube. To this supernatant, 1/10 volume of 3M NaAc and 2 volume of absolute ethanol were added and mixed gently. Subsequently, the suspension was cooled at -80˚C for 30min and then centrifuged at the highest speed for 10min at 4˚C. Pellet was washed twice with 70% ethanol, air dried and resuspended in 20µl 1X TE buffer.

3.1.4.3. Isolation of plasmid DNA (Mini prep)

The selected single colony was used to inoculate 4ml LB medium, provided with antibiotics and incubated overnight at 37˚C. When medium became turbid due to bacterial growth, 2ml of bacterial culture were taken out into a separate tube and centrifuged at 6000 rpm for 10min at 4˚C. Supernatant was discarded and pellet was resuspended in pre-chilled 300µl of EDTA containing PI solution (Qiagen) to solublize the pellet. Followed by the addition of 300µl of P2 solution and incubation at RT for 5 min to lyse the bacterial cells with alkaline NaOH and SDS. Then 300µl of P3 solution (low pH acetic acid) were added to precipitate the cellular

debris and placed them on ice for 10-15min until a milky pellet was visible. After centrifugation at high speed for 15min at 4˚C, the plasmid resolved in supernatant, was tranferred to the new tube to improve the purity. 600µl of chilled isopropanol were added, invertly mixed and then centrifuged at 14000rpm for 30min at 4˚C to precipitate DNA. Pellet was washed by adding 200µl 75% ethanol and centrifuged for 10min at 4˚C. After discarding the supernatant, pellet was air dried for 20min and resuspended in 20µl water. 2-4µl of DNA were used for control restriction digestion analysis and also for commercial sequencing (Eurofins MWG Operon).

3.1.4.4. Isolation of purified plasmid DNA (Midi prep)

The selected candidate clones after restriction digestion and sequencing were used for Midi prep purification. For Midi prep, 100µl of bacterial culture were added to inoculate 100ml LB medium containing antibiotics and incubated at 37˚C for overnight with shaking at 110rpm. Next day, 600µl of bacterial culture were frozen as a backup in LB medium containing 25% (V/V) glycerol at -80˚C. The remaining bacterial cells were harvested by centrifugation at 5000rpm for 15min at 4˚C. Furthermore, Qiagen kit was used for plasmid DNA isolation by following the manufacturer's protocol (QIAGEN Plasmid Midi Kit, cat# 12145).

3.1.5. Polymerase Chain Reaction (PCR)

Target genes were amplified from MHV68 viral DNA. PCR was performed by using Phusion High Fidelity DNA polymerase kit (*BioL*abs, cat# M0530L) protocol. Recipe used for mastermix is as follows:

5x Phusion buffer	10µl
dNTPs (10mM)	1µl
DNA polymerase	0.5µl
Forward Primer (10 mM)	2.5µl
Reverse Primer (10 mM)	2.5µl
Template DNA (~200ng/µl)	1.25µl
Total	50µl

Thermocycler program was set according to the primer and template length. Following are the standard cycle conditions:

Steps	Temperature	Time	No. of Cycles
Initial denaturation	98˚C	30 sec	1x
Denaturation	98˚C	5 sec	35x
Annealing	68˚C	30 sec	
Elongation	72˚C	1-2.5 min	
Extended elongation	72˚C	5 min	1x
Cooling	4˚C	Pause	

PCR to amplify long DNA sequences

To amplify long DNA sequences of target genes, 2 step hybridization PCR protocol was used:

Step	Temperature	Time	No. of Cycles
Initial denaturation	98˚C	2 min	1x
Denaturation	98˚C	10 sec	10x
Annealing	50˚C	30 sec	
Elongation	72˚C	2 min	
Extended elongation	72˚	5 min	1x
Initial denaturation	98˚C	30 sec	1x
Denaturation	98˚C	10 sec	25x
Annealing	68˚C	30 sec	
Elongation	72˚C	2 min	
Extended elongation	72˚C	5 min	1x
Cooling	4˚C	Pause	

Colony PCR

The colony PCR was used to verify the insertion of DNA into the target region. To screen the positive clones after Red recombination in MVA-BAC genome, 20µl reaction mix for each sample were prepared by adding Dream Taq green PCR Master Mix (2X) and specific primers of target gene. A part of a colony was used to innoculate fresh LB agar plate containing antibiotics, marked with numbers and incubated at 37˚C. Using the same pipette tip, rest of the colony was transferred to the PCR tube containing reaction mixture. The PCR reaction was performed in thermocycler and PCR products were analyzed by agarose gel electrophoresis. The colonies with correct fragment size were selected for further use and processed according to the respective number on new agar plates.

3.1.6. DNA precipitation

After the gel extraction, vector DNA and insert (DNA fragment) was precipitated to remove chaperon proteins. In 200µl eluted DNA, 20µl of 3M NaAc and 300µl of isopropanol were added, five times mixed by gentle inversions and incubated for 20min at -80˚C. The mixture was thawed and centrifuged at 13000rpm for 5min. Supernatant was discarded and pellet was washed with 70% ethanol by following centrifugation at 13000rpm for 10min. The pellet was air dried and resuspended in 30µl H_2O.

3.1.7. Dephosphorylation and ligation

Dephosphorylation was done to prevent the self-re-ligation of vector by the removal of phosphate bond at 5´ end of vector DNA. For 50µl of reaction mix, 2µl of CIP (alkaline phosphatase; Biolabs) were added to the digested vector and incubated for 1hr at 37˚C. After the dephosphorylation, DNA was purified by using QIAquick PCR purification kit following the manufacturer's instruction.

For ligation, 100ng of vector were used. The molar ratio used for vector to insert (DNA fragment) was 1:4 for sticky end ligation, which was further incubated at 16˚C with shaking overnight. Next day, ligated product was purified by butanol precipitation. For butanol precipitation, ligation reaction was incubated at 65˚C for 10min to deactivate enzymatic activity. Then 40µl H_2O, 3µl Yeast tRNA and 500µl butanol were added and vortexed. Followed by centrifugation at maximum speed for 10min. Supernatant was discarded and pellet was washed by 500µl of 70% ethanol. Final pellet was air dried and resuspended in 10µl H_2O.

3.1.8. Generation of recombinant MVA in GS1783 cells

3.1.8.1. Construction of transfer plasmids expressing MHV68 genes

The selected genes of MHV68 virus were PCR amplified by using modified primers. All the primers for generating the cDNAs of full length genes of interest were designed to have additional Kozcak sequence and HA tag sequence (Hemagglutinin (HA) protein tag) at the C-terminal end of the transgenes. The deletion VI region of transfer plasmid PH5-dVI-MVA was utilized for cloning target genes into genome of MVA under the control of modified PH5-vaccinia virus early and late strong promotor. The cloning strategies were based on different restriction enzyme combinations which are *Bam*HI+*Afl*II (*BioLabs*) for M2, M3, ORF6 and ORF61 genes, *Swa*I+*Afl*II for ORF8 gene and *Swa*I+*Bgl*II (*BioLabs*) for ORF51 gene of

MHV68 (Fig.1a). After cloning, results were confirmed by control restriction digestion, sequencing and transient protein expression analysis.

3.1.8.2. Red-recombinase homologous recombination

GS1783 cells containing bacmid MVA-BAC (self excisable) were grown in the presence of 30µg/ml chloramphenicol (Cm). Initially, the linearized DNA fragment was obtained by restriction digestion of transfer plasmid by *Pac*I enzyme. The expression cassette contained homologous recombination sites at the right flank (Fl1) and left flank (Fl2) to recombine into MVA-BAC genome, including the kanamycin selection marker gene (*aph*AI) which was also right and left flanked by an internal homologous recombination site (DX‘‘) and the gene of interest under the control of the modified PH5 vaccinia virus promotor.

For 1st recombination, the linearized DNA fragment was transferred into GS1783 cells by electroporation, following the same protocol of transformation as mentioned under the heading 3.1.3. The transformed cells were grown on agar plates containing 30µg/ml chloramphenicol (Cm) and 30µg/ml kanamycin for 24h. The isolated single colonies were picked and identified by colony PCR (mentioned under heading 3.1.5.1). In order to remove *I-Sce*I-*aph*AI cassette, 2nd recombination was performed by inoculating 1ml of LB broth containing 30µg/ml Cm with positive cointegrates from 1st recombination. The inoculum were incubated at 32˚C for 2-4h with shaking at 220rpm until the media became turbid. Then 1ml of pre-warmed LB broth having 30µg/ml Cm and 2% L-arabinose were added. The bacteria were further incubated at 42˚C (water bath shaker) for 30min with shaking at 220rpm, then for another 2-3h at 32˚C and shaking at 220rpm. The bacterial growth density was measured at $O.D_{600}$ nm and 10µl of bacterial suspension from 1:100 ($O.D_{600}$ nm < 0.5) or 1:1000 ($O.D_{600}$ nm > 0.5) dilution were used to streak on LB agar plates containing 30µg/ml Cm and 1% L-arabinose. The plates were incubated for 1-2 days at 30-32˚C until the isolated colonies appeared in visible sizes. The positive co-integrates were further verified by colony PCR.

3.2. Virology Methods

3.2.1. BAC rescue

BAC rescue was done in order to remove the BAC cassette from final recombinant MVA (rec-MVA) by using rabbit fibroma virus (RFV) as helper virus in DF-1 cells. Initially, transfection mix was prepared by adding 5µg of recombinant MVA-BAC DNA, with 6µl of turbofect transfection reagent (Thermo scientific, cat#R0531) to the 400µl of DMEM, without FCS and antibiotic. Transfection mix was incubated for 20min at room temperature.

From each well, media was aspirated from growing DF-1 cells (70-80% confluent cells monolayer). Then transfection mix was added to the DF-1 cell monolayer in each well of 6-well plate with additional 4ml DMEM, having 10% FCS but no antibiotics and incubated for 3h at 37˚C. For infection, RFV virus MOI 0.1 was diluted in 100µl of DMEM, and directly added to the cells in each well without removing the transfection medium. After 72h, viral plaques were monitored for the GFP flourescence. Cells were harvested and centrifuged at 4000rpm for 10min at 4˚C. Supernatant was removed and pellet was resuspended in 1ml medium. After three times freeze-thaw and sonification for 30sec, supernatant was stored at -80˚C.

3.2.1.1. Limiting dilution assay

After sub passaging rescued virus in DF-1 cells (from 6-well → T25 flask → T75 flask), rec-MVA-BAC self excising constructs were monitored under microscope for the reduction of fluorescence with the increased cytopathic effect (CPE) in the infected cells. The viral suspension from 3rd passage (from T75 flask) was used for the limiting dilution assay to select the GFP negative and CPE positive clones. In limiting dilution assay, 10-fold serial dilution of each rec-MVA-BAC construct was used to infect the DF-1 cells (60-70% confluent) in two 96-well plate. After 3 days of virus growth, GFP negative and CPE positive clones from higher dilutions were selected and further subcultured in 6-well plates. The selected clones were also examined for the loss of GFP marker and RFV contaminants from final rec-MVA by means of PCR, using specific primer.

3.2.2. Virus amplification and crude stock preparation

For virus amplification, 10 T-175 flasks were seeded with DF-1 cells for each rec-MVA. Monolayer of 80-90% confluent DF-1 cells were infected with rec-MVA with low MOI. After 72h, there was a clear cytopathic effect and cells were harvested and centrifuged at 1700rpm, 4˚C for 5min. Cell pellets (from each T-175 flask) were resuspended in 1ml 10% FCS DMEM medium followed by 3 times freeze/thawing and sonification, each for 30sec. To prepare the virus at large scale, CEF cells were grown in 35 T-175 flasks for each rec-MVA. Virus stocks from each previous T-175 flask were used to infect CEF cells monolayer in 35 T175 flasks. After 72h, cells having a clear CPE were then harvested and centrifuged at 1700rpm, 4˚C for 5min. After discarding the supernatant, cell pellet was resuspended in 35ml 10mM Tris pH.9 buffer (1ml/flask) and stored at -80˚C for further purification.

3.2.3. Purification of rec-MVA on sucrose cushion

For purification, crude stocks of virus (resuspended in 35ml 10mM Tris buffer) were subjected three times to freezing and thawing. Ultrasonic device was disinfected by 70% ethanol and the needle was irradiated with UV light for 10min. The cell suspension was placed on ice and passed through ultrasonication three times, each for 15sec. After centrifugation at 4000rpm, 4˚C for 5min, supernatant was transferred to another 50ml tube and 35ml of fresh 10mM Tris buffer pH.9 were added to the cell pellet and again repeat the ultrasonication 3 times, centrifuged and transferred the supernatant to new falcon tube. The supernatant containing virions was slowly dropped on sterile 36% sucrose cushions (25ml) in SW32 centrifuge tubes and ultracentrifuged at 13500rpm, 4˚C for 60min. The cell pellet was again resuspended and pooled by using 10mM Tris buffer pH.9 and then dropped on 36% sucrose cushion (10ml) in SW28 centrifuged tubes. After ultra centrifugation at 13500rpm, 4˚C for 60min, the cell pellets were resuspended and pooled in 1mM Tris buffer pH.9 for long term storage at -80˚C.

3.2.4. Virus titration and low multiplicity growth kinetics by determining the $TCID_{50}$

DF-1 or CEF cells were seeded in 96-well plates. A serial dilution (from $10e^{-1}$ to $10e^{-12}$) of viral suspension was used to infect the cells grown in 96 well-plates and incubated for 1 week. From day 4 to day 7, cells in each well were monitored for CPE under the microscope and CPE positive wells were marked.

$TCID_{50}$ was determined according to the following formula:

Log10 50% endpoint dilution= x-d/2 + (dΣ r/n)

x = highest dilution in which all eight well (8/8) are counted positive

d = log10 of dilution factor (d=1 when 10-fold serial dilutions were used)

r = number of positive wells per dilution

n = total number of well per dilution (n=8 when dilutions are plated out in replicates of eight)

To analyse the low multiplicity growth kinetics of rec-MVA, CEF cells were infected using MOI 0.01 and harvested at 0, 24, 48 and 72h time points (whereas at 0h, cells were infected for 45min at 4˚C, used as a control for virus attachment). The viral suspensions harvested at each time point were back titrated by $TCID_{50}$ assay.

3.2.5. Plaque assay

The number of infectious viral particles from virus stocks or lung homogenates were determined by staining plaques. Viral plaques of replicating virus in adherent cells were visible after staining with crystal violet. Initially serial dilution of virus (1:10) was prepared in volume of 1ml by using M2 medium. Then 900µl of each dilution of virus was added to BHK-21 cells (60% confluent cell monolayer) in each well of 24-well plate and incubated for 90min at 37˚C for infection. After incubation, infection medium was removed and 1ml of preheated methylcellulose containing medium was added to each well to prevent spread of virus. The cells were further incubated for 5 days and then medium was aspirated. Staining of plaques was performed by adding 500µl of 1x crystal violet solution for 15min at RT. Number of viral plaques in each well were observed by using light microscope and viral titre was determined by using following formula:

Number of plaques in highest dilution x highest dilution x Input factor* = _____PFU/ml

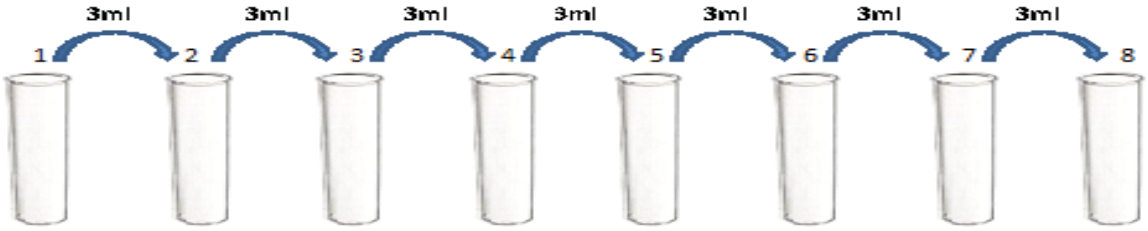
*Input factor = 1.1

3.2.6. Latent virus reactivation assay

The replication frequency of latent virus can be determined in a reactivation assay. The latent virus is present in an episomal form in the spleen. When virus-containing splenocytes are transferred to NIH3T3 cells, the virus starts its lytic replication cycle that is referred as reactivation of virus. Therefore, NIH3T3 cells were seeded in a tissue culture 96 well plates (2 plates for each group) by using 7000-7500 cells in 200µl medium/well containing 5%FBS and 1%pen/strep. Next day, spleens were harvested from infected mice to prepare splenocytes. After counting the cells, splenocytes were pooled from each group of mice up to 13.5million cells (total in 9ml volume). Then 1:3 serial dilution of splenocytes was prepared, starting from a concentration of 1.5M cells/ml (1.5x10e5 cells/ wells) (Fig.3.1). Each dilution of splenocytes was plated in triplcate (3 lanes = 24 wells) by using 100µl/well in a 96-well plate. Therefore, the total volume in each well was equal to 200µl (having pre-seeded NIH3T3 cells) + 100µl of splenocytes. The cells were incubated at 37˚C, 5% CO_2. At day 7 and 14, CPE in each well was determined under the microscope. The percentage CPE positive wells in each dilution was calculated using the following formula:

No. of CPE positive wells x 24 / 100

Fig. 3.1



total 13.5Mcells							
in 9ml media	6ml media	6ml media	6ml media	6ml media	6ml media	6ml media	6mlmedia
↓ 100ul/well	↓	↓	↓	↓	↓	↓	↓
(150000cells)	(50000cells)	(16,666cells)	(5,555cells)	(1,851cells)	(617.3cells)	(205.8cells)	(68.6cells)

3.3. Cell Culture

All the cell culture work was done under sterile conditions. The cells were grown in an incubator at 37˚C, with a supply of 5% CO2 atmosphere and 95% humidity providing optimal growth conditions.

All the cell lines were grown in M2 medium (RPMI 1640 supplemented with 1% penicillin streptomycin, 10% fetal calf serum and 0.05M β-2mercaptoethanol). Cell lines were grown in cell culture-graded flasks, plates or dishes. Cells were sub-passaged from 1:2 to 1:10 dilution (when reached upto 80-90% confluency) according to the growth kinetics and requirement.

For adherent cell lines medium was removed and cells were washed with PBS, trypsinized and incubated at 37˚C for 2 min. Cells were detached from the surface and singularized by adding 5ml M2 medium. Furthermore, cells were seeded into culture flasks or plates according to the requirements.

For semi-adherent cells like EL-4, cells were detached from surface with the help of cell scraper without removing the medium and splitted in a respective split ratio. While DC2.4 cells were strongly flushed in the flask as they are semi-adherent population and cell suspension was used for further experiment or sub passaging.

3.3.1. Cryo-preservation of cells

Cells in growing phase were used for storage. Adherent cells cultured in T-185 culture flask were harvested by trypsinization. Cells were pellet down at 1500rpm, 4˚C for 5 min. The pellet was resuspended in a freezing medium ($1x10^6$ cells/ml) and transferred to sterile cryo-tubes. The cells were frozen slowly by placing them into cryo-box at -80˚C. Next day, tubes were transferred to liquid nitrogen storage (-196˚C) for long term storage.

Furthermore, T cells after four days of restimulation, were pooled and centrifuged at 1500rpm, 4˚C for 5min. The cells were then resuspended in a freezing medium (T cells from 2 wells/ ml of freezing medium) and processed as described above.

3.3.2. Culturing of cryo-preserved cells

To re-cultivate the cryo-preserved cells, the cells were taken out from liquid nitrogen and thawed at 37˚C in water bath. The cells were immediately washed with 10ml of M2 medium. After centrifugation, cell pellet was resuspended with fresh M2 medium, transfered into T-75 cell culture flask and placed in an incubator at 37˚C with 5% CO_2.
For T cells, cryo-preserved cells from each tube were recultivated in four wells of 24 well-plate.

3.3.3. Primary chicken embryo fibroblast (CEF) culture

Chicken embryos were incubated for 10-11 days in a breeder at 37˚C with optimal humidity. Later on, the eggs were disinfected with 70% ethanol. A small crack was made on the egg (at blunt round side) and shell covering was removed with sterile forceps and scissor. Then chorionallantois membrane was removed by forceps to open allantoic cavity. The embryo was carefully taken out and transferred to the petri dish. The eyes and splanchna were removed and embryo was transferred to the beaker containing PBS. In 50ml syringe, plunger was removed and tissue chunks in PBS were poured into the barrel from beaker and forced with plunger to pass through into another beaker. The crushed embryo was washed 3 times with PBS to remove red blood cells and transferred to the conical flask containing 100ml pre-warmed trypsin solution (0.25%). The magnetic bar was added to the flask and placed on hot plate magnetic stirrer at temperature 37˚C, for 25 min. The lysate was filtered through double-layered sterile guaze into a 500ml beaker. After three times trypsinization and filteration, the filterate was divided into two 50ml falcon tubes and centrifuged at 1800rpm for 6min. The pellet was washed three times with 50ml PBS/tube by following centrifugation at 1800rpm for 6min. The cell pellets were pooled, resuspended in a desired CEF's medium and transferred in T185 culture flasks for cultivation in a normal cell culture incubator

3.4. Immunological Methods

3.4.1. Immunization

For all immunization experiments, 6-8 weeks old C57BL/6N female mice were used. Mice were infected with wild type MVA-F6 or recombinant MVA (rec-MVA) with selected dose of $1x10^8$ IU/200µl (TCID50 dose) through intraperitoneal (i.p) application. Virus was diluted in 1x Tris buffer.

For MHV68 challenge experiments, mice were first given anasthesia via intraperitoneal application. For anasthesia, mixture of ketamin and xylazin were used. Afterwards, mice were infected with MHV68 virus using dose of $5x10^4$ PFU/20µl through intranasal application. Virus was diluted in RPMI having 5% FCS.

Calculation for Ketamin (100mg) and Xylazin (20mg/ml)

Ketamin: 0.1ml ketamin + 0.9ml NaCl (1:10 dil) → take 0.05ml/10g (a)

Xylazin: 0.1ml Xylazin + 0.9 ml NaCl (1:10 dil) → take 0.0125ml/10g (b)

Mixed the diluted ketamin (a) and xylazin (b) and applied accordingly to the weight of mice.

3.4.2. Preparation of splenocytes

The spleen was removed from the mouse under sterile conditions and homogenized by using syring plunger over cell strainer in a petri dish. Homogenized cell suspension was pellet down at 1500rpm, for 5min. Erythrolysis was done by adding 3ml TAC buffer to the pellet for 2min at 37˚C and filled upto 50ml PBS for washing. The cells were passed through nylon filter and centrifuged again. Final cell pellet was resuspended in 2ml M2 medium and counted for further experiments.

3.4.2.1. Cell counting

Splenocytes were counted in a dilution of 1:40. First the cells were diluted at ratio of 1:10 by using 450µl RPMI medium and 50µl of cells. From this pre-dilution, cells were further diluted at a ratio of 1:4 by using 50µl cells, 50µl trypan blue and 100µl 4% acetic acid. Neubauer chamber was used to count the cells by using following formula:

n (cells/ml) = mean of two quadrants x dilution factor x 10^4

3.4.3. Generation of antigen specific CD8+ and CD4+ T cells

3.4.3.1. Lipopolysaccharide blast

Splenocytes from naive C57BL/6 mice were prepared as described in 3.4.2. Total volume was adjusted to the no. of splenocytes $1x10^6$/ml in M2 medium, provided with 25µg/ml LPS and 7µg/ml Dextran SO4. In each T-75 culture flask, 40ml cell suspension was added and incubated in standing position at 37˚C, 5% CO2 and 90% humidity for three days.

3.4.3.2. Primary culture

The LPS treated splenocytes were harvested and irradiated at 3000 rad (30 Gray). The splenocytes were first divided into falcon tubes for different peptides. The cells were washed with 20ml RPMI 1640 medium followed by centrifugation and the pellet was resuspended in 1ml RPMI 1640 medium. To this cell suspension 250ng of MHC class-I specific peptide (2.5µl from 100µg/ml) were added for CD8+ T cells, while for CD4+ T cells 500ng MHC class-II specific peptide (5µl from 100µg/ml) were used. Peptide stocks (1mg/ml) were diluted with DMSO to the desired concentration. The peptide pulsed splenocytes were incubated for 30min at 37˚C and then washed twice with 10ml of M2 medium. The cells were adjusted to the volume having $3x10^6$ cells/ml by using M2 medium.

The splenocytes from C57BL/6 mice (pre-vaccinated) were prepared as described previously. The splenocytes were adjusted to the volume having $7x10^6$ cells/ml with M2 medium and 1ml was added per well of a 24-well plate containing 1ml of LPS treated cells. For CD8+ and CD4+ T cells, primary cultures were incubated for 7 days and 14 days, respectively.

3.4.3.3. T cell restimulation

For maintenance, CD8+ T cells were restimulated every 7th day, CD4+ T cells every 14th day, by following the below procedure:

For CD8+ T cells, EL-4 cells were irradiated with 10000rad (100 Gray) and divided into falcon tubes for each peptide. The cells were washed with 20ml RPMI 1640 medium. The cell pellet was resuspended in 1ml of RPMI 1640 medium and 1µl of each peptide, from a working peptide stock of 1µg/ml was added and incubated for 30min at 37˚C. Cells were then washed twice with 10ml M2 medium and were adjusted to the volume having $1x10^6$ cell/ml with M2 medium.

The splenocytes from naive mice were prepared and irradiated with 3000rad (30 Gray). After washing once with M2 medium, splenocytes were adjusted to the volume having $12x10^6$ cell/ml with M2 medium.

For CD4+ T cells, splenocytes were pulsed with peptide instead of EL-4 cells. As previously described, irradiated splenocytes were resuspended in RPMI 1640 medium and divided into falcon tubes. After washing twice with RPMI 1640, cells were resuspended in 1ml RPMI 1640 medium, 2µl of peptide from 1mg/ml peptide were added to them and incubated for 30min at 37˚C. Cells were washed and adjusted to the volume having $12x10^6$ cell/ml with M2 medium.

T cells from primary culture were collected, centrifuged and resuspended with fresh M2 medium. For CD8+ T cells, 0.5ml of peptide pulsed EL-4 cells, 0.5ml of splenocytes, 0.5ml of 5% TCGF (conditioned medium as supernatant from rat splenocytes stimulated with 5µg/ml concanavalin A; Beeton *et al*.,2007) and 0.5ml CD8+ T cells were added in one well of 24 well plate. While for CD4+ T cells, 0.5ml of peptide pulsed splenocytes, 0.5ml of 5% TCGF and 1ml of CD4+ T cells were added in one well. The cultures were incubated at 37˚C till the next round of restimulation.

3.4.4. Preparation of BMDC's

Murine tibia and femur bones from both legs were prepared. Bones were sterilized with 70% ethanol, washed with PBS and then placed into M2 medium. The end joining part of bones were cut away and bone marrow was flushed three times into a dish by using 1ml syringe filled with M2 medium. The suspension was collected in a falcon tube, and centrifuged (1500rpm, 5min). Erythrocytes were lysed by adding 5ml TAC buffer (0.144M NH_4Cl and 0.017M Tris pH 7.65) and filtered through a 100µm cell strainer. In each 94x16mm petri dish, $5x10^6$ cells were plated to grow BMDCs in 10ml M2 medium including 10% GM-CSF (conditioned medium; a supernatant from B16 cells expressing GM-CSF cells gifted by Georg Hacker, Freiburg, Germany). The culture was incubated at 37˚C, 5% CO_2 in a humid atmosphere.

On day 3, additional 10ml M2 medium containing 10% GM-CSF were added to the cells. On day 6, 10ml medium from cells was replaced with a fresh 10ml M2 containing 10% GM-CSF. The cells were ready for experiment on day 7.

3.4.5. *In vitro* antigen presentation assays

3.4.5.1. For exogenous antigen presentation assay

A 100µl of each diluted peptide were used to pulse $4x10^5$ BMDCs/ DC-2.4 cells in 100µl and further incubated for 30min at 37˚C in 96 well plate (for each sample). After incubation, cells were washed with M2 medium. Peptide-pulsed cells were then resuspended in 100µl of

Brefeldin-A with a concentratoin of 1µg/ml and co-cultured with $2x10^5$ antigen-specific T cells /100µl in M2 medium, incubated for 4hr at 37˚C. Whereas, cells pulsed with irrelevant peptide (1ng/ml) were used as peptide control and with no peptide as mock control to access the background activity of T cells. After 4hr of incubation, intracellular cytokine staining was carried out as described in 3.4.6.

For CD8+ T cell antigen recognition analysis, M2 medium was used to prepare serial dilution of peptide from $10e^{-8}$ M(1ng/ml) to $10e^{-11}$M (0.001ng/ml). For control, 10^{-8}M (1ng/ml) diluted peptide was used.

For CD4+ T cell antigen recognition analysis, M2 medium was used to prepare serial dilutions of peptide from $10e^{-5}$ M(1000ng/ml) to $10e^{-8}$M (1ng/ml). For control, 10^{-5}M (1000ng/ml) diluted peptide was used.

3.4.5.2. For endogenous antigen presentation

BMDCs were infected in each well of 6-well plate with MVA-F6 (MOI 5) and rec-MVA (MOI 5) for 15h, and MHV68 virus (MOI 10) for 48hrs. Infected BMDCs $4x10^5$/100µl were incubated with 100µl of Brefeldin-A (1µg/ml) and co-cultured with $2x10^5$/100µl of antigen-specific T cells in M2 medium, incubated for 4h at 37˚C. While for the peptide-pulsed control cells 100µl of 1ng/ml peptide was used for CD8+ T cell assay,100µl of 1000ng/ml peptide was used for CD4+ T cells assay. Uninfected cells were used a negative control. After incubation for 4h with CD8+ T cells or 15h with CD4+ T cells, intracellular cytokine staining was carried out.

3.4.6. Intracellular cytokine staining

3.4.6.1. EMA staining

EMA staining is used for live/dead differentiation of cells, since the photo-activated molecule can enter only dead cells devoid of an intact membrane. Cells were transferred into a V-bottom 96 well plate and washed with FACS buffer. Then cells were incubated with 1µg/ml EMA (1:2000 dilution) for 20 min on ice under bright light to stain dead cells. The cells were washed twice by using FACS buffer (180µl) for 2min, 1400rpm at 4˚C.

3.4.6.2. Surface and intracellular marker staining

After EMA staining, all steps were performed on ice under dark condition. Cells were stained with surface markers like anti-CD8 or anti-CD4 labelled with PB (1:300 dilution) by using 50µl/well for 30min. The cells were then fixed and permeablized by using 100µl of Cytofix/Cytoperm™ fixation (BD Pharmingen™) for 15min. The cells were washed with 1x

perm-wash and incubated with anti-IFNγ labelled with APC (1:300 dilution) by using 50µl/well for 30min. Lastly, the cells were again washed by perm-wash and fixed by using 2% PFA diluted with FACS buffer in 1:2 ratio.

3.4.6.3. Flow cytometry

Flow cytometry measures the characteristics of single cells passing through the laser beam and scatters the fluorescence light that differentiate the cells on the base of physical parameters like size, granularity and density (by forward light scatter or FSC and sideward light scatter or SSC). These cells are further exposed to fluorochrome-conjugated antibodies (e.g PB/APC). These fluorochromes can absorb high energy photon and upon returning to their resting state, they emit light energy at higher wavelenght called as fluorescence. The use of multiple fluorochromes having similar excitation and different emission wave lenghts (colours), allow to measure several cell properties simultaneously.

Following the gating protocol, cell populations were first separated by using FSC and SSC. Next, living cell populations were determined by gating for absence of EMA detected in the PerCP and PE channels. Finally, the respective surface markers and intracellular cytokine markers were detected according to the their labelled fluorochrome.

3.5. Protein expression analysis

3.5.1. Western blot

Western blot is an immunobloting technique to determine protein expression by using specific antibodies. This process is carried out by sodium dodecyle sulphate polyacrylamide gel-electrophoresis (SDS-PAGE) to separate proteins according to their molecular weight.

3.5.2. Preparation of cell lysates

Protein expression analysis was done either after coinfection/transfection with MVA and transfer plasmids (containing MHV68 genes) or after infection with rec-MVA. For infection, the cell monolayer was infected with virus (MOI 10 $TCID_{50}$) for 1hr at 37˚C. After the removal of infection medium, either fresh medium was added to prepare cell lysate after 24h incubation or transfection was done. For transfection, 1.5µg of plasmid DNA in 250µl medium and 3µl fugene transfection reagent in 250µl medium without FCS were mixed and incubated at RT for 20min. This mix was added to the cells with additional 1ml medium with FCS (in one well of 6-well plate) and incubated for 4h at 37˚C. After the removal of

transfection medium, 2ml fresh medium was added and incubated for further 24hrs to prepare cell lysates.

For preparation of cell lysates, cells were detached from the surface with a cell scraper, transferred into 1.5ml tube and centrifuged at 1500rpm for 5min at 4˚C. The cell pellet was further washed with PBS to remove FBS and resuspended in 80µl of tyrosinase lysis buffer. Following three times freeze/thawing, the cells were lysed by sonification three times at interval of 1min placing on ice. Lastly, the cells were centrifuged at 13000rpm for 5min at 4˚C and supernatant was transferred into new tube, stored at -80˚C.

3.5.3. SDS PAGE

To prepare sample for loading on the gel, 20µl of protein were mixed with 4µl of 5X loading buffer (reducing agent) and heated at 95˚C for 5min to denature proteins by breaking disulfide bonds. The same procedure was applied for the protein ladder (molecular weight marker), used 8µl/well. Two small sized gels were run at 60 milliAmp for 80-90min in a vertical electrophoresis chamber. Following are the recipes for 12% SDS gel preparation.

Resolving Gel (12%)	**Volume for small gel**
Bis/ Acrylamide 30%	4.8ml
2M Tris pH 8.8	2.5ml
SDS (20%)	60µl
TEMED	24µl
APS (10%)	144µl
H_2O	5.3ml

Stacking Gel (12%)	**Volume for small gel**
Bis/ Acrylamide 30%	1.5ml
0.5M Tris pH 6.8	1.2ml
SDS (20%)	45µl
Saccharose (60%)	2.1ml
TEMED	12µl
APS (10%)	120µl
H_2O	4.2ml

The gels were removed from the electrophoresis chamber. The stacking gel was carefully removed and transferred into the blotting buffer. The nitrocellulose membrane (0.45µm pore size) was cut according to the size of the gel and equilibrated by using blotting buffer. The gel and membrane were sandwiched in 6 layers of whatman paper, pre-wet in blotting buffer and placed in a transblotter to run for 60 min at 60volts, 1000mAmp and 300watt.

After blotting, the nitrocellulose membrane was placed in blocking solution (5% milk powder in 1X TBST) for 40min. After washing the membrane 3 times with 1X TBST buffer, the membrane was incubated with the primary antibody (Anti-HA monoclonal rabbit antibody;

dilution 1:2000) for 1hr on a shaker. Followed by 3 washings, the membrane was incubated with the secondary antibody (Anti-rabbit; dilution 1:2000) for 45min on a shaker and washed subsequently. ECL substrate solution 1ml (mixture of Lumi-Light solution A and B, ratio 1:1) was added to cover the whole membrane for 2min. The membrane was dried with whatman paper and placed in a dark casset to further detect the protein expression signals by using photographic film.

4. Results

4.1. Construction and characterization of MVA transfer plasmids encoding MHV68 genes

In order to generate the transfer plasmids having each of the selected MHV68 genes, we used the MVA transfer plasmid PH5_dVI_MVA that contains right and left flanked homologous sequences adjacent to the deletion VI (dVI) region of MVA genome. All MHV68 genes selected (ORF6, ORF61, ORF8, ORF51, M2 and M3) were cloned in single transfer plasmids by using gene-specific cloning strategies (Fig.4.1). MHV68 genes were expressed under the control of the modified PH5 strong early and late promoter of vaccinia virus (explained in method 3.1.5.4a). After cloning, positive clones were selected through control DNA restriction digestion and results were analyzed by agarose gel electrophoresis (Fig.4.2A). Furthermore, transient protein expression was determined through the detection of a HA-tag attached to the C-terminus of all MHV68 genes by western blot analysis (SDS-PAGE). All constructs showed strong recombinant protein expression (Fig.4.2B). Additionally, all final transfer constructs were sequenced to proof the genomic integrity of the integrated DNA.

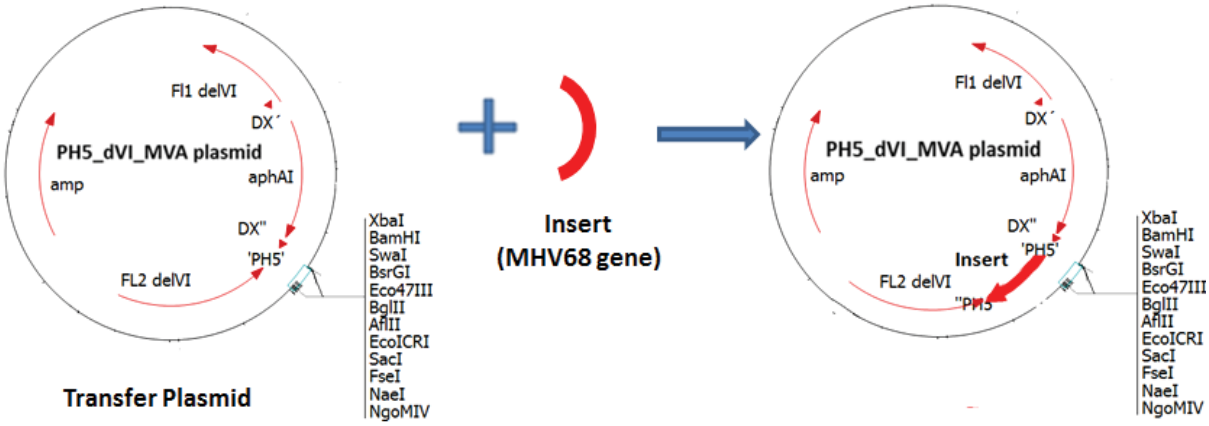


Figure.4.1 Schematic map of the MVA transfer plasmid vector and the cloning strategy
For construction of the transfer plasmids encoding MHV68 genes, the MVA transfer plasmid (PH5_dVI_MVA) and cDNA generated from MHV68 viral DNA by RT-PCR for each MHV68 gene were digested by combination of different restriction enzymes (BamHI+AflII for ORF6, ORF61, M2 and M3 genes, SwaI+AflII for ORF8 gene and SwaI+BglII for ORF51 gene) allowing a site-directed cloning. Each MHV68 gene (insert) was cloned into an expression cassette allowing the target gene expression under the control of PH5 early and late promoter and the targeting of the cassette into the deletion VI (dVI) region of MVA.

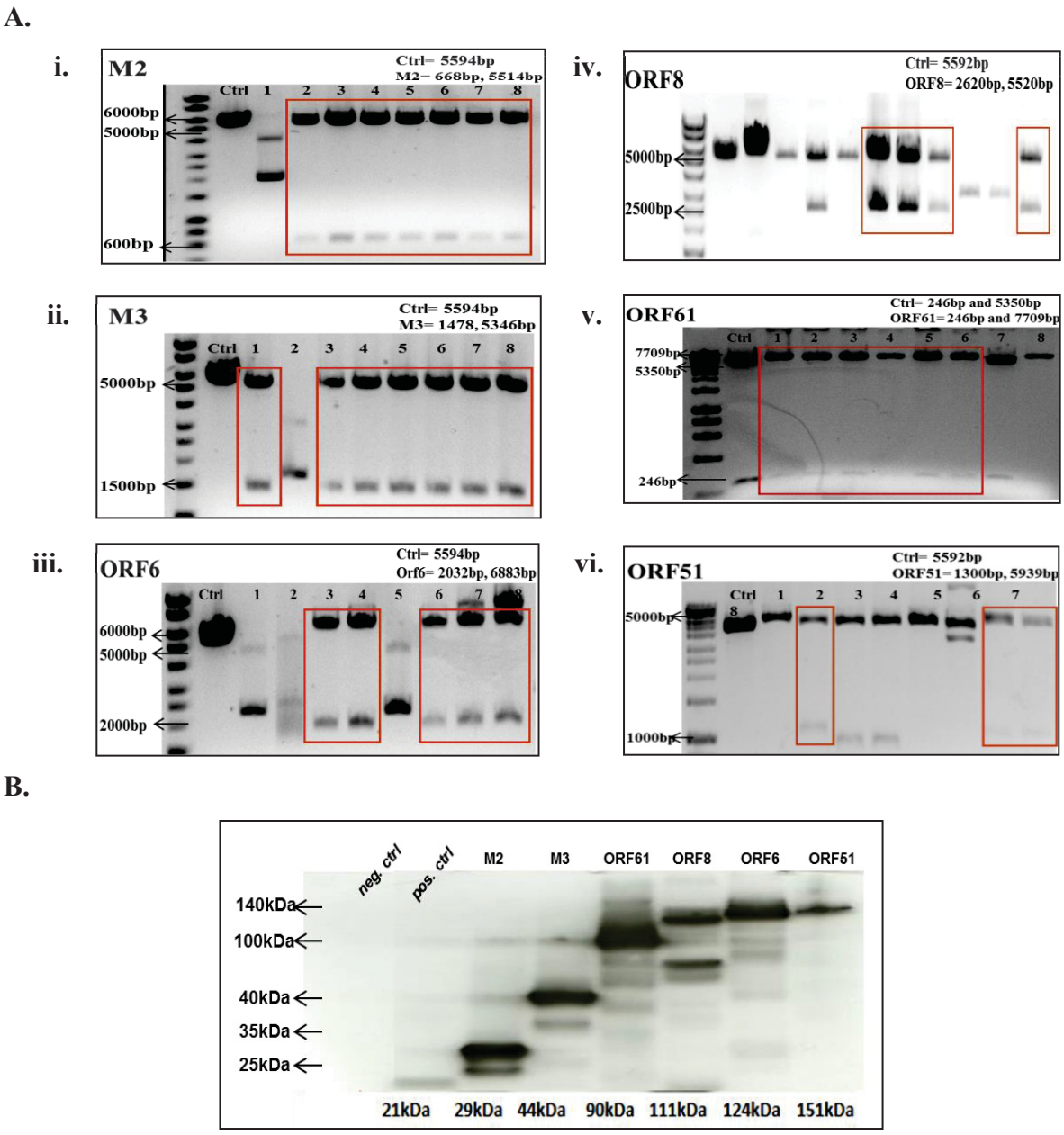


Figure.4.2 Characterization of MVA transfer plasmid having MHV68 gene

A. Integration of target genes in the expression cassette of the transfer vector. Agarose gel electrophoresis (2%) after DNA restriction digestion showing positive clones with correct fragment sizes for the indicated MHV68 antigen (red rectangles). In i) M2 ii) M3 iii) ORF6 iv) ORF8 v) ORF61 vi) ORF51, uncut plasmids were used as controls. **B. Westernblot analysis for protein synthesis.** In BHK-21 cells transient transfection of empty MVA transfer vector (as a negative control), pIRES-EGFP plasmid endoding a HA tagged US6 gene of HCMV (as a positive control) and MVA transfer plasmids encoding indicated MHV68 genes in BHK-21 cells for 4h, followed by infection with MVA-F6 for 24h. After 24h, cells were harvested to prepare cell lysates and transient expression of protein was detected by using an anti-HA monoclonal rabbit antibody in western blot.

4.2. Generation of recombinant viruses by using MVA-BAC homologous recombination

4.2.1. Preparation of the linearized transgene expression cassettes by *Pac*I digestion

After construction and characterization, each MVA transfer plasmid was digested by *Pac*I (restriction enzyme), resulting in a linear DNA fragment. This fragment contained homologous sites on right and left flank of the expression cassette to recombine with MVA-BAC genome in the del VI region. The expression cassette included an *aph*AI gene (kanamycin selection marker), which was flanked right and left by an internal homologous recombination site as well as the respective MHV68 target genes under the control of PH5 vaccinia virus promoter (Fig.4.3A). Results demonstrating the correctly sized linear DNA fragments obtained after restriction digestion of transfer plasmids are shown in Fig.4.3B.

A. **B.**

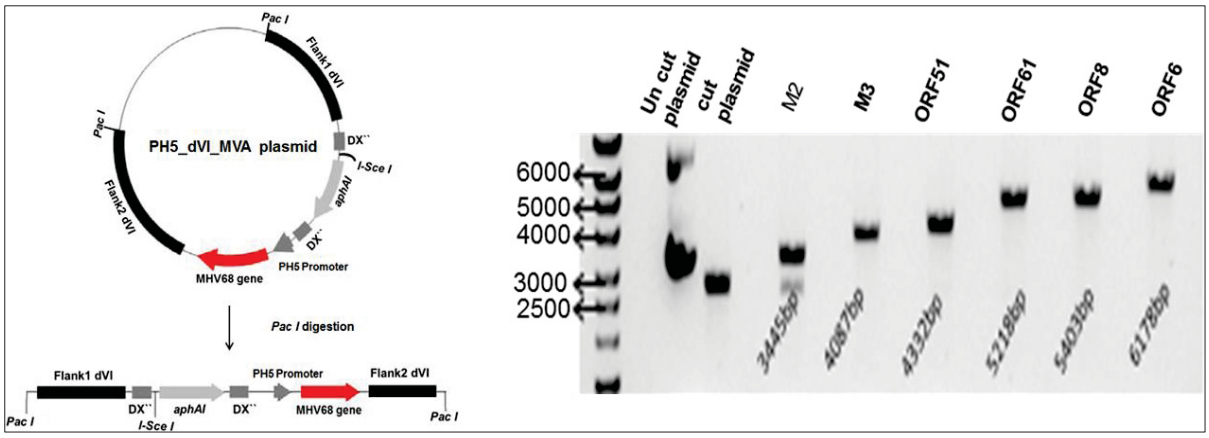


Figure.4.3 Restriction digestion of MVA transfer plasmids

A. Schematic map of linear expression cassettes after *Pac*I DNA restriction digestion. **B.** Agarose gel electrophoresis (2%) after DNA restriction digestion of PH5_dVI_MVA transfer plasmids (encoding for each MHV68 antigen) showing correct fragment sizes for the indicated MHV68 antigen**.** Cut and un-cut empty PH5_dVI_MVA plasmids were used as a positive and negative control respectively.

4.2.2. *En passant* recombination of transgene expression cassettes in deletion-VI region of MVA-BAC genome

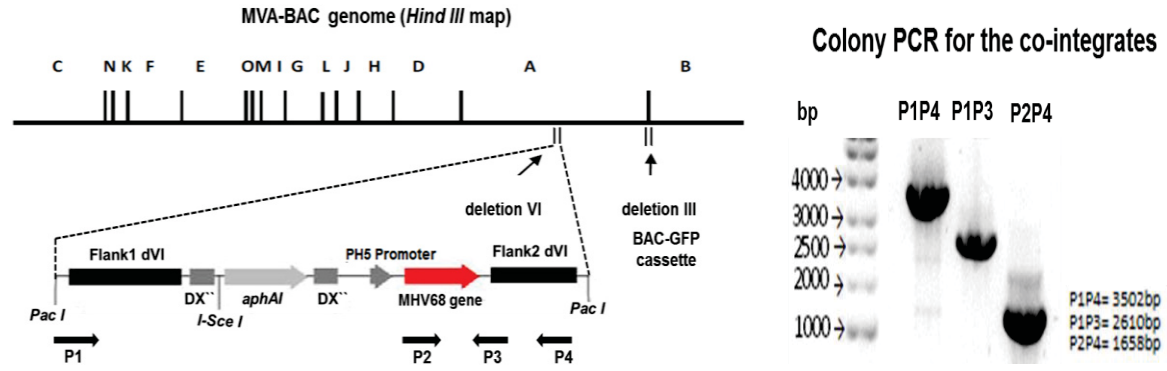
To construct the self-excising recombinant MVA-BACs, linearized transgene expression cassettes after *Pac*I digestion were transferred into GS1783 *E.coli* cells by electroporation.

GS1783 cells belong to an artificially engineered E.coli strain, in which the genomic DNA possesses the λ-red recombinase gene under the control of a temperature sensitive promoter as well as the *I-Sce*I homing endonuclease gene under the control of a substrate inducible promoter. In particular, GS1783 cells contain MVA-BAC genome in cytoplasm. The BAC-GFP cassette targets the deletion-III region of the wild type MVA genome. The BAC-GFP cassette comprises a chloramphenicol acyltransferase gene (Cm), origin of replication (Ori2) of the mini-F backbone and a GFP gene under the control of a poxviral promoter (driven by the late FP4B gene promoter).

In the 1st recombination step, each linear transgene expression cassette was recombined into the deletion-VI region of the MVA-BAC genome. The recombinant clones were selected on agar plates due to simultaneous kanamycin and chloramphenicol resistance. The efficacy of recombination (integration of the complete expression cassette) was determined by a colony PCR using primers P1, P2, P3 and P4 in different combinations (mentioned in Fig.4.4A). The results shown in Fig.4.4 for M2 gene are representative for all other MHV68 genes.

In the 2nd recombination step, positive co-integrates from the 1st recombination were grown in medium with L-arabinose to induce the *I-Sce*I endonulease gene expression, leading to the formation of double stranded breaks adjacent to the *aph*AI gene (kanamycin marker). The temperature was raised shortly up to 42˚C which facilitates the 2nd recombination after the removal of *aph*AI gene and the DNA fragments were re-joined through the recombination of internal homologous regions (DX''). The recombination efficacy was again analyzed using the same colony PCR strategy as decribed above. As expected, fragments reduced in size were observed for both primer combinations spanning the whole dVI region and the region of *aph*AI gene (as shown for M2 in Fig. 4.4B). Similar results were obtained for ORF6, ORF61, ORF8, ORF51 and M3 MHV68 genes (data not shown). Both 1st and 2nd recombination was highly efficient and more than 90% of the tested clones for each recombinant MVA-BAC were correct. Furthermore, the accuracy of genomic integration and the integrity of the MHV68 target gene sequence were confirmed through DNA sequencing for each of the final rec-MVA-BAC constructs.

A. 1st recombination



B. 2nd recombination

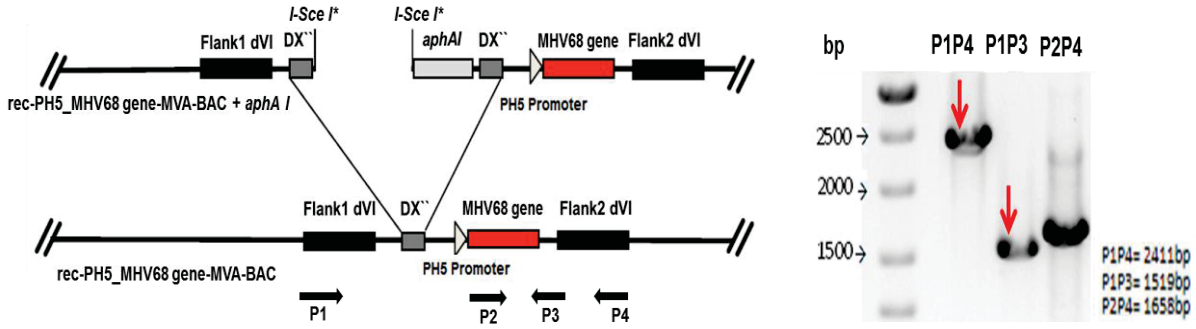


Fig.4.4 Insertion of transgene expression cassettes into the deletion VI region of MVA-BAC

A. Schematic map of the 1st recombination, showing the linear transgene expression cassette containing MHV68 gene (e.g ORF6, ORF61, ORF51, ORF8, M2 and M3) to be inserted into deletion-VI of MVA-BAC. Colony PCR for the correct integration denoted 3.5kb, 2.6kb and 1.6kb fragments, corresponding to the PCR products obtained by the combinations of primers: P1+P4, P1+P3 and P2+P4. **B.** Schematic map of the 2nd recombination, showing cleavage of *aph*AI by *I-Sce*I endonucleases. Colony PCR denoted 2.4kb, 1.5kb and 1.65kb, corresponding to the PCR product with reduced size for the *aph*AI gene-deleted cassette obtained by the combination of primers: P1+P4, P1+P3 and P2+P4.

4.2.3. Generation of recombinant MVA vaccines by recombineering of a self-excisable BAC

DNA from each recombinant MVA-BAC was isolated from GS1783 *E.coli* cells and transfected into the DF-1 cells. Additionally, rabbit fibroma virus (RFV) was used as a helper virus for infection. Although unable to replicate in DF-1 cells, the helper virus provided the poxviral initial transcriptional machinery to initiate the replication cycle using the rec-MVA-BAC as a template. The self-excising BAC cassette resides in deletion-III region of MVA, which contains flanking sequences at both sides (right and left) of the inserted mini F cassette. The BAC cassette may be efficiently removed by intermolecular homologous recombination by utilizing reverse complement duplications of the right and left flanks inserted between the chloramphenicol acetyltransferase gene (Cm) and origin of replication (Ori2). We initially amplified all rec-MVA-BAC in DF-1 cells, which were identified by GFP fluorescence (Fig 4.5A). The BAC cassette in the deletion-III region including the GFP marker gene expressed by a poxvirus promoter was almost completely lost after 3 rounds of passaging, resulting in markerless rec-MVA (Fig.4.5B). After sub-passaging rec-MVA viruses, we performed limiting dilution assays and singularized the rec-MVA viruses on DF-1 cells (70-80% confluent) in 96 well plates. BAC self-excised clones of rec-MVA viruses, which showed a clear cytopathic effect on the cell monolayer work without GFP signal, were selected after 6 days of infection (Fig.4.6A).

Since MVA is able to replicate in CEFs, MVA infection of that cell line leads to a higher virus yield. Therefore, we amplified the virus crude stock in the immortalized CEF cell line called DF-1, switched then to CEFs for preparation of pure virus stocks. Given that helper virus RFV could replicate in DF-1 or CEF cells, it was necessary to analyzed whether the RFV has been cleared from the rec-MVA before crude stock preparation. For this reason, DNA of each rec-MVA was extracted for PCR analysis by using specific primers. We found that RFV DNA was undetectable after 5 rounds of sub- passage of each rec-MVA in DF-1 cells (Fig.4.6C). At the same time, viral DNA was also tested for the GFP contaminants in final rec-MVA viruses, by using specific set of primers. All the selected clones were free from GFP sequence, showing that all rec-MVA viruses have been efficiently rescued from BAC (Fig.4.6B).

The final rec-MVAs were sequenced again to check if the transgene expression cassette in deletion VI region of MVA was inserted in the right orientation, and if the sequence of recombinant gene was correct. Furthermore, each of the final rec-MVA was titrated by $TCID_{50}$ assay to know the exact amount of infectious virus particle.

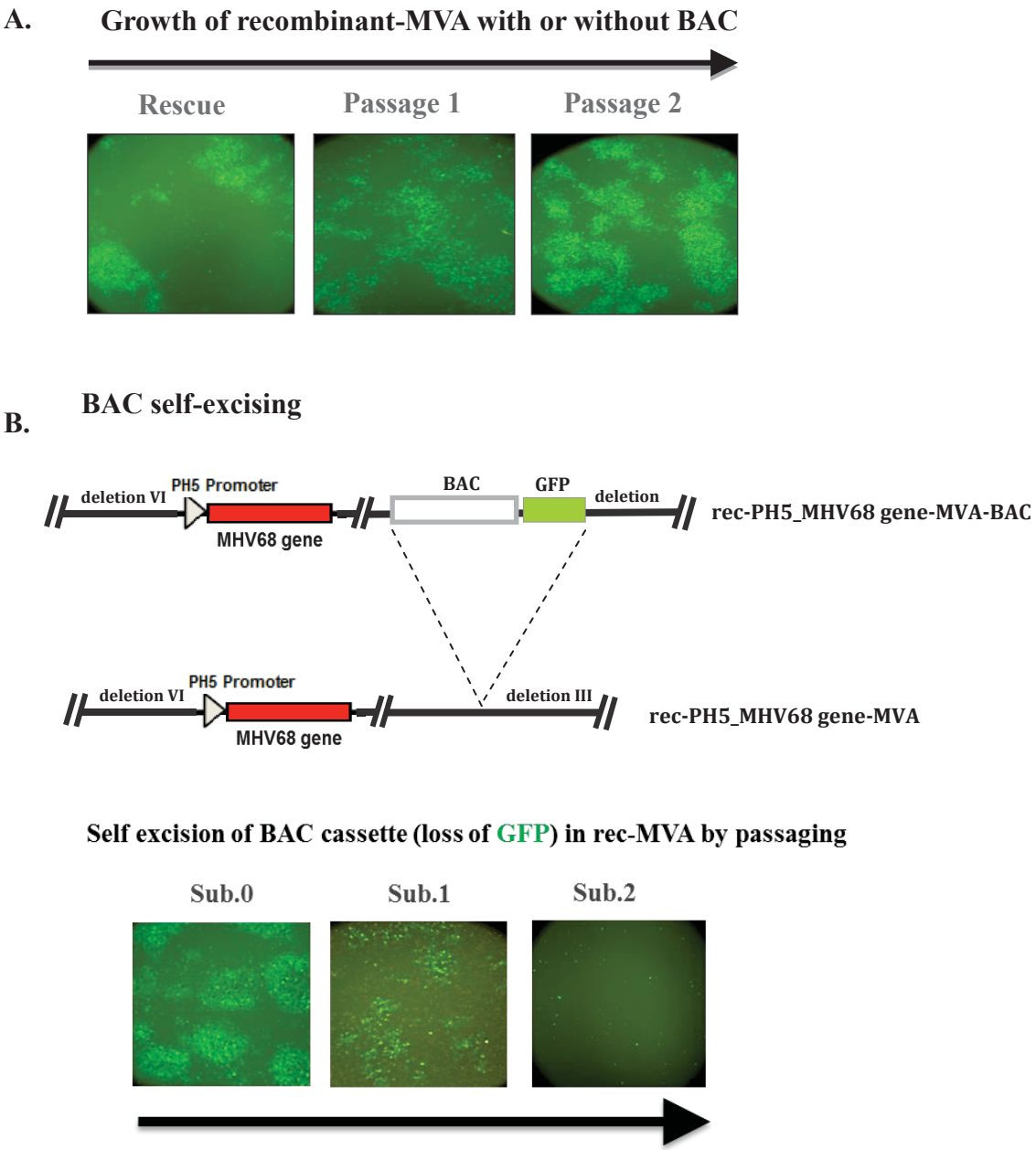
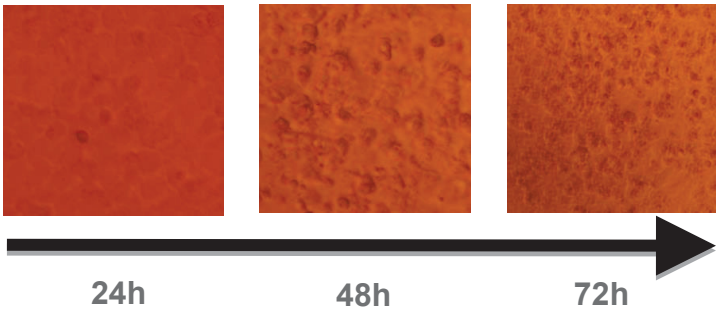


Fig.4.5 BAC rescue and amplification of rec-MVA

A. Each rec-MVA encoding one of the selected MHV68 genes was rescued from BAC as described in methods (3.2.1). The BAC rescue was done in DF-1 cells, which were transfected by using 5µg of rec-MVA-BAC with 6µl of turbofect transfection reagent. After 3h of incubation, DF-1 cells were infected with helper virus (rabbit fibroma virus) RFV (MOI 0.1). Rescued rec-MVA formed clear green fluorescent plaques after 72h and became more prominent in further passages. **B.** Schematic map of self excising BAC and the BAC miniF-GFP cassette in deletion III. The BAC cassette was subsequently lost from the viral genome following 3 rounds of sub-passaging in DF-1 cells. The resulting rec-MVA lost the GFP fluorescence with high efficacy.

A. Selection of GFP-/ CPE+ viral plaques



B. Viral clones without GFP marker gene **C. Viral clones without RFV**

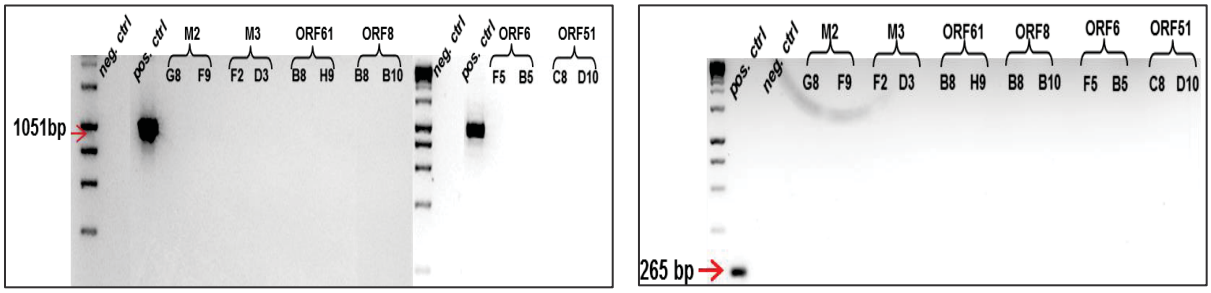


Fig.4.6 Selection of rec-MVA, Aim for viral plaques without GFP and RFV contaminants

A. Limiting dilution assay. Each rec-MVA was diluted from 10^{-1} to 10^{-12} and added to a monolayer of DF-1 cells in 96 well plates. After 6 days of infection, the wells were monitored and the clones selected having cytopathic effect, but no GFP fluorescence. **B and C.** Selected BAC-excised rec-MVA viruses, MVA-BAC virus (having GFP gene) and RFV were used to infect the DF-1 cells for 72h. Total viral DNA was extracted using phenol-chloroform method. **B.** GFP-specific primers were used for PCR analysis. The GFP fragment of 1051bp from isolated MVA-BAC viral DNA was used as a positive control and wild type MVA-F6 viral DNA was used as a negative control. **C.** RFV-specific primers were used for PCR analysis. The RFV gene fragment of 265bp contained in the isolated RFV DNA was used as a positive control and MVA-F6 viral DNA used as a negative control. Gel electrophoresis was done by using 1% agarose gel.

4.3. *In vitro* characterization of recombinant MVA vaccines

4.3.1. Western blot analysis of recombinant protein synthesis

To analyze the recombinant protein synthesis in rec-MVA, HeLa cells were infected with each of the recombinant viruses. After 24h of infection, cell lysates were prepared for western blot analysis. Antibodies specific for the recombinant MHV68 antigens were not available so far. Therfore, the recombinant protein production was analysed by utilizing an HA-specific

antibody, since HA was contained as a tag present at the C-terminal end of all recombinant MHV68 proteins. The results indicated that each of the rec-MVA was expressing the recombinant protein efficiently and in the expected size (Fig.4.7).

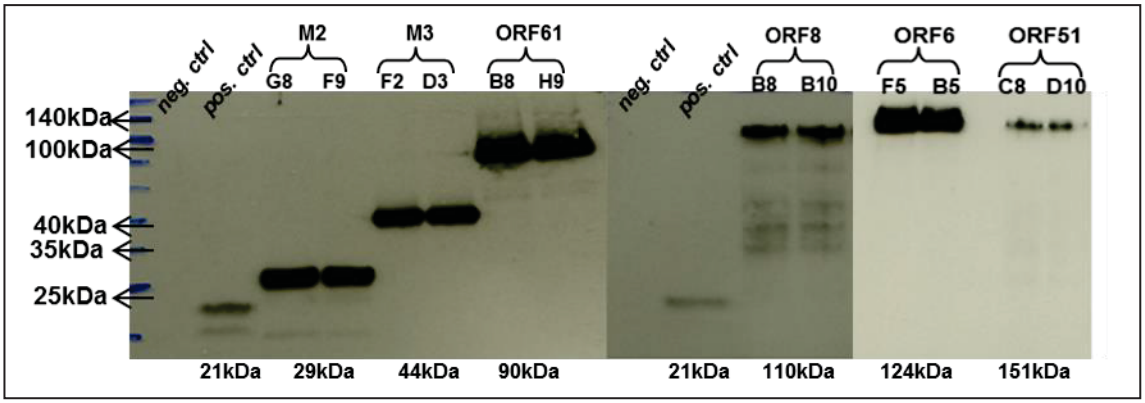


Fig.4.7 Synthesis of recombinant MHV68 proteins upon infection with rec-MVA

Western blot analysis of Hela cells infected with rec-MVA (MOI-10) encoding MHV68 antigens with HA-tag and wild type MVA (as negative control). For positive control, the cells were first transfected by using pIRES-EGFP plasmid having HA tagged US6 gene of HCMV and then infected by MVA wt. After 24h of infection, cell lysates were prepared and subjected to SDS-PAGE. Immunobloting was performed followed by detection using anti HA monoclonal rabbit antibody.

4.3.2. Low multiplicity growth kinetics of rec-MVA in CEF cells

Previously, it has been reported that some mutant rec-MVA, which were unable to express foreign antigens due to genomic instability, showed a suppressive effect on viral replication (Wyatt, Earl *et al*. 2009). To exclude this concern, we infected CEFs with each rec-MVA respectively and analysed their ability to replicate compared to wild-type MVA. Infection was done by using low MOI of 0.01 to perform multi step growth kinetics. The amount of progeny virus was determined at 0, 24, 48 and 72h of post infection. The viral titers at each time point were measured by titration ($TCID_{50}$ assay). All rec-MVA showed efficient growth properties compared to the growth of wild-type MVA (Fig.4.8).

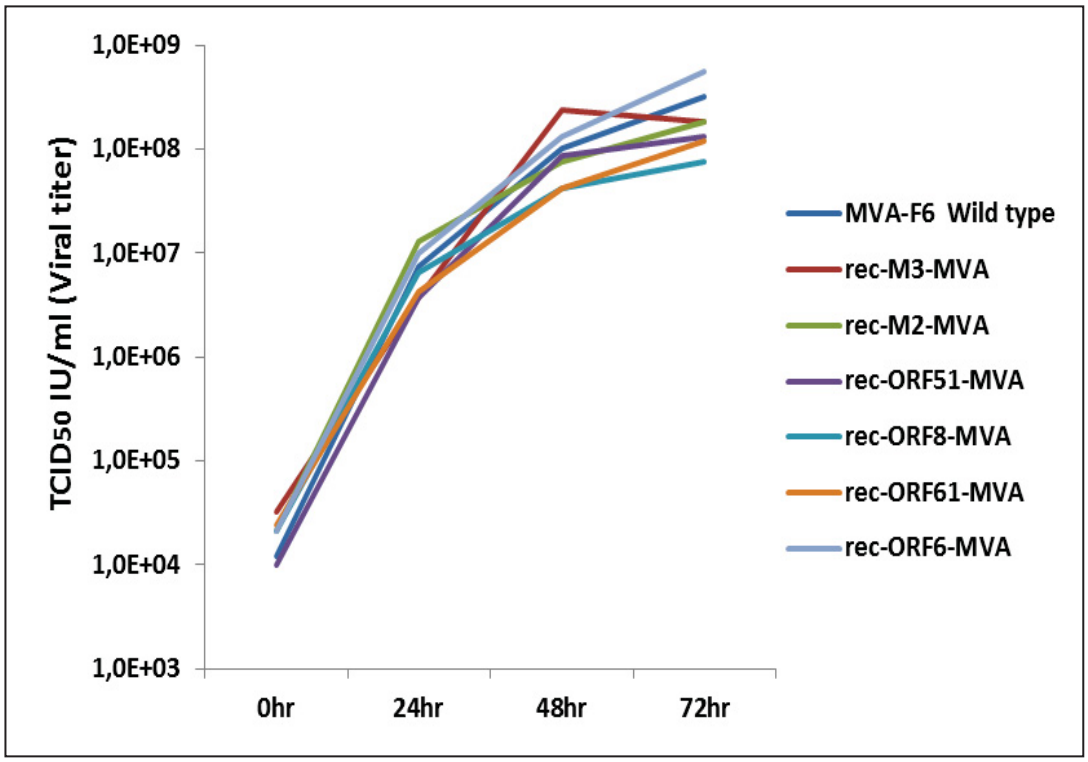


Fig.4.8 Growth kinetics of rec-MVA

All rec-MVA showed exponential viral growth as compared to wild-type MVA. By using low MOI 0.01, CEF cells were infected with each of the indicated rec-MVA respectively and harvested at indicated time points. Growth curves were determined by measuring rec-MVA titers ($TCID_{50}$) at indicated time points.

4.4. Generation of MHV68 antigen-specific CD8+ and CD4+ T cell lines

MVA is known to carry and express recombinant antigens which are able to elicit immune reponses efficiently. For this reason, we have generated T cell lines to characterize each rec-MVA for their ability to stimulate a MHV68 antigen-specific T cell response *in vitro*. Each rec-MVA contains MHV68 antigens which contain MHC class-I/II-restricted epitopes, already published in literature (Table.4.1). We have generated both, CD8+ and CD4+ T cell lines, specific for MHV68 antigens, MVA antigens as well as unrelated antigens. The T cell lines D13 or B5 (MVA-specific antigens) and βgal or Ova (unrelated antigens) were used as a read out system for determining the strenght and quality of exo- or endogenous antigen presentation.

For the generation of the CD8+ T cell lines, C57BL/6 mice (8 to 10 weeks) were vaccinated with rec-MVA via the intraperitoneal (i.p.) application route. Mice were sacrificed at 8 days of post infection (dpi) and spleens were harvested to prepare splenocytes. As antigen presenting

cells, splenocytes from naive C57BL/6 mice were incubated with LPS for 3 days to generate MHC class-I/II high stimulator cells (LPS blasts). Thereafter, LPS blasts were incubated with MHV68- or MVA-specific MHC class-I restricted peptides. For the so-called primary culture, LPS blasts (loaded with respective MHC class-I restricted peptides) were added to splenocytes derived from rec-MVA vaccinated mice. For maintenance of CD8+ T cells, the cultures were restimulated at every 7th day by using peptide-pulsed EL-4 cells as antigen presenting cells, splenocytes from naive mice as feeder cells and T cell growth factor (TCGF) (see method 3.4.3) (Fig.4.9). In case of CD4+ T cell lines, C57BL/6 mice were i.p. vaccinated with rec-MVA and received a second boost vaccination at day 5 corresponding to a short term prime boost regimen (Kastenmuller *et al* 2007). Mice were sacrificed at day 6 post boost vaccination and spleens were harvested to prepare splenocytes. For primary culture, splenocytes from vaccinated mice were co-cultured with LPS blasts (loaded with respective MHC class-II restricted peptides) in order to expand antigen-specific CD4+ T cells (Fig.4.9). For the maintenance of CD4+ T cell lines, the cultures were restimulated at every 14th day by using peptide-pulsed splenocytes as antigen presenting and feeder cells, including TCGF.

Table 4.1 MHV68 antigens, corresponding T cell epitopes and MHC restriction

Recombinant antigens	Expressing epitopes	MHC class	T cells	References
ORF6	**ORF6$_{487}$**	**D^b**	**CD8+**	(Freeman, Burkum *et al*.2012)
	ORF6$_{593}$	**I-A^b**	**CD4+**	(Freeman, Burkum *et al*. 2014)
ORF61	**ORF61$_{524}$**	**K^b**	**CD8+**	(Freeman, Burkum *et al*.2012)
	ORF61$_{343}$	**I-A^b**	**CD4+**	(Freeman, Burkum *et al*. 2014)
	ORF61$_{691}$	**I-A^b**	**CD4+**	(Freeman, Burkum *et al*. 2014)
ORF51	**ORF51$_{111}$**	**I-A^b**	**CD4+**	(Freeman, Burkum *et al*. 2014)
M2	**M2$_{124}$**	**I-A^b**	**CD4+**	(Freeman, Burkum *et al*. 2014)

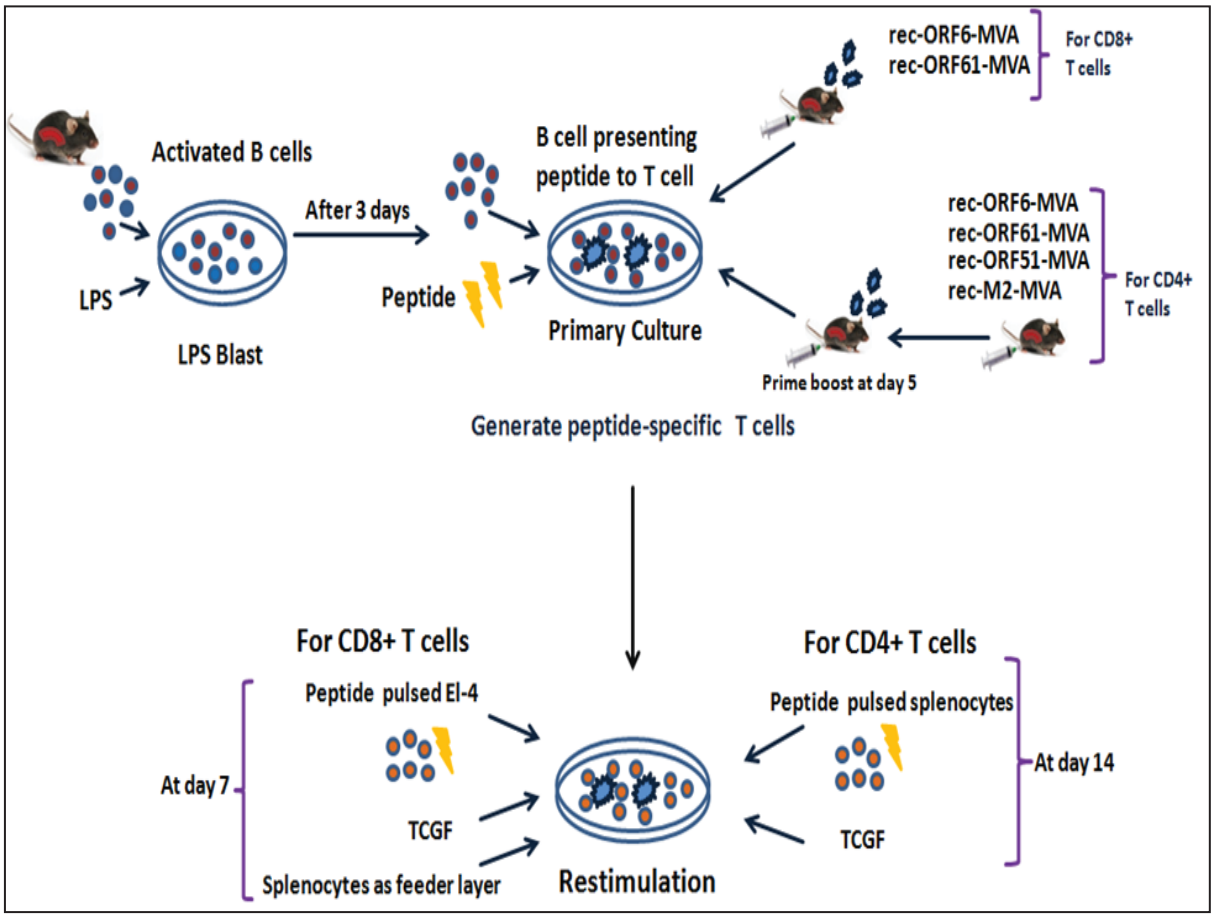


Fig.4.9 Generation of antigen-specific CD8+ and CD4+ T cell lines

Schematic map showing the strategy to generate the T cell lines. Splenocytes from naive C57BL/6 mice were treated with LPS for 3 days, harvested after 3 days (LPS blasts mainly consisting of B lymphoblastoid cells) and incubated with specific MHC class-I- (for CD8+ T cells) or MHC class-II-restricted peptides (for CD4+ T cells). For the primary culture, peptide-pulsed LPS blasts were added to splenocytes derived from rec-MVA-vaccinated mice. For restimulation, primary culture CD8+ T cells, peptide-pulsed EL-4 cells, naive splenocytes (as feeder cells) and TCGF were cocultivated at day 7. For primary culture CD4+ T cells, peptide-pulsed splenocytes and TCGF were added for restimulation at day 14.

4.5. Exogenous and endogenous antigen recognition analysis by using epitope specific CD8+ T cell lines

CD8+ T cell lines specific for the MHV68 ORF6$_{487}$ and ORF61$_{524}$ antigens were successfully generated. D13$_{118}$ as MVA-specific and β-gal$_{499}$ as unrelated antigens were used to generate control T cell lines. After 4 to 6 weeks of periodical restimulation, CD8+ T cells were tested for their ability to get activated through recognition of exogenous (peptide-pulsed) antigens,

presented by DC2.4 cells, a DC-like cell line. For activation, we measured IFNγ and TNFα cytokine release by intracellular cytokine staining (ICS). We used MHC class-I restricted peptides at a concentration of 10^{-8} (1ng/ml) to 10^{-11} M (0.001ng/ml). Each CTL line showed a concentration-dependent activation upon high to low amounts of peptide used for stimulation. Irrelevant peptides were used as negative control (ctrl peptide) and mock received no peptide. The results indicated that both $ORF6_{487}$ and $ORF61_{524}$ antigen-specific CD8+ T cells were producing high levels of IFNγ (Fig.4.10A) and TNFα (Fig.4.10B). The irrelevant peptides and mock control showed no activation indicating absence of unspecific background reactivity.

A. **B.**

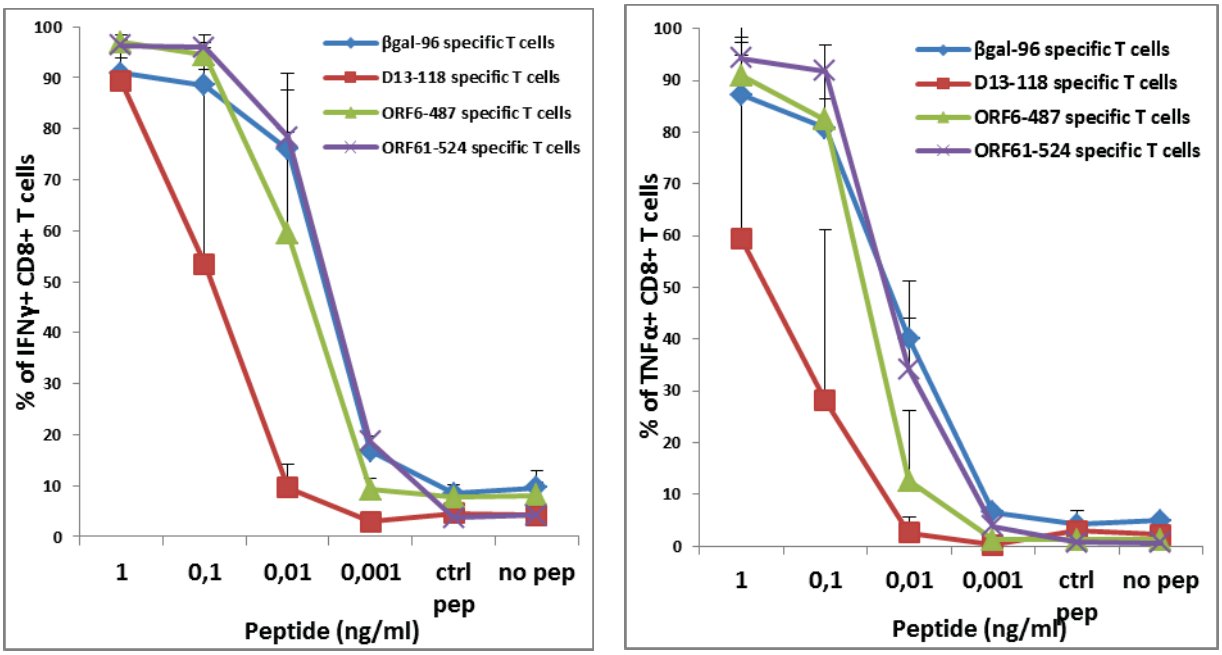


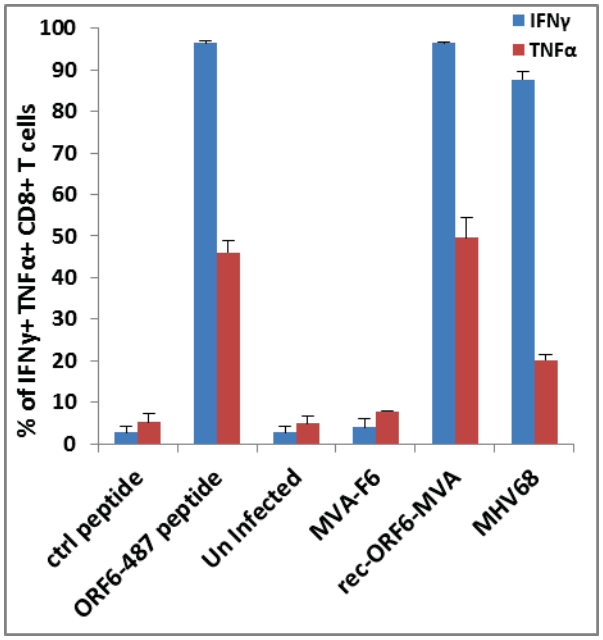
Fig.4.10 Activation of CD8+ T cells upon exogenous antigen stimulation

DC2.4 cells were pulsed at different concentrations of peptide ranging from 10^{-8} M (1ng/ml) to 10^{-11} M (0.001ng/ml). Indicated antigen-specific CD8+ T cells were added to DC2.4 cells pulsed with either the cognate peptide at declining concentration, the control peptide at highest concentration or no peptide and incubated for 4h. **A.** relative numbers of IFNγ and **B.** TNFα producing CD8+ T cells as quantified by ICS and FACS. Data are mean ± SEM (n=2, pooled from two experiments).

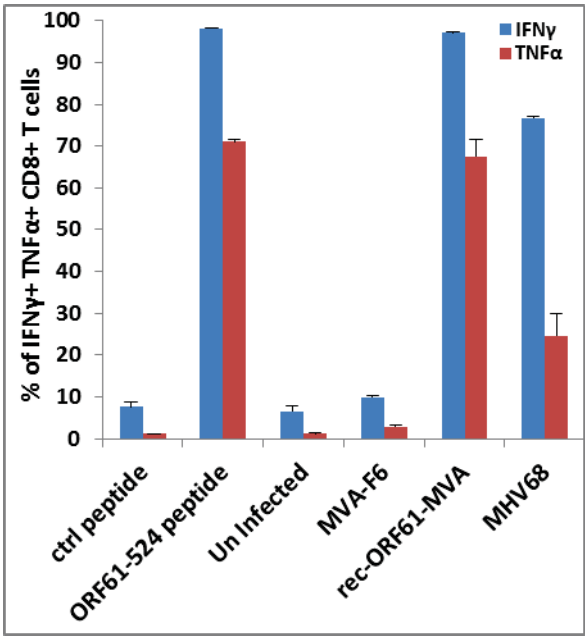
We further investigated the endogenous MHC class-I antigen presentation after rec-MVA infection by using an *in vitro* culture assay (method 3.4.5.2). The rec-MVA-infected BMDCs used as a target cells (presenting endogenous antigen) were co-cultivated with respective antigen-specific CD8+ T cells. After 4h of incubation, the activation of $ORF6_{487}$ or

$ORF61_{524}$ epitope-specific CD8+ T cells was measured by IFNγ and TNFα production through ICS. The results indicate that rec-ORF6-MVA, rec-ORF61-MVA and also MHV68 virus infected BMDCs allow for endogenous antigen presentation and stimulation of $ORF6_{487}$ (Fig.4.11A) and $ORF61_{524}$ (Fig.4.11B) specific CD8+ T cells at a magnitude, that was comparable to the stimulation of peptide-pulsed BMDCs, used as a positive control. Irrelevant peptide (ctrl peptide)-pulsed BMDCs, uninfected or wildtype MVA-F6-infected BMDCs were used as negative controls showing minimal unspecific background activity. The results for experimental control βgal_{96} (Fig.4.11C) and $D13_{118}$ (Fig.4.11D) antigen specific CD8+ T cells indicated the efficient infection of BMDCs and antigen presentation by rec-MVA and wild type MVA-F6, with no background reactivation.

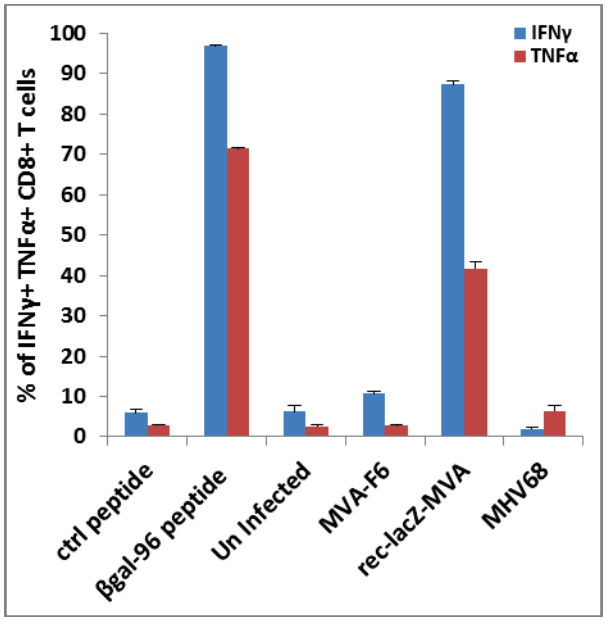
A. ORF6$_{487}$ specific CD8+ T cells



B. ORF61$_{524}$ specific CD8+ T cells



C. βgal$_{96}$ specific CD8+ T cells



D. D13$_{118}$ specific CD8+ T cells

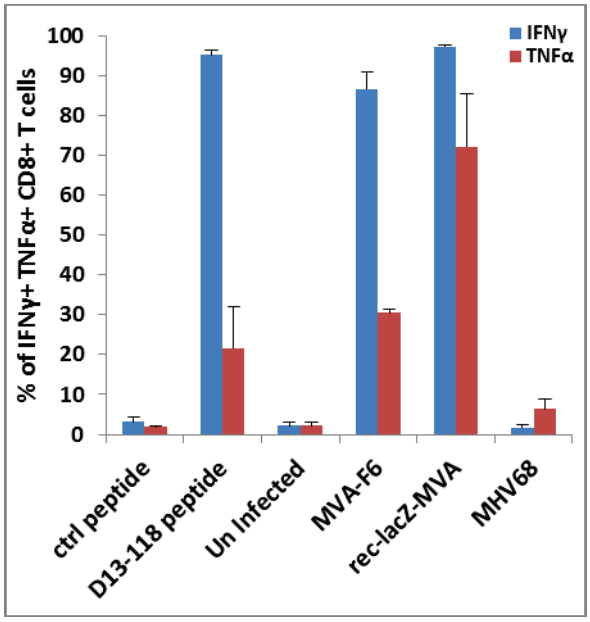


Fig.4.11 Activation of CD8+ T cells upon endogenous antigen stimulation

BMDCs were infected with MVA-F6, rec-ORF6-MVA or rec-ORF61-MVA at MOI 5 for 15h and MHV68 virus at MOI 10 for 48h, then co-cultured with CD8+ T cells specific for: **A.** ORF6$_{487}$, **B.** ORF61$_{524}$, **C.** βgal$_{96}$ and **D.** D13$_{118}$ for 4h. Peptides at 10^{-8}M (1ng/ml) were used to pulse BMDCs. IFNγ and TNFα production was determined by ICS. Data are mean ±SEM (n=2, pooled from two experiments).

4.6. Exogenous and endogenous antigen recognition analysis by using epitope specific CD4+ T cell lines

Similarly, we have generated MHC class-II restricted CD4+ T cell lines specific for MHV68 $ORF6_{593}$, $ORF61_{343}$, $ORF61_{691}$, $ORF51_{111}$ and $M2_{124}$ antigens. Here, $B5_{46}$ and Ova_{265} served as epitopes for MVA- and irrelevant antigen-specific control T cell lines. CD4+ T cells were tested for their ability to recognize and get activated by exogenous (peptide-pulsed) antigen presenting cells (BMDCs) as measured by IFNγ production followed by ICS. For pulsing, we used 10^{-5}M (1000 ng/ml) to 10^{-8}M (1 ng/ml) peptide dilutions. Each antigen-specific CD4+ T cell line showed a concentration-dependent activation upon high to low amounts of peptide used for stimulation. Irrelevant peptides were used as negative controls and mock received no peptide. The results indicate that high numbers of Ova_{265}, $ORF6_{593}$, $ORF61_{343}$, $ORF61_{691}$ and $B5_{46}$ specific CD4+ T cells were producing IFNγ (<90%) at highest peptide concentration. Whereas, less $ORF51_{111}$ and $M2_{124}$ specific CD4+ T cells were producing IFNγ (<60%), but comparatively had less background reactivity. However, CD4+ T cells with other specificities showed unspecific background activation (10-40%) against irrelevant peptide and mock controls. Nevertheless, despite partly higher unspecific backround, each CD4+ T cell line demonstrated a clear specific IFNγ production against its cognate epitope (Fig.4.12).

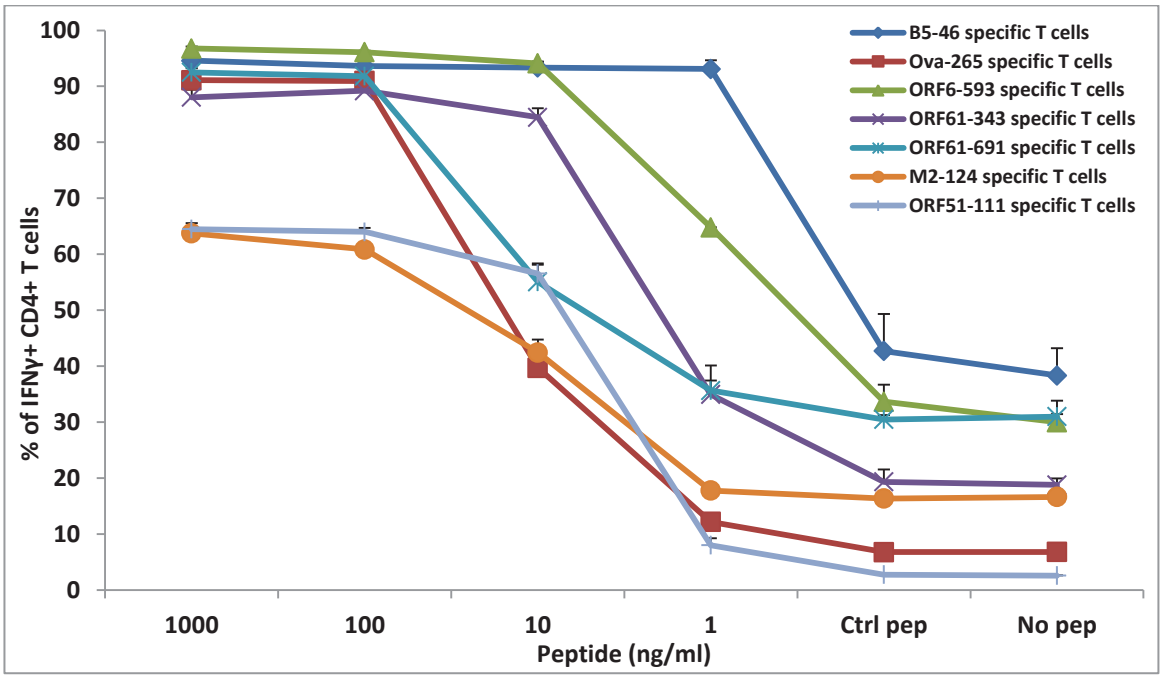


Fig.4.12 Activation of CD4+ T cells upon exogenous antigen stimulation

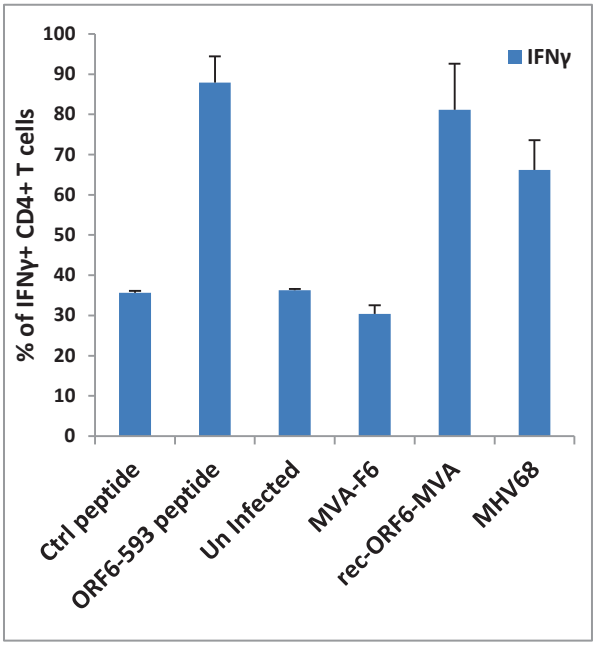
BMDCs were pulsed with different concentrations of peptide 10^{-5}M (1000 ng/ml) to 10^{-8}M (1 ng/ml). Antigens-specific CD4+ T cells were added to peptide-pulsed BMDCs and incubated for 4h. The numbers of IFNγ producing CD4+ T cells were determined by ICS and analyzed by FACS. Data are mean ± SEM (n=2, pooled from two experiments).

We further investigated MHC class-II-restricted endogenous antigen presentation after infection of rec-MVA by using the *in vitro* culture assay. Therefore, after 20 weeks of periodical restimulation, CD4+ T cell lines were used to determine the strength and quality of antigen presentation by infected antigen presenting cells (APCs) like BMDCs. rec-MVA-infected BMDCs were used as target cells (presenting endogenous antigens) and co-cultivated with respective antigen-specific CD4+ T cells. After 15h of incubation, the activation of epitope-specific CD4+ T cells as compared to those stimulated with peptide-pulsed BMDCs (positive control) was analyzed by measuring IFNγ production by ICS followed by FACS. The results indicate an efficient specific antigen presentation by BMDCs upon infection of rec-ORF6-MVA, rec-ORF51-MVA and also upon infection with wild type MHV68 virus. $ORF6_{593}$ (Fig.4.13A) and $ORF51_{111}$ (Fig.4.13B) specific IFNγ+CD4+ T cells were detectable at a magnitude that was comparable to the one obtained by stimulation with peptide-pulsed BMDCs. In case of rec-ORF61-MVA or rec-M2-MVA infected BMDCs, there was very low activation of respective $ORF61_{343}$ (Fig.4.13C), $ORF61_{691}$ (Fig.4.13D) and $M2_{124}$ (Fig.4.13E)

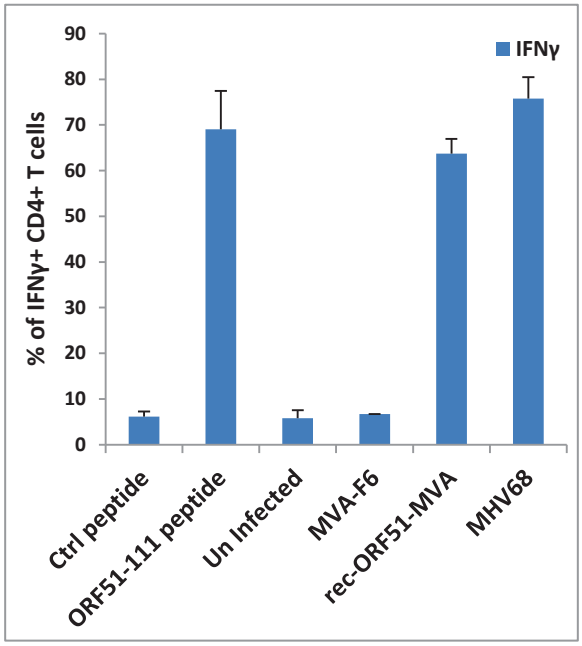
specific CD4+ T cells. In addition, BMDCs infected with MHV68 virus were unable to activate these three T cell lines.

Irrelevant peptide (ctrl peptide)-pulsed BMDCs, uninfected or wildtype MVA-F6-infected BMDCs used as negative controls stimulated unspecific background activity in $B5_{46}$, $ORF6_{593}$, $ORF61_{343}$, $ORF61_{691}$ and $M2_{124}$ specific CD4+ T cells. Both experimental controls, $B5_{46}$ (Fig.4.13F) and Ova_{265} (Fig.4.13G) antigen specific CD4+ T cells, indicated efficient infection of BMDCs as well as antigen presentation by rec-MVA (Ova and B5) and wild type MVA-F6 (B5 only) with compromised unspecific background reactivation.

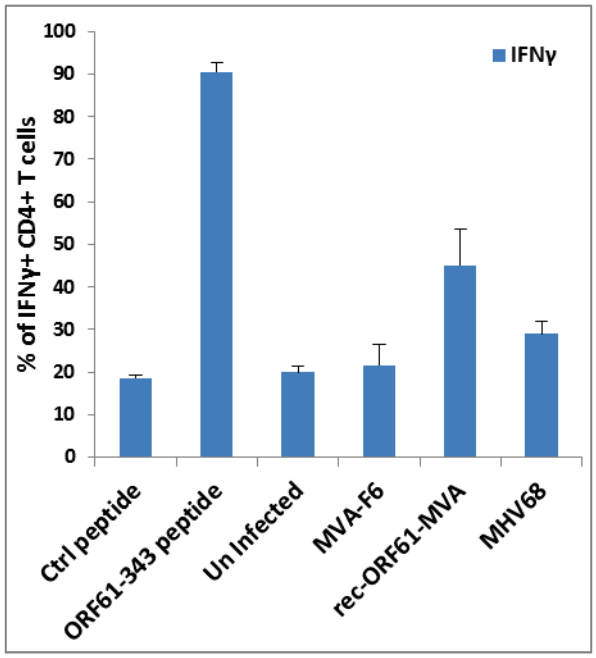
A. $ORF6_{593}$ specific CD4+ T cells



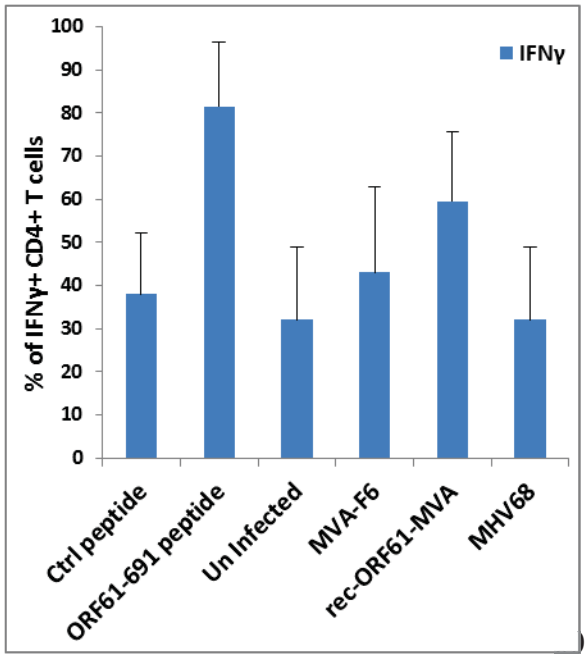
B. $ORF51_{111}$ specific CD4+ T cells



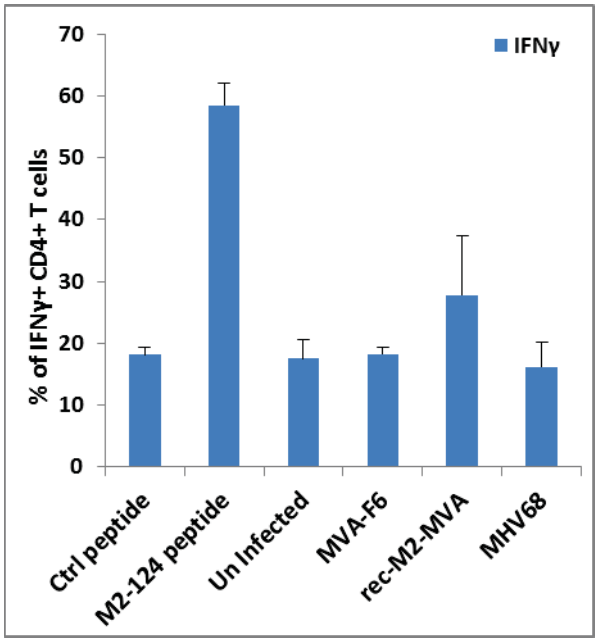
C. $ORF61_{343}$ specific CD4+ T cells



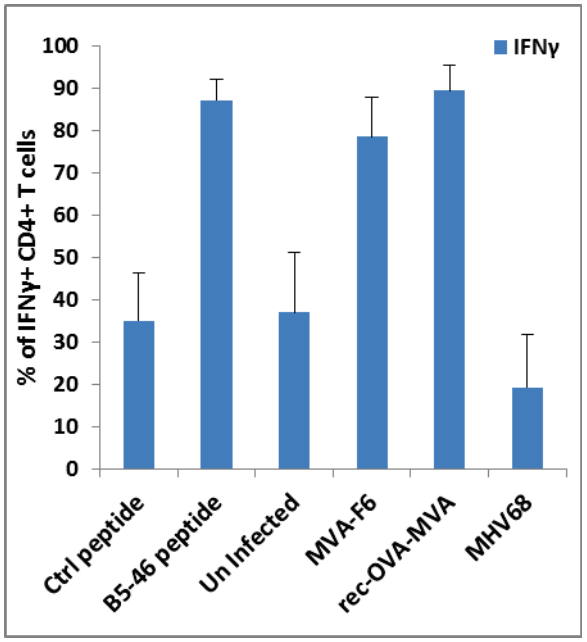
D. $ORF61_{691}$ specific CD4+ T cells



E. $M2_{124}$ specific CD4+ T cells



F. $B5_{46}$ specific CD4+ T cells



G. Ova_{256} specific CD4+ T cells

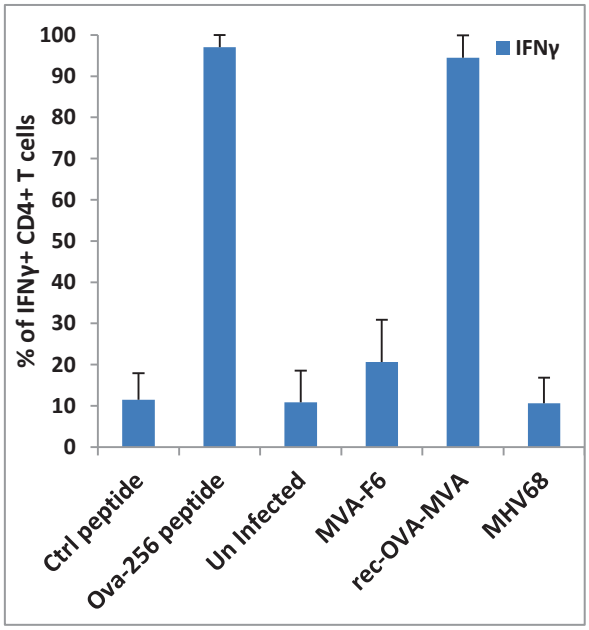


Fig.4.13 Activation of CD4+ T cells via endogenous antigen presentation

BMDCs were infected with MVA-F6, rec-ORF6-MVA or rec-ORF61-MVA, rec-ORF51-MVA and rec-M2-MVA at MOI 5 for 15h and MHV68 virus at MOI 10 for 48h and then co-cultured with CD4+ T cells specific for: **A.** $B5_{46}$, **B.** Ova_{256}, **C.** $ORF6_{593}$, **D.** $ORF51_{111}$, **E.** $ORF61_{343}$, **F.** $ORF61_{691}$ and **G.** $M2_{124}$ for 15h. Peptides were used at 10^{-5}M (1000ng/ml) to pulse BMDCs. Irrelevent peptide were used as specificity control, and uninfected cells as infection control. IFNγ production was determined by ICS. Data are mean ±SEM (n=2, pooled from two experiments).

4.7. *Ex vivo* CD8+ T cell response analysis of recombinant MVA vaccines

The MHV68 ORF6 antigen has an early and late gene expression profile, while ORF61 shows a late and late latent gene expression. Both of these antigens are known to induce strong immune responses after infection of MHV68. Therefore, we generated recombinant MVA vaccines carrying each of the MHV68 ORF6/ ORF61 antigens thereby covering both, the early and late phase of the virus infection cycle. To monitor the CD8+ T cell specific response against MHV68 antigens upon vacination with rec-MVA, we used specific MHC class-I restricted epitopes encoded by ORF6 and ORF61 MHV68 antigens. For experimental setup, C57BL/6 mice were vaccinated through intraperitoneal application of rec-MVA (either rec-ORF6-MVA or rec-ORF61-MVA) or MVA-F6 as vaccination control and PBS as a negative control. For *ex vivo* analysis, mice were sacrificed at specific time points to take out spleens. Splenic T cells were stimulated with peptide epitopes βgal_{499} (as a non specific anitgen control), $B8_{20}$ (as a MVA specific antigen control) or $ORF6_{487}$/$ORF61_{524}$ (recombinant antigens). Production of IFNγ, TNFα and IL2 by CD8+ T cells was determined by ICS followed by FACS analysis.

For the acute phase of infection, mice were sacrificed at 8 dpi (Fig.4.14A) to prepare splenocytes for *ex vivo* analysis. The results indicated an antigen-specific production of cytokines by CD8+CD62L- (activated) T cells (Fig.4.14B). Interestingly, after rec-ORF6-MVA vaccination, we observed very strong $ORF6_{487}$-specific CD8+CD62L- T cell production of IFNγ (2%) and TNFα (Fig.4.14C). Likewise, $ORF61_{524}$-specific CD8+CD62L- T cells were also effectively producing IFNγ (1%) (Fig.4.14D). The B8 is the most dominant MHC class-I-restricted antigen of MVA, leading to strong production of cytokines, demonstrating the efficacy of rec-MVA infection, comparable to wild type MVA-F6 infection.

For the memory phase of infection, mice were sacrificed at 35 dpi and spleens were harvested for *ex vivo* analysis (Fig.4.14A). We observed that during memory phase, there was a 10-fold reduced IFNγ production (0.2%) for $ORF6_{487}$ specific CD8+ T cells (Fig.4.15C), similarly for $ORF61_{524}$ specific CD8+ T cells producing 0.1% IFNγ (Fig.4.15D), as compared to the acute phase response. Interestingly, B8-specific CD8+ T cells also showed a 10-fold reduction in cytokine production. However, the T cell response has been reduced but still showed a concised population of antigen specific CD8+ T cells. These results have given a clear idea that increasing the priming of ORF6 and ORF61 specific CD8+ T cells could increase the expansion of T cell response during memory phase.

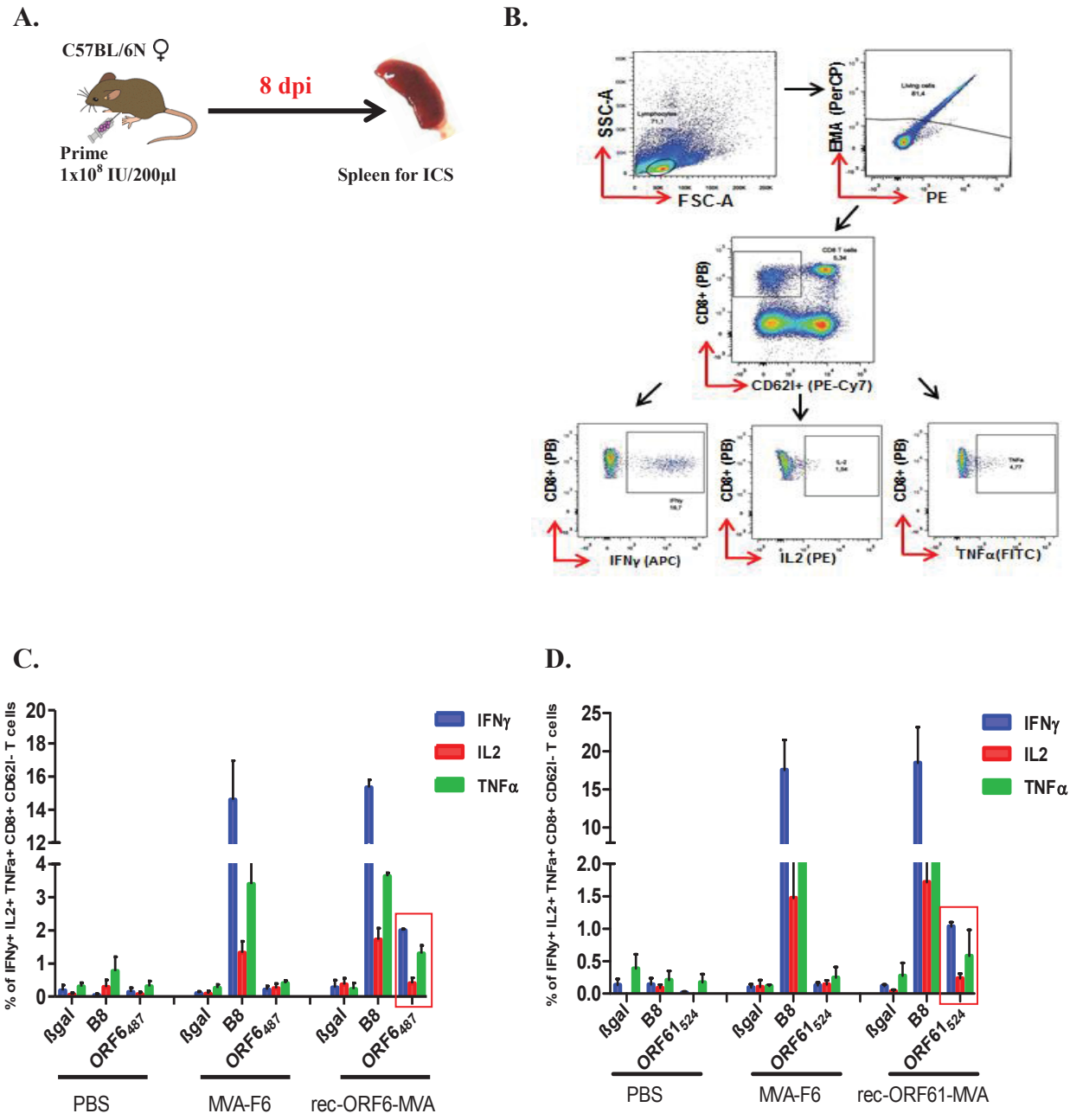


Fig.4.14 CD8+ T cell immune responses induced by rec-MVA vaccines in the acute phase (8 dpi). A. C57BL/6 mice (6-8 weeks old) were immunized with 1x10e^8 IU/200µl rec-MVA, MVA-F6 or PBS (200µl) control by intraperitoneal application. At 8 dpi, mice were sacrificed, spleens were harvested, and splenocytes were prepared for *ex vivo* immune response analysis, carried out by ICS followed by FACS analysis. **B.** FACS gating strategy **C.** Detection of ORF6$_{487}$ specific- **D.** Detection of ORF61$_{524}$ specific cytokine producing CD8+CD62L- T cells. βgal$_{499}$ was used as unrelated peptide and B8$_{20}$ used as MVA-specific control peptide. Data shown are mean ±SEM of n=6 pooled from 3 experiments in both C and D.

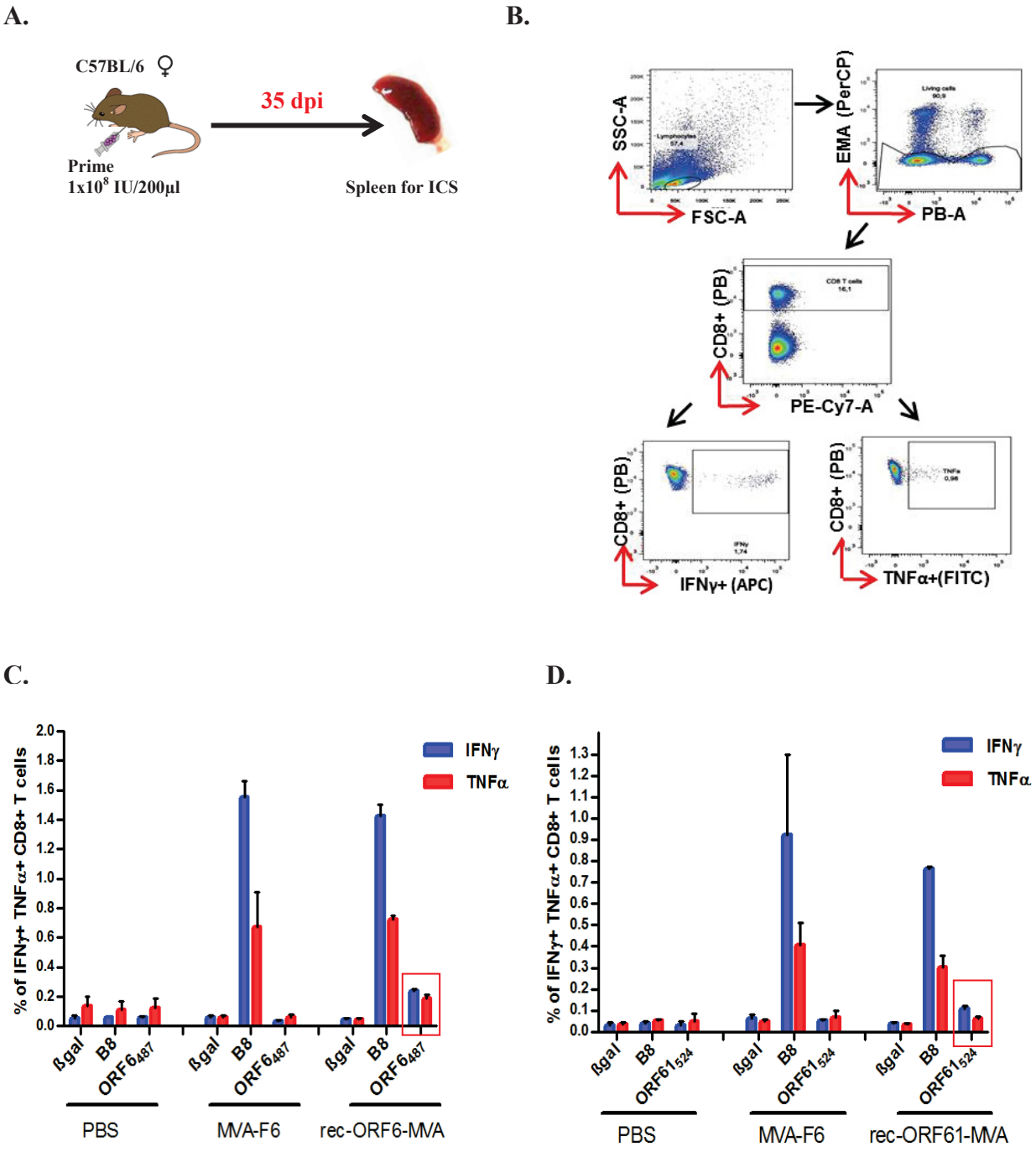


Fig.4.15 Immune response induced by rec-MVA vaccines in the memory phase of infection (35 dpi). A. C57BL/6 mice (6-8 weeks old) were immunized with $1x10^8$ IU/200µl rec-MVA, MVA-F6 or PBS (200µl) control by intraperitoneal application. At 35 dpi, mice were sacrificed, spleens were harvested, and splenocytes were prepared for *ex vivo* immune response analysis, carried out by ICS followed by FACS analysis. **B.** FACS gating strategy **C.** Detection of $ORF6_{487}$ specific- **D.** Detection of $ORF61_{524}$ specific cytokine producing CD8+ T cells. βgal_{499} was used as unrelated peptide and $B8_{20}$ used as MVA-specific control peptide. Data shown are mean ±SEM of n=9 pooled from 2 experiments in both C and D.

Previously, it has been published that rec-MVA vaccines are able to elicit strong T cell-mediated secondary immune responses upon boost vaccination (Fournillier, Frelin *et al*. 2013). Therefore, we also established an experiment to analyse the expansion of T cell responses upon prime/boost vaccination of rec-MVA having MHV68 antigens. We used C57BL/6 mice for vaccination through intraperitoneal application using MVA-F6, rec-ORF6-MVA or rec-ORF61-MVA viruses. After priming, mice received a second boost vaccination at day 5 corresponding to a short term prime boost regimen (Kastenmuller, Gasteiger *et al*. 2007). At 6 days after boost vaccination, the mice were sacrificed to harvest the spleens for *ex vivo* analysis. We followed the same procedure as previously described. After prime/boost vaccination with rec-ORF6-MVA, we observed higher levels of activated CD8+CD62L- T cells producing IFNγ (6%) specific to the $ORF6_{487}$ epitope. In case of rec-ORF61-MVA, $ORF61_{524}$ epitope-specific CD8+CD62L- T cells producing IFNγ (1.5%), showed only a slight increase in the secondary response (Fig.4.16B), compared to only prime vaccination in Fig.4.14D. These results indicated that rec-ORF6-MVA, as well as rec-ORF61-MVA has a strong potential to elicit memory responses which can be reactivated and expanded upon second boost vaccinantion.

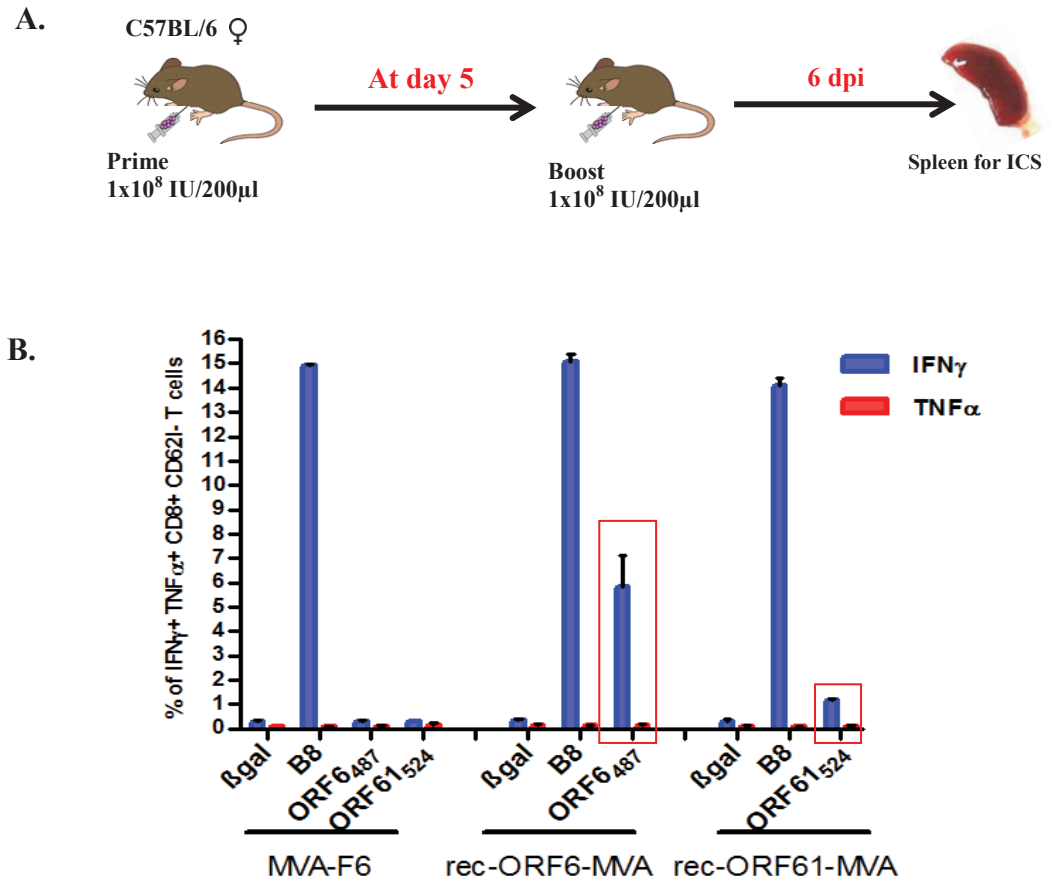


Fig.4.16 CD8+ T cell immune responses induced by rec-MVA vaccines after prime/boost vaccination. A. C57BL/6 mice (6-8 weeks old) were immunized with $1x10^8$ IU/200µl rec-MVA or MVA-F6 by intraperitoneal application in a prime /boost (at day 5 post prime) regimen. After 6 days of boost vaccination, mice were sacrificed, harvested spleens to prepare splenocytes for *ex vivo* immune response analysis, carried out by ICS followed by FACS. **B.** Detection of $ORF6_{487}$ specific and $ORF61_{524}$ specific CD8+CD62L- T cells producing cytokines. βgal_{499} was used as unrelated peptide and $B8_{20}$ used as MVA-specific control peptide. FACS strategy is explained in (Fig.4.14B). Data shown are mean ±SEM of n=15 pooled from 2 experiments.

4.8. *Ex vivo* CD4+ T cell response analysis of recombinant MVA vaccines

Multiple MHV68 antigens have been identified in context of CD4+ T cell responses. However, the detailed functional characteristics of antiviral CD4+ T cell responses have not been elucidated. Including MHV68 ORF6 and ORF61, we have utilized additional antigens such as MHV68 M2 (early/late expressed) and ORF51 (late expressed antigen) to generate rec-MVA. By utilizing MHC class-II restricted peptide epitopes, we have analyzed CD4+ T cell responses upon vaccination with rec-MVA. For experimental setup, C57BL/6 mice were vaccinated through intraperitoneal application using rec-MVA (either for M2, ORF51, ORF6 or ORF61 antigen) or MVA-F6 or PBS as a control. For *ex vivo* analysis, mice were sacrificed at specific time points to take out spleens. Splenic T cells were stimulated with Ova_{265} specific peptide (as a non specific antigen control), $B5_{46}$ (as a MVA specific antigen control), $M2_{124}$, $ORF51_{111}$, $ORF6_{593,}$ $ORF61_{343}$ and $ORF61_{691}$ (specific to recombinant antigens). Production of IFNγ and IL2 by CD4+ T cells were determined by ICS followed by FACS analysis.

For acute phase of infection, mice were sacrificed at 8 dpi to prepare splenocytes for *ex vivo* analysis (Fig.4.17A). Interestingly, the results indicated excellent antigen specific CD4+CD62L- T cell responses, with high levels of IFNγ production for each of the MHC class-II restricted epitopes (Fig.4.17B-E), in response to their respective rec-MVA vaccination, which have also elicited a considerable production of IL2. B5 is the dominant MHC class-II restricted antigen of MVA. Efficient production of cytokines in response to dominant $B5_{46}$, demonstrated the efficacy of rec-MVA infection compared to the infection with wild type MVA-F6 for each experimental setup.

Regarding memory phase infection, mice were sacrificed at 35 dpi to harvest spleens for *ex vivo* analysis (Fig.4.18A). In response to each rec-MVA, we observed around 10-fold reduced IFNγ production by antigen-specific CD4+ T cells. The dotted line shows cutoff for IFNγ and IL-2 producing cells, because of the higher unspecific background activity in response to each antigen. Interestingly, $ORF6_{343}$ and $ORF51_{111}$ specific T cells showed production of IFNγ above this cutoff line upon infection with the respective rec-MVA (Fig.4.18B and C), which was not the case for ORF61 and M2 antigen. Overall, the data suggest the four antigens ORF6, ORF61, ORF51 and M2 possess some activity to elicit antigen-specific CD4+ T cell population during memory phase.

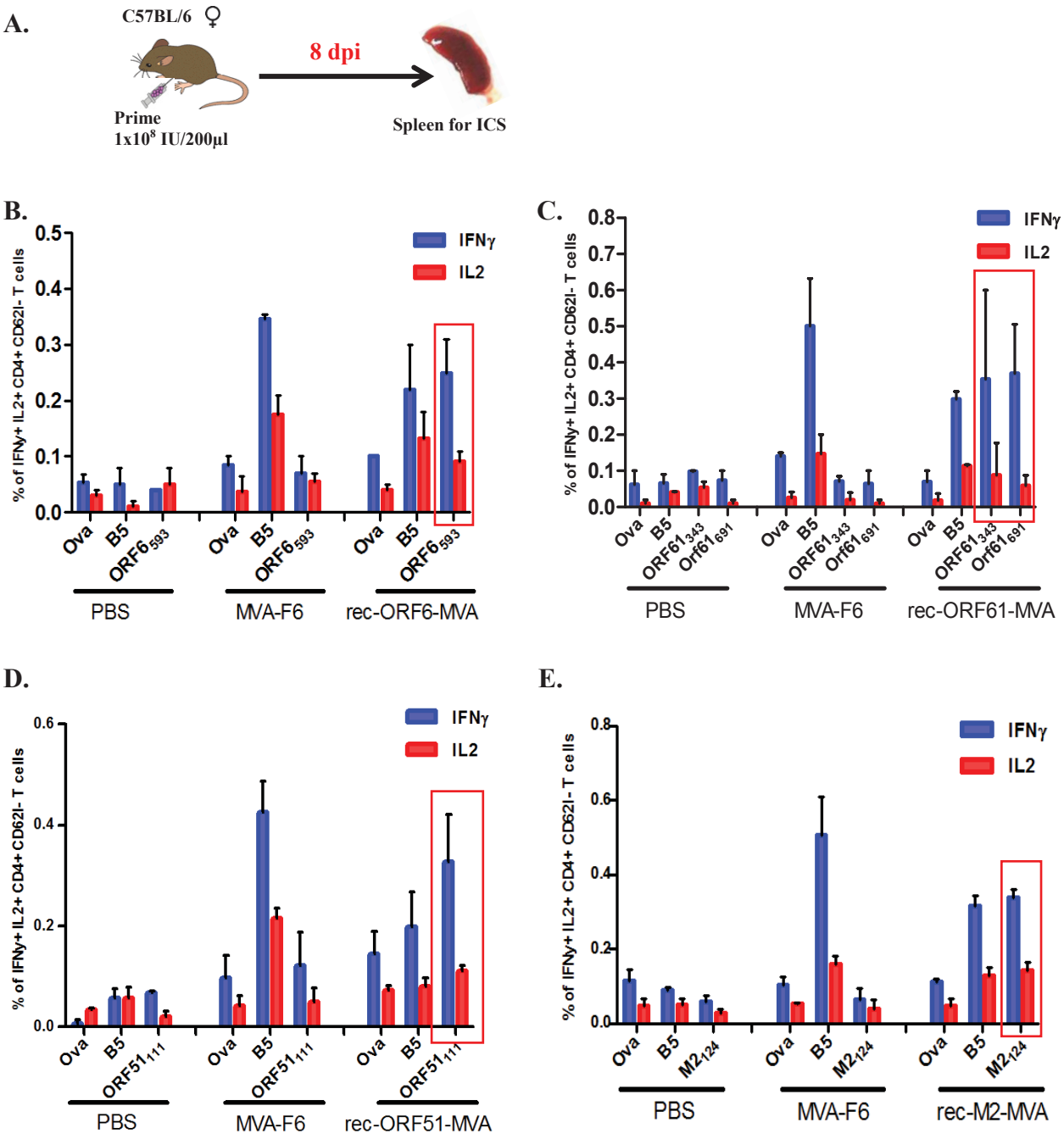


Fig.4.17 CD4+ T cell immune responses induced by rec-MVA vaccines in the acute phase (8dpi). A. C57BL/6 mice (6-8 weeks old) were intraperitoneally immunized with $1x10^8$ IU/200µl rec-MVA or MVA-F6 or PBS (200µl) as controls. At 8 dpi, mice were sacrificed, spleens harvested, and splenocytes prepared for *ex vivo* immune response analysis by ICS followed by FACS analysis. Quantitation of **B.** $ORF6_{593}$-specific, **C.** $ORF61_{343}$ and $ORF61_{691}$ specific, **D.** $ORF51_{111}$-specific and **E.** $M2_{124}$-specific CD4+CD62L- T cells producing cytokines. Ova_{265} was used as unrelated control peptide and $B5_{46}$ as MVA-specific control peptide. FACS strategy is depicted in Fig.4.14B. Data shown are mean ±SEM of n=6 pooled from 3 experiments in all B, C, D and E.

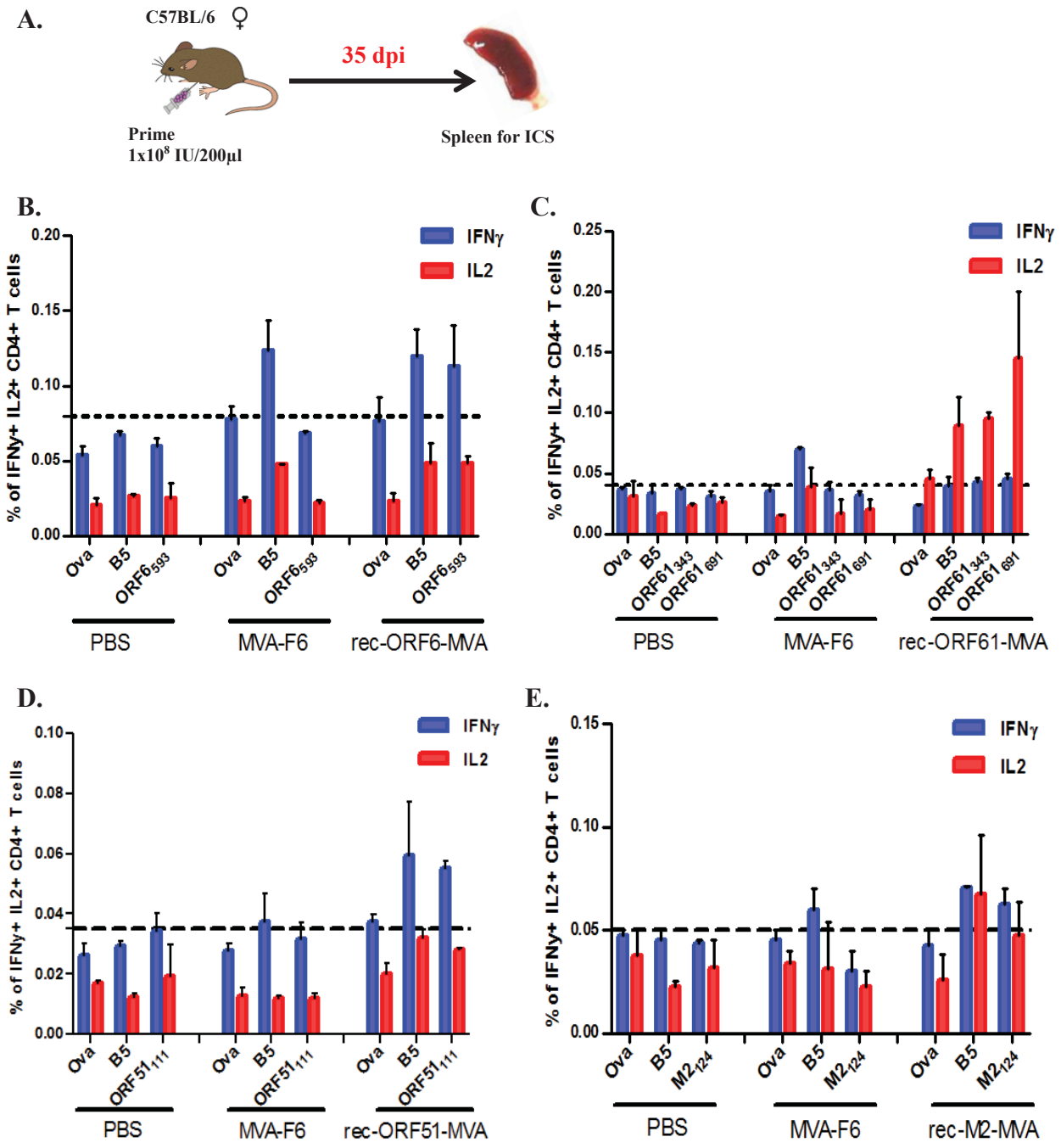


Fig.4.18 CD4+ T cell immune responses induced by rec-MVA vaccines in the memory phase (35dpi). **A**. C57BL/6 mice (6-8 weeks old) were intraperitoneally immunized with 1x10^8 IU/200µl rec-MVA or MVA-F6 or PBS (200µl) as controls. At 35 dpi, mice were sacrificed, spleens harvested, and splenocytes prepared for *ex vivo* immune response analysis by ICS followed by FACS analysis. Quantitation of **B.** $ORF6_{593}$-specific, **C.** $ORF61_{343}$ and $ORF61_{691}$ specific, **D.** $ORF51_{111}$-specific and **E.** $M2_{124}$-specific CD4+ T cells producing cytokines. Ova_{265} was used as unrelated control peptide and $B5_{46}$ as MVA-specific control peptide. FACS strategy is depicted in Fig.4.15B. Data shown are mean ±SEM of n=9 pooled from 2 experiments in all B, C, D and E.

It has been reported previously that rec-MVA vaccines can efficiently enhance the immunogenicity of CD4+ T cell mediated responses upon prime/boost vaccination of rec-MVA (McShane, Brookes *et al*. 2001). Therefore, we analysed the CD4+ T cell immunity after using prime/boost regimens in rec-MVA vaccination encoding MHV68 antigens. We used C57BL/6 mice for intraperitoneal vaccination with rec-ORF6-MVA, rec-ORF61-MVA, rec-ORF51-MVA or rec-M2-MVA virus or MVA-F6 virus as a control, with a second vaccination following at day 5 (Thiele, Tao *et al*. 2015). Mice were sacrificed 6 days after boost vaccination, and spleens were harvested to prepare splenocytes for *ex vivo* immune response analysis, following the same procedure as described previously. After prime/ boost vaccination, we observed high levels of CD4+CD62L- T cells producing IFNγ (0.25-0.35%) specific to $ORF6_{593}$, $ORF61_{343}$ and $ORF61_{691}$ epitopes (Fig.4.19B) as well as $ORF51_{111}$ and $M2_{124}$ epitopes (Fig.4.19C) in response to the rec-MVA expressing the corresponding MHV68 antigens. Interestingly the T cell response determined by IFNγ was only comparable to prime vaccination (Fig.4.17). The results of the response in the memory phase have shown very small populations of antigen specific CD4+ T cells without boost vaccine. Notably, after prime/boost regimen vaccination, we observed enhanced activation of CD4+ T cells, producing both IFNγ and IL2. These results demonstrate that rec-ORF6-MVA, rec-ORF61-MVA, rec-ORF51-MVA and rec-M2-MVA have the capacity to enhance antigen-specific CD4+ T cell immunogenicity upon boost vaccination.

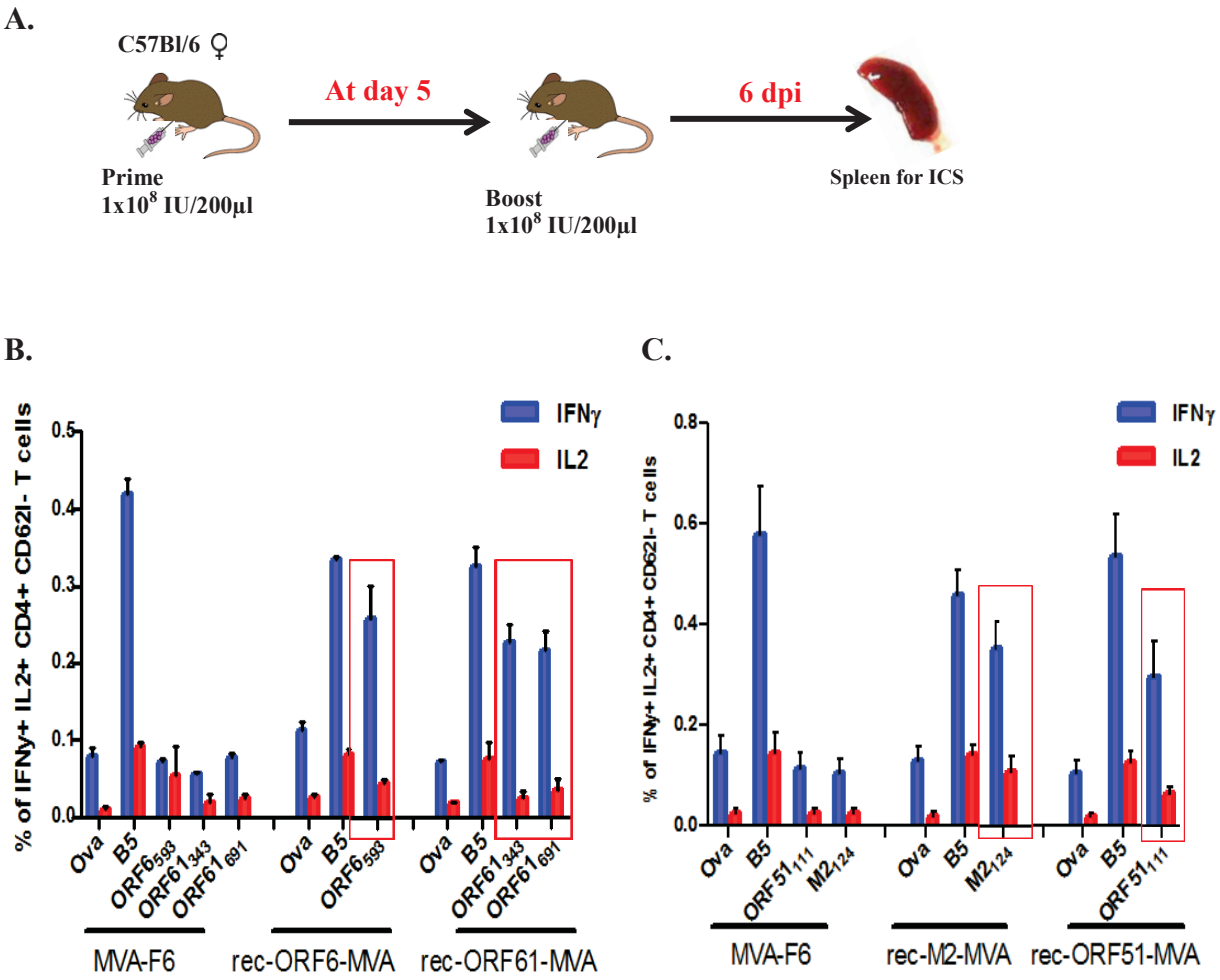


Fig.4.19 CD4+ T cell immune responses induced by rec-MVA vaccines after prime/boost vaccination A. C57BL/6 mice (6-8 weeks old) were immunized with 1x10^8 IU/200µl rec-MVA or MVA-F6 by intraperitoneal application in a prime /boost (at day 5 post prime) regimen. After 6 days of boost vaccination, mice were sacrificed, harvested spleens to prepare splenocytes for *ex vivo* immune response analysis, carried out by ICS followed by FACS. Quantitation of **B.** ORF6$_{593}$-specific, ORF61$_{343}$ and ORF61$_{691}$-specific, **C.** ORF51$_{111}$-specific and M2$_{124}$-specific CD4+CD62L- T cells producing cytokines. Ova$_{265}$ was used as unrelated control peptide and B5$_{46}$ as MVA-specific control peptide. FACS strategy is depicted in Fig.4.14B. Data shown are mean ±SEM of n=15 pooled from 2 experiments.

4.9. Protective efficacy of prophylactic recombinant vaccines

After infection, herpesviruses spread from cell to cell within the host (Peeters, Pol *et al*. 1993, Dingwell, Brunetti *et al*. 1994). Therefore, virions are a less exposed to antibodies (Roth and Compans 1980) and antibody-dependent cytotoxicity is considered to be more important for host defence than neutralization (Balachandran, Bacchetti *et al*. 1982, Kohl, Loo *et al*. 1986).

So far, we have shown the efficacy of all recombinant MVAs tested for generating an target antigen-specific T cell reponse by *in vitro* and *ex vivo* analysis. Furthermore, we performed an experiment to analyze the protective capacity of each rec-MVA based on their efficacy in terms of induction of T cell immunity. After infection, gamma herpesviruses cause an acute infection that lasts from 7 to 10 days with a peak of lytic virus replication at 7 dpi. After the acute phase, the viral particles spread to the lymphoid organs and start seeding the virus in the spleen from day 13 to 42 causing chronic infection, with a peak of latent virus replication at day 17. Therefore, we primed mice with rec-MVA followed by a boost vaccination at day 5. Four weeks after the boost vaccination, when short lived effector T cells are contracted and mainly T cell memory cells are present, we challenged the mice with MHV68 intranasally (i.n.). Then, we analyzed the *ex vivo* CD8+ and CD4+ T cell responses at day 7, corresponding to the lytic phase (section 4.9.1) and at day 17, corresponding to the latent phase of infection (section 4.9.2). Additionally, we performed a so-called reactivation assay at day 17 to analyze the latent virus reactivation capacity (section 4.9.3). These experiments should allow us to determine the protective capacity of prophylactic rec-MVA vaccination (Fig.4.20).

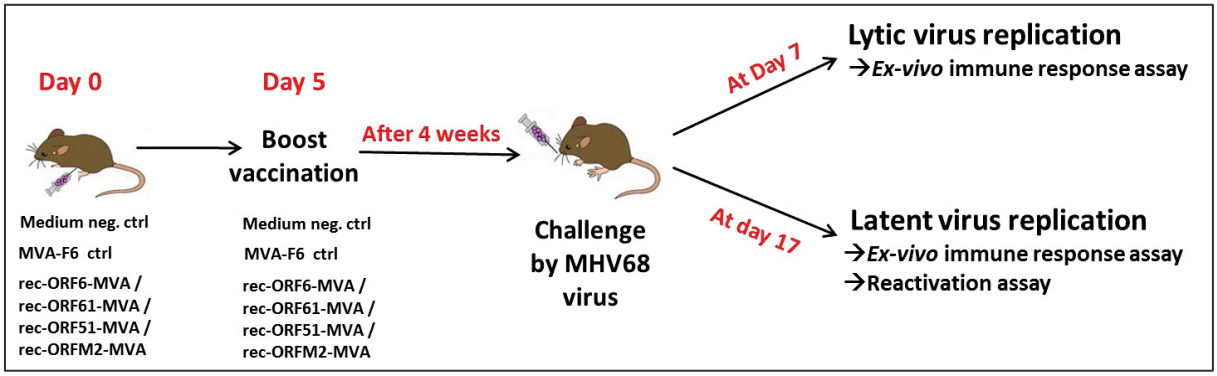


Fig. 4.20 Schematic experimental outline to analyze protective efficacy of rec-MVA C57BL/6 mice were vaccinated i.p. in a prime /boost (at day 5 post prime) regimen using PBS, MVA-F6 or rec-MVAs ($1x10^8$ IU/200µl). After 4 weeks, mice were challenged i.n. with MHV68 ($5x10^4$ PFU/20µl). After 7 and 17 days post challenge, mice were sacrificed and spleens were harvested to analyse *ex vivo* immune responses. Additionally, spleen weight was determined. Reactivation assays were performed at day 17 post MHV68 challenge.

4.9.1. *Ex vivo* T cell analysis during lytic infection

After prime/boost vaccination of rec-ORF6-MVA, rec-ORF61-MVA, rec-ORF51-MVA or rec-M2-MVA, mice were challenged intranasally by MHV68 (Fig.4.20). Initially, we analysed the immune response *ex vivo* upon in the lytic virus replication phase. We sacrificed mice at 7 days post challenge to harvest the spleen and splenic T cell analysis was carried out by ICS. To monitor CD8+ T cell responses, splenocytes were incubated with MHC class-I restricted peptide epitopes such as $ORF6_{487}$/ $ORF61_{524}$ peptide (specific for recombinant antigens) or βgal_{499} (negative control) or $B8_{20}$ (vaccination control). We used anti-CD3 as a positive control for non-antigen-specific T cell stimulation. For CD4+ T cell response analysis, splenocytes were incubated with MHC class-II restricted peptide epitopes such as $ORF6_{593}$/ $ORF61_{343}$/ $ORF61_{691}$/ $ORF51_{111}$ or $M2_{124}$ (specific for recombinant antigens) or Ova_{265} (negative control) or $B5_{46}$ (vaccination control). Production of cytokines by activated (CD62L negative) CD8+/ CD4+ T cells was determined by ICS followed by FACS analysis.

The results for the CD8+ T cell response after MHV68 challenge showed, that mice which were pre-immunized with rec-MVA had higher levels of CD8+CD62L- T cells producing IFNγ (12-13%) specific to $ORF6_{487}$ and $ORF61_{524}$ epitopes, compared to mice which were not primed with these antigens before (pre-immunized with PBS or MVA-F6 (immunization control)) (Fig.4.21). The results indicate that MHV68 infection reactivated pre-existing memory T cells *in vivo* which had been generated by prophylactic vaccination with rec-ORF6-MVA and rec-ORF61-MVA vaccines.

The results for CD4+ T cell responses after challenge indicated, that mice which were pre-immunized with MVA-F6 or PBS had very low levels of CD4+CD62L- T cells producing IFNγ or IL2 in the specific response to $ORF6_{593}$, $ORF61_{343}$/ $ORF61_{691}$ and $ORF51_{111}$ and $M2_{124}$ epitopes. Interestingly, the ORF6 and ORF61 epitope-specific CD4+CD62L- T cell responses were higher in mice which were pre-immunized with rec-ORF6-MVA and rec-ORF61-MVA, respectively (Fig.4.22A). In contrast, $ORF51_{111}$ and $M2_{124}$ epitope-specific responses were only slightly increased in mice which were pre-immunized with rec-ORF51-MVA and rec-M2-MVA, respectively, as compared to MVA-F6 or PBS immunized ones (Fig.4.22B). Overall, rec-ORF6-MVA and rec-ORF61-MVA, rec-ORF51-MVA and rec-M2-MVA were able to generate efficient CD4+ T cell responses, which were enhanced after i.n. challenge with MHV68.

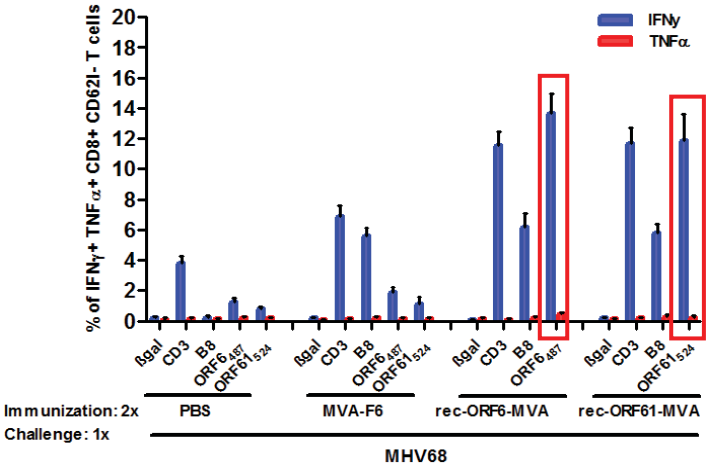


Fig.4.21 CD8+ T cell responses against MHV68 antigens in pre-immunized mice during lytic infection of MHV68

Experimental setup as in Fig.4.20. C57BL/6 mice were sacrificed at 7 days post MHV68 challenge and spleens were harvested to prepare splenocytes. *Ex vivo* T cell analysis for $ORF6_{487}$ and $ORF61_{524}$-specific IFNγ+ or TNFα+ CD8+ CD62L- T cells by ICS/FACS. βgal_{499} as unrelated and $B8_{20}$ as MVA-specific controls. Anti-CD3 used as non-antigen-specific control. Data shown are mean ±SEM of n=11 pooled from 2 experiments.

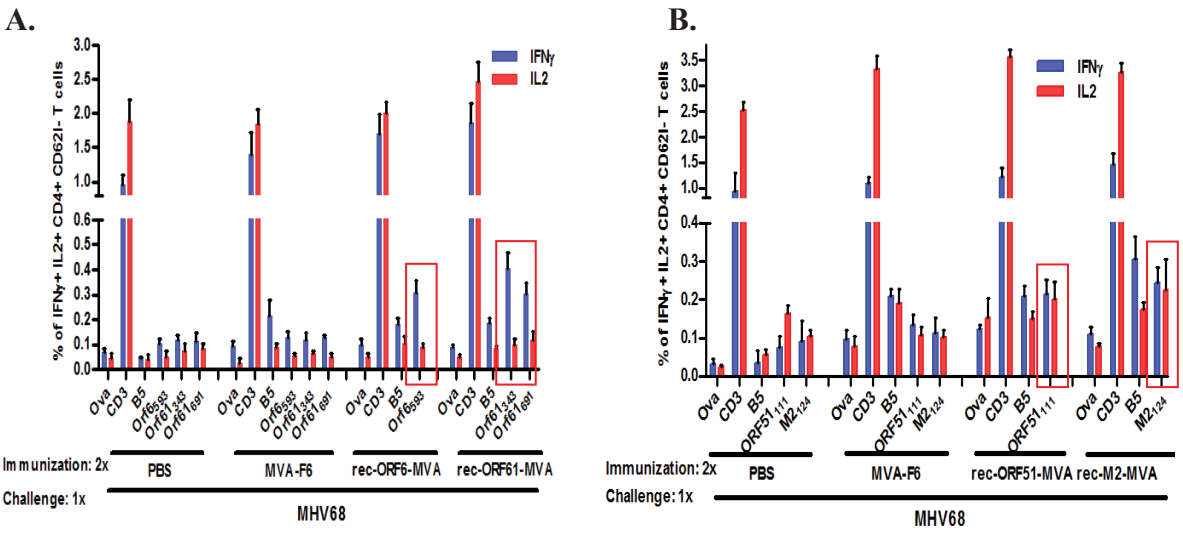


Fig.4.22 CD4+ T cell responses against MHV68 antigens in pre-immunized mice during lytic infection of MHV68

Experimental setup as in Fig.4.20. C57BL/6 mice were sacrificed at 7 days post MHV68 challenge and spleens were harvested to prepare splenocytes. *Ex vivo* T cell analysis for; **A.** $ORF6_{487}$-specific, $ORF61_{343}$- and $ORF61_{524}$-specific and, **B.** $ORF51_{111}$- and $M2_{124}$-specific IFNγ+ or IL2+ CD4+CD62L- T cells by ICS/FACS. Ova_{265} as unrelated and $B5_{46}$ as MVA-specific control. Anti-CD3 used as non-antigen-specific control. Data shown are mean ±SEM of n=11 pooled from 2 experiments.

4.9.2. *Ex vivo* T cell analysis during latent virus infection

After lytic infection, we further analysed the T cell response during latent virus replication after MHV68 virus challenge in rec-MVA pre-immunized mice. For *ex vivo* analysis, we sacrificed mice at 17 days post MHV68 challenge and harvested the spleens to prepare splenocytes. We used splenic T cells to monitor both CD8+ and CD4+ T cell responses by using MHC class-I and MHC class-II-restricted peptides specific for ORF6, ORF61, ORF51 and M2 antigens for restimulation respectively, as described in section 4.9.1. Production of cytokines by activated CD8+/ CD4+ T cells was determined by ICS followed by FACS analysis.

The results for CD8+ T cell responses after MHV68 challenge indicated, that mice which were pre-immunized with PBS or MVA-F6 had less CD8+CD62L- T cells producing IFNγ upon stimulation with $ORF6_{487}$ and $ORF61_{524}$ peptides as compared to mice which were pre-immunized with respective rec-MVA ($ORF6_{487}$ (nearly 2 fold higher); $ORF61_{524}$ (nearly 2 fold higher)) (Fig.4.23). These results support the efficiency of rec-ORF6-MVA and rec-ORF61-MVA to induce memory CD8+ T cell responses which may be expanded by MHV68 also during latent infection.

The results for CD4+ T cell responses after MHV68 challenge proved to be very interesting. Mice, which were pre-immunized with PBS, showed higher levels of CD4+CD62L- T cells producing IFNγ specific to all recombinant antigens. However, mice which were pre-immunized with MVA-F6 showed a relatively lower response of activated CD4+ T cells specific to $ORF6_{593}$, $ORF61_{343}$ and $ORF61_{691}$ epitopes which was not the case for $ORF51_{111}$ and $M2_{124}$ epitopes. Surprisingly, $ORF6_{593}$, $ORF61_{343}$, $ORF61_{691}$ (Fig.4.24A) and $ORF51_{111}$ (Fig.4.24B) epitope specific CD4+CD62L- T cell responses were lower in mice which were pre-immunized with respective rec-MVA as compared to the PBS or MVA-F6-immunized mice. This was not the case for $M2_{124}$-specific responses upon pre-immunization with rec-M2-MVA (Fig.4.24B). Before taking a final conclusion, we performed latent virus reactivation assays to correlate the T cell responses obtained for each rec-MVA to the reactivation capability of latent MHV68 viruses.

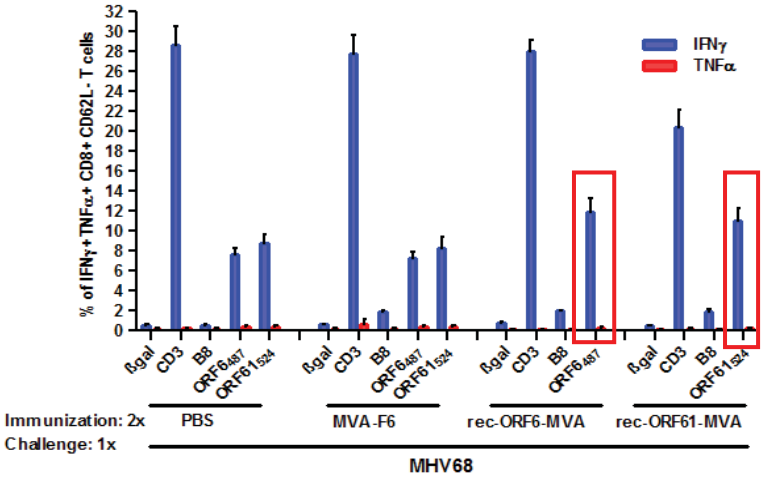


Fig.4.23 CD8+ T cell responses against MHV68 antigens in pre-immunized mice during latent infection of MHV68

Experimental setup as in Fig.4.20. C57BL/6 mice were sacrificed at 17 days post MHV68 challenge and spleens were harvested to prepare splenocytes. *Ex vivo* T cell analysis for $ORF6_{487}$ and $ORF61_{524}$-specific IFNγ+ or TNFα+ CD8+ CD62L- T cells by ICS/FACS. βgal_{499} as unrelated and $B8_{20}$ as MVA-specific controls. Anti-CD3 used as non-antigen-specific control. Data shown are mean ±SEM of n=11 pooled from 2 experiments.

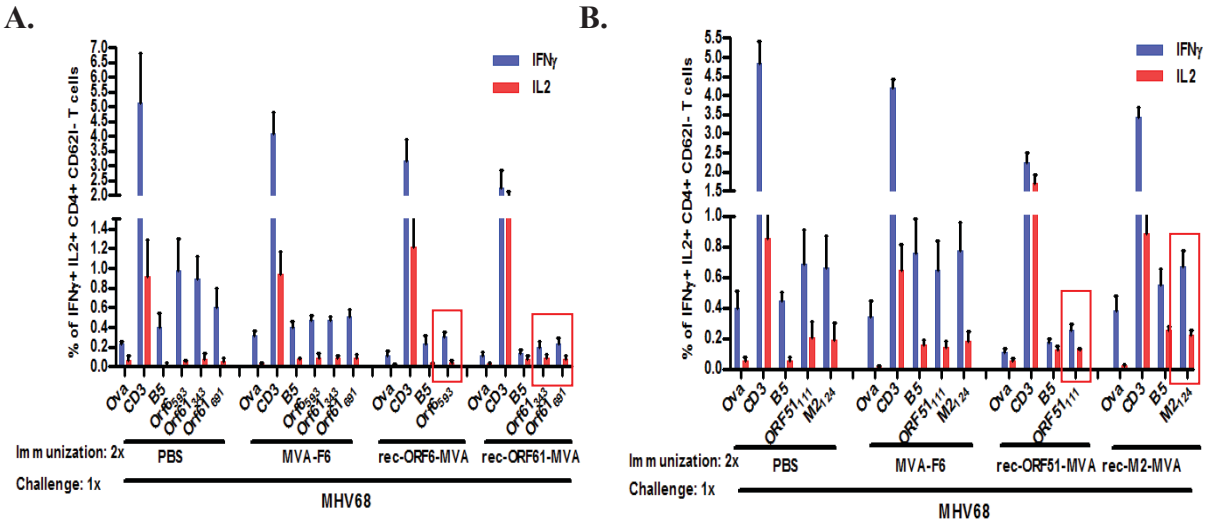


Fig.4.24 CD4+ T cell responses against MHV68 antigens in pre-immunized mice during latent infection of MHV68

Experimental setup as in Fig.4.20. C57BL/6 mice were sacrificed at 17 days post MHV68 challenge and spleens were harvested to prepare splenocytes. *Ex vivo* T cell analysis for; **A.** $ORF6_{487}$-specific, $ORF61_{343}$- and $ORF61_{524}$-specific and, **B.** $ORF51_{111}$- and $M2_{124}$-specific IFNγ+ or IL2+ CD4+CD62L- T cells by ICS/FACS. Ova_{265} as unrelated and $B5_{46}$ as MVA-specific control. Anti-CD3 used as non-antigen-specific control. Data shown are mean ±SEM of n=11 pooled from 2 experiments.

4.9.3. Reactivation efficiency of latent virus after prophylactic vaccination

After determining the efficacy of vaccination for the induction of antigen-specific T cell immunity, we investigated another parameter namely the reactivation capacity of latent viruses established after challenge with MHV68. For this purpose, we analyzed the frequency of latent virus to reactivate from infected spleens upon MHV68 challenge. For experimental setup, C57BL/6 mice were i.p. immunized (prime/boost regimen) with rec-MVA or MVA-F6 or PBS as controls. At four weeks after the boost vaccination, mice were challenged i.n. with MHV68 (see also section 4.9). At 17 days post challenge, spleens were harvested to measure weights and also to prepare splenocytes which were plated out on NIH3T3 cells in serial dilutions in 96 well plates. The cytopathic effect caused by reactivated latent virus was determined and the reactivation frequency of MHV68 in each vaccination group was calculated.

Mice immunized with rec-ORF6-MVA and rec-ORF61-MVA showed a significant reduction in spleen weight ($P<0.05$) (Fig.4.25A) and also a reduction in reactivation (Fig.4.25B), when compared to the control groups. The frequency of reactivating splenocytes was 1 in $\pm$ 12,558 cells for the PBS/MHV68 and 1 in $\pm$ 10,193 cells for the MVA-F6/MHV68 immunized control group (reaching to the point 63.2% dotted line, the point at which one reactivation event is likely to occur per well). The number of *ex vivo* reactivating splenocytes in the rec-ORF61-MVA/MHV68 immunized group was 1 in $\pm$ 40,061 cells and 1 in $\pm$ 52,317 cells in the rec-ORF6-MVA/MHV68 immunized group. These results clearly demonstrate a lower reactivation frequency of latent virus in mice pre-immunized with rec-MVA as compared to the controls.

Furthermore, in contrast to rec-M2-MVA, mice immunized with rec-ORF51-MVA showed a reduction in spleen weights ($P<0.05$) (Fig.4.25C) and also a reduction in reactivating splenocytes (Fig.4.25D) in comparison to the control groups. The frequency of reactivating splenocytes was 1 in $\pm$ 9,882 cells for the PBS/MHV68 and 1 in $\pm$ 6,179 cells for MVA-F6/MHV68 immunized control groups. The number of reactivating splenocytes in the rec-M2-MVA/MHV68 immunized group was 1 in $\pm$ 11,797 cells, whereas the frequency of reactivating splenocytes in the rec-ORF51-MVA/MHV68 immunized group was remarkably low with 1 in $\pm$ 90,285 cells. The data clearly indicate a lower reactivation frequency of latent virus only in the group of mice vaccinated with rec-ORF51-MVA, but not for those vaccinated with rec-M2-MVA.

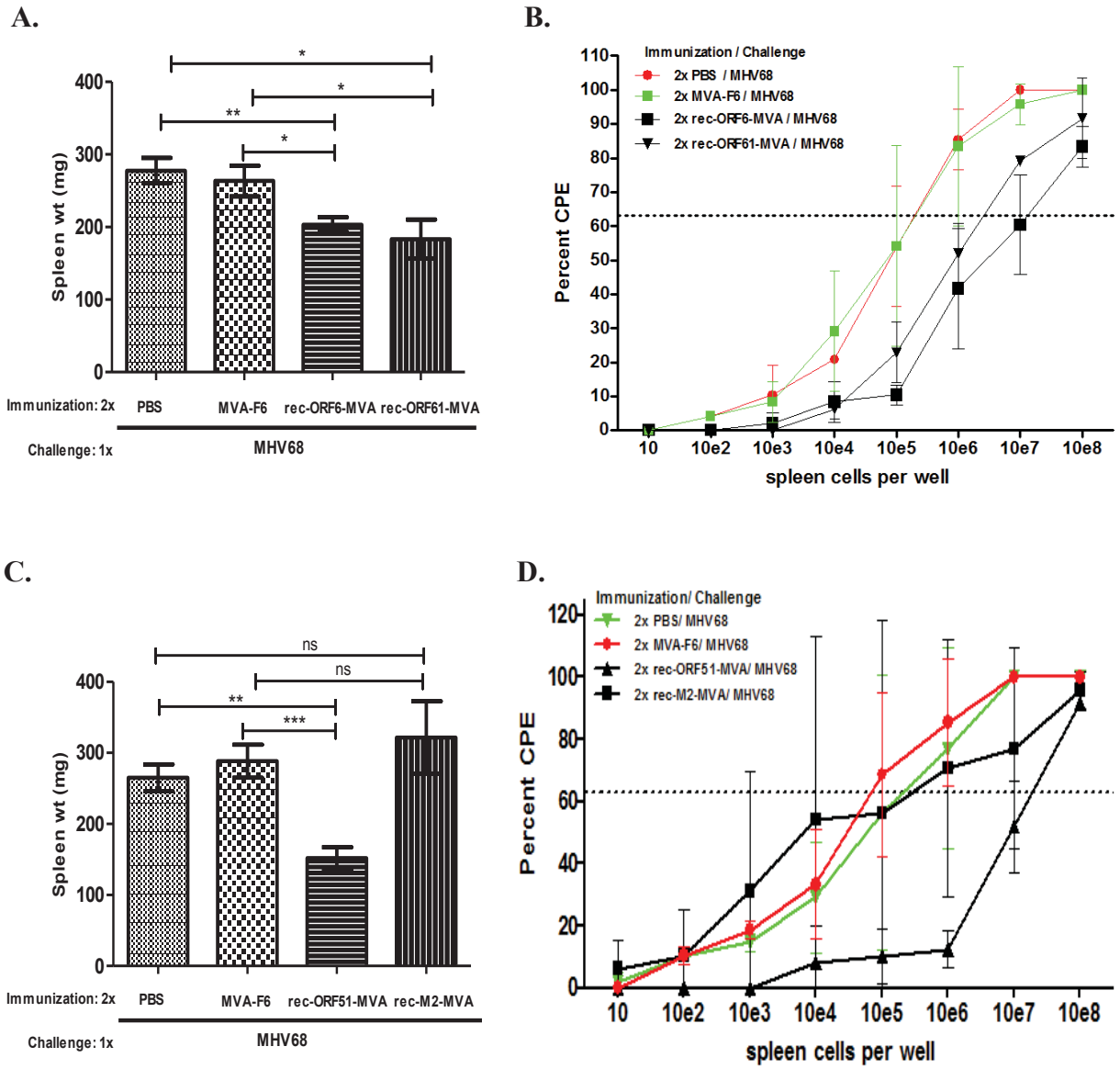


Fig.4.25 Latent virus reactivation and spleen weight after challenge of vaccinated mice

For *ex vivo* reactivation of splenocytes, mice were challenged with MHV68, 4 weeks after prime-boost immunization with rec-MVA or MVA-F6 or PBS controls (experimental setup is explained in Fig. 4.20). Spleens were harvested at 17 days after challenge. **A.** Mean weight of spleens or **B.** reactivating splenocytes in rec-ORF6-MVA and rec-ORF61-MVA vaccinated mice. **C.** Mean weight of spleens or **D.** reactivating splenocytes in rec-ORF51-MVA and rec-M2-MVA vaccinated mice. Data shown are mean ± S.E.M pooled from two independent experiments. In each experiment, splenocytes from three mice per group were pooled. The horizontal line indicating the point 63.2% poisson distribution, which is used to calculate the frequency of splenocytes for reactivation (containing the latent virus).

5. Discussion

5.1. Generation of recombinant MVA vaccines by *en passant* recombineering of MVA-BAC

In this project, we have generated recombinant MVA (rec-MVA) vaccines to provide and enhance the T cell immunity against gamma herpesvirus infection. MHV68 has been utilized as a model to study human gamma herpesviruses e.g EBV and KSHV, because of genetic similarities. We selected MHV68 antigens ORF6, ORF61, ORF8, ORF51, M2 and M3 covering the lytic and latent phase of infection. Single-stranded DNA binding protein encoded by ORF6 and ribo-nucleo reductase protein encoded by ORF61 are known to induce T cell reponses during early and late phase of infection dominantly. Previously, ORF6 and ORF61 peptide pulsed DCs have been utilized as vaccines against MHV68 which resulted in a reduction of viral titers during acute infection but not during long term infection. Similarly, ORF8 encoding for glycoprotein-B (gB-110) a sub-dominant latent antigen is also known to reduce viral lytic replication, but did not contribute in virion neutralization after recombinant vaccinia virus (Vac-gB) vaccination (May and Stevenson 2010). Additionally, rec-MVA-gp150 (gp150 encoded by ORF51) have shown already to induce MHV68-neutralzing antibodies and reduced viral infectivity at 14dpi providing a model system for MHV68 vaccination (Stewart, Micali *et al*. 1999). MHV68 M2 and M3 antigen-specific T cell responses have been also characterized in a DNA vaccination model and proved to be effective against early latent infection (day 14) but not at later time points (Hoegh-Petersen, Thomsen *et al*. 2009; Obar, Donovan *et al*. 2004). MVA is already used as a recombinant live viral vector vaccine due its highly immunogenic properties and its safety record allowing efficient targeting of antigens to both, MHC class-I and class-II pathways (Dudek, Tim *et al*., 2006). Therefore, we generated rec-MVA vector vaccines expressing MHV68 antigens.

For rec-MVAs, we initially generated transfer plasmid encoding each of the recombinant antigens. The transfer plasmid PH5_dVI_MVA was already generated and characterized by Ingo Drexler's group. In this transfer plasmid, deletion VI region of MVA has been targeted for cloning of recombinant antigens. Each of the cloned genes were expressed under the modified PH5 promoter that has a strong activity for early and late gene expression, which was anticipated to enhance the expression of recombinant antigens in MVA vectors leading to enhanced immunogenicity (Kastenmuller, Gasteiger *et al*. 2007, Baur, Brinkmann *et al*. 2010). We amplified the cDNA of ORF6, ORF61, ORF8, ORF51, M2 and M3 genes by using long

modified primers to additionally introduce cloning sites and a HA-tag sequence at the C-terminus of each MHV68 gene. We introduced the HA-tag to fascilitate the detection of recombinant antigens during western blot analysis because monoclonal antibodies for each antigen were not available. The cDNAs and PH5_dVI_MVA plasmid were digested by using compatible restriction enzymes and ligated. The ligated product was purified and clones of correct size were selected by agarose gel electrophoresis. All the transfer plasmids allowed for strong recombinant protein expression with correct size as detected by anti-HA in western blot (section 4.1). Additionally, the correct sequence for all six transfer plasmids was cofirmed by sequencing excluding mutations after cloning.

Furthermore, we utilized the established method of *enpassant* recombination MVA-BAC technology to accelerate and facilitate the generation of rec-MVAs compared to the traditional transient-dominant selection of plaques for markerless constructs (Cottingham and Gilbert 2010). The transgene insertion was carried out by homologous recombination of the transgene expression cassette contained in the transfer plasmid with MVA-BAC DNA, employing deletion VI region of MVA. This recombination was done in GS1783 *E.*coli cells harboring the MVA-BAC DNA. The kanamycin marker gene present in the transgene expression cassette facilitated the selection of recombined positive clones (efficiency about 50%). However, in a second recombination step this marker gene was removed efficiently (80-100%) by endonuclease *ISec*-I and fragments were rejoined by λ red-recombination. Consequently, all the resulting rec-MVA-BAC clones (for each MHV68 antigen) were markerless.

In spite of removal of kanamycin marker gene, all rec-MVA-BAC still contained the BAC cassette at deletion III locus of the MVA genome which does not affect the immunogenecity in mice for pre-clinical work But for clinical purpose, site specific recombination is required for the removal of BAC as it could interfere with the immune system (Cottingham, Andersen *et al*. 2008). Therefore, we utilized a self-excisable MVA-BAC for generating rec-MVAs following virus rescue. Although, BAC cassette withing the viral genome does not affect the viral growth (Cottingham, Andersen *et al*. 2010). We established a protocol to rescue rec-MVA from self excising MVA-BAC (section.3.2.1) in which the rec-MVA-BAC virus remarkably lost its GFP (a marker gene present in the BAC cassette) (Tischer, Kaufer *et al*. 2007), indicating the loss of the BAC cassette. The resulting rec-MVAs had a clear cytopathic effect with no GFP signal in microscopy. The rec-MVAs were also tested by PCR for the presence of the GFP maker gene and the RFV (helper virus) by using specific primers. Although RFV can not grow in DF-1 or CEF cells, contaminations needed to be excluded. The resulting rec-MVAs were free of GFP marker and RFV contaminants. Additionally, all rec-

MVAs were characterized for recombinant protein expression, sequenced and also the growth kinetic pattern proved to be comparable to wild type MVA.

This efficient and reliable genetic engineering method by using self-excisable MVA-BAC provided us a tool for generating rec-MVA viruses which can be used as vaccines. The MVA-BAC in *E.coli* cells are stable showing no intramolecular recombination due to the low copy number of BAC, and recombination results in the removal of Ori2, that is essential for BAC propagation (Tischer, Kaufer *et al*. 2007). Additionally, instability of rec-MVA has been reported only due to the mutation in the transgene rather than in the MVA backbone (Wyatt, Belyakov *et al*. 2008, Wyatt, Earl *et al*. 2009). By combining all the parameters of established MVA-BAC mutagenesis system, we accomplished the generation of six rec-MVAs, each encoding for ORF6, ORF61, ORF8, ORF51, M2 and M3 MHV68 antigens. These rec-MVA vaccines were further utilized to investigate the T cell mediated immune responses against the recombinant antigens.

5.2. Generation of CD8+ and CD4+ T cell lines and their activation *in vitro* by exogenous or endogenous antigen presentation

T cells are one of the key players of the adaptive immune response. They mediate their function upon specific recognition of antigens, presented on the surface of antigen presenting cells (APCs) on MHC class-I/ II complexes. CTLs mediate the killing of tumor cells or virus-infected cells e.g by releasing toxic granules or cytokines activated by antigen presentation via MHC class-I molecule (Andersen, Schrama *et al*. 2006). Viral infections which have resistance to antibody-mediated protection like HIV or have less neutralization sensitivity like EBV/KSHV depend on CD8+ T cells to be controlled efficiently (Doherty, Christensen *et al*. 2001, Kitchen, Levin *et al*. 2012). Along with CD8+ T cells, CD4+ T cells have more immune regulatory functions activated by antigen presentation on MHC class-II. Activated CD4+ T cells are further differentiated into various effector subtypes mediating the immune response through secretion of subtype-specific cytokines. CD4+ T cells carry out a variety of functions, including activation of the innate immune system, B lymphocytes, CTLs and also non-immune cells, as well as playing a critical role in suppression of immune reactions (Luckheeram, Zhou *et al*. 2012). A synergistic role of CD8+ and CD4+ T cells in controlling infections increased the inclination towards developing vaccines in context of T cell therapy.

MVA are known to infect BMDC and allows for antigen expression, processing and presentation efficiently via MHC class-I and class-II pathway (Guzman, E *et al*., 2012,

Bohnen, C *et al*., 2013) In the current project, we have generated MHV68 antigen specific CD8+ or CD4+ T cells *in vitro*. These T cell lines have been utilized to test their antigen specificity upon activation by peptide pulsed BMDC. Additionally, T cells specificity were also tested upon activation by endogenous antigen after infection of BMDC with rec-MVA and also with WT MHV68 virus, indicating the strenght and quality of antigen processing and presentation by BMDC *in vitro*.

Moreover, MVA is widely used in heterologous prime-boost vaccinations as a boost vector. We wanted to imitate the boost situation *in vivo* explained by the presence of activated antigen-specific memory T cells. Therefore, we generated CD8+ and CD4+ T cell lines which resemble memory cells as a readout system *in vitro*. Mice were given single vaccination with no boost to generate CD8+ T cells, which was enough for efficient CD8+ T cell priming. CD8+ T cells restricted to βgal_{96}, $B8_{20}$, $ORF6_{487}$ or $ORF61_{524}$ epitopes proved to be highly sensitive and specific *in vitro*. Upon peptide stimulation, antigen-specific CD8+ T cell lines were able to produce cytokines even at low antigen density. However, rec-MVA and MHV68 were able to infect BMDCs efficiently and allowed to present the endogenous recombinant antigens to a similar extent as indicated by strong activation of CD8+ T cells producing cytokines.

Moreover, CD4+ T cells were generated by using splenocytes from prime-boost vaccinated mice, to enhance the priming efficacy and expansion of T cells. Each CD4+ T cell line was found to be highly specific and sensitive upon stimulation with peptide. Previously, it has been reported that rec-MVA infected BMDCs are able to present endogenous antigen and are recognized by CD4+ T cells (Thiele, Tao *et al*. 2015). In our study, $ORF6_{593}$ and $ORF51_{111}$-specific CD4+ T cells were able to recognize and be activated efficiently by endogenous antigens presented by BMDCs after infection with respective rec-MVA and MHV68. Conversely, ORF61 and M2-specific MHC-II-restricted epitopes were not presented efficiently after infection of BMDCs with respective rec-MVA or after MHV68 infection, resulting in reduced activation of specific CD4+ T cells *in vitro* (section.4.6). Interestingly, both ORF61 and M2 specific CD4+ T cells showed specificity and sensitivity upon activation with peptide pulsed BMDC, showing the functionality of T cells producing cytokines. But this functionality was impaired for endogenous antigen recognition after infection of both rec-MVA and MHV68 infection. In case of ORF61 antigen, the MHC class-I epitope was efficiently presented but MHC class-II epitope might not be processed or presented efficiently, or may be not enough endogenous antigen is available to activate the CD4+ T cells. The nature of ORF61 protein for epitope processing and presention to elicit specific CD4+ T cell

activation and response *in vitro* have not been particularly discussed in literature which is required to elucidate the role of this antigen. Moreover, M2 is a latent antigen that is responsible for dowregulating MHC class-II molecule expression by inducing IL-10 production (Siegel, Herskowitz *et al*. 2008). Nature of M2 protein could involve in modulating the antigen presentation upon infection of BMDC with rec-M2-MVA and MHV68. Interestingly, *in vitro* analysis M2-specific CD4+ T cells stimulated with BMDC infected with MHV68 was relatable with the result of *ex vivo* T cell analysis after MHV68 challenge.
Notably, rec-ORF8-MVA was not able to elicit either CD8+ or CD4+ T cell responses upon vaccination (data not shown). Therefore, we did not characterize this vaccine. Secondly, no MHC class-I/ II-restricted antigenic epitope is reported for the M3 antigen in C57BL/6 mice. Therefore, we did not use rec-M3-MVA in this study. Additionally, we have done experiments to screen for epitopes derived from the M3 antigen in a separate project.

5.3. Induction of antigen-specific T cell responses after immunization with recombinant MVA

5.3.1. CD8+ T cell responses induced by rec-MVA

CD8+ T cells have major role in limiting the lytic phase of MHV68 infection (Ehtisham, Sunil-Chandra *et al*. 1993, Weck, Barkon *et al*. 1996, Stevenson, Cardin *et al*. 1999), but are not considered potent enough to control MHV68 latent infection (Cardin, Brooks *et al*. 1996). CD8+ T cell responses are well characterized for MHV68 infection concerning $ORF6_{487}$ and $ORF61_{524}$ epitopes (Freeman, Lanzer *et al*. 2010, Freeman, Burkum *et al*. 2012).
In principle, MVA are involved in direct priming as well as cross priming of T cells and are able to produce efficiently viral and recombinant antigens in APC and non-APC (McConkey, Reece *et al*. 2003). Interestingly, MVA induces CD8+ T cell immunity activated by so-called late viral antigens which are not synthesized within infected DC due to early block of viral life cycle, therefore appeared to be cross primed (Ann,Chahroudi *et al*.2006, Drexler, Ingo *et al*.2003). In context of viral vaccine-mediated T cell immunity against MHV68, we investigated the efficacy of rec-MVA to induce CD8+ T cell responses in the acute and memory phase of infection in C57BL/6 mice by *ex vivo* T cell analysis. MVA vectors are highly immunogenic and generate strong cellular immune responses e.g dominated by MVA-encoded $B8_{20}$ epitope-specific CD8+ T cells (Baur, Brinkmann *et al*. 2010). However, our study has shown that primary immunization with rec-ORF6- or rec-ORF61-MVA strongly induced antigen-specific effector CD8+ T cells during the acute phase of infection (8 dpi), that

can be categorized as subdominant according to the hierarchy of other MVA antigens (Kastenmuller, Gasteiger *et al*. 2007), Croft, Nathan P. *et al*., 2013). In line with this, after immunization of rec-ORF6-MVA and rec-ORF61-MVA antigen-specific CD8+ T cell responses contracted to about 10% of the acute plateau phase in the memory phase of infection (35 dpi). Interestingly, each of MHV68 antigen expression is controled by PH5 early/late MVA promoter which induces the overexpression of recombinant antigen during early phase but also preserve the priming efficiency of antigen (Becker, PabloD. *et al*., 2014). Therefore, enhanced expression of MHV68 antigens might lead to the improved CD8+ T cells immunity during memory response which could be further characterized for T cell proliferation and expansion capabilities.

MVA is known to induce strong immune responses upon boost immunization (Joachim, Nilsson *et al*. 2015, Ewer, Rampling *et al*. 2016). We also investigated the efficacy of each vaccine to enhance the immune response upon second immunization. Following priming, the boosting vaccine was given at day 5 when T cells are highly activated. The rec-ORF6-MVA and rec-ORF61-MVA induced strong effector CD8+ T cell response upon boost vaccination (Reyes-Sandoval, A *et al*. 2010). Taken together, the overall CD8+ T cell response was strongly induced and showed the capacity of rec-ORF6-MVA and rec-ORF61-MVA to enhance T cell immunity by boosting at day 5 after priming in accordance with previous reported studies (Kastenmuller, Gasteiger *et al*. 2007).

5.3.2. CD4+ T cell responses induced by rec-MVA

The role of CD4+ T cells for controlling MHV68 lytic infection (Weck, Barkon *et al*. 1996, Stewart, Usherwood *et al*. 1998, Stevenson, Belz *et al*. 1999, Nash, Dutia *et al*. 2001) as well as long term infection (Molloy, Zhang *et al*. 2011, Hu, Zhang *et al*. 2013) is well established. As a helper and regulator of antiviral immunity, effector CD4+ T cells can directly kill virus-infected cells and serve as cytolytic CD4+ T cells (Cheroutre and Husain 2013). CD4+ T cells contribute to immunity against MHV68 infection by more direct mechanism (Christensen, Cardin *et al*. 1999, Stuller and Flano 2009, Stuller, Cush *et al*. 2010).

We also investigated the use of rec-MVA fort he induction of antigen-specific CD4+ T cell responses during the acute and memory phase afetr vaccination by performing *ex vivo* T cell analysis. We utilized $ORF6_{593}$. $ORF61_{343}$, $ORF61_{691}$, $ORF51_{111}$ and $M2_{124}$ peptide epitopes for the CD4+ T cell analysis as reported in literature (Freeman, Burkum *et al*. 2014). Concerning MVA, CD4+ T cell responses are dominated by the MVA B5 antigen that is also known to induce strong antibody responses (Viner, Girgis *et al*. 2007, Meseda, Atukorale *et al*. 2016). In

our findings, primary immunization with rec-ORF6-MVA, rec-ORF61-MVA, rec-ORF51-MVA or rec-M2-MVA strongly induced antigen-specific effector CD4+ T cells during the acute phase (8 dpi). Conversely, during the memory phase (35 dpi) only rec-ORF6-MVA and rec-ORF51-MVA retained some potential to generate measurable antigen-specific CD4+ T cell responses. Interestingly, wild type MVA-F6 was also not consistent to allow for measurable $B5_{46}$ epitope-specific CD4+ T cell responses after 35 dpi. This implicated that CD4+ T cells specific to each antigen are more activated and proliferative that differentiate into effector cells ($CD62L^{low}$) during acute infection but memory CD4+ T cell responses are contracted with single prime vaccine. Strikingly, during memory response only ORF61-specific CD4+ T cells were producing high level of IL-2 (with very low detection of IFNγ), reflecting the properties of CD4+ central memory cells (T_{cm}) which also express CD62L conferring self-renwable and strong protection capacity (MacLeod, M. *et al*, 2009). These ORF61-specific CD4+ T cells could be further investigated for CCR7 marker specific for IL-2 producing T_{CM} cells.

Interestingly, MVA shows enhanced immunogenicity concerning CD4+ T cell responses upon prime-boost regimen vaccinations (McShane, Brookes *et al*. 2001). After the primary immunization, boost vaccines at day 5 is considered to be most effective to enhance the immune response in early prime boost regimens (Kastenmuller, Gasteiger *et al*. 2007). Our data also showed increased antigen specific CD4+ T cell responses after boost vaccinations at day 5. All rec-MVA encoding for ORF6, ORF61, ORF51 and M2 antigens, were consistently producing increased amounts of effector CD4+ T cells at day 6 after boosting (Thiele, Tao *et al*. 2015). Comparing prime-boost responses to responses at 35 dpi without boost vaccine, we could speculate the enhanced CD4+ T cell memory responses upon boost vaccination with each rec-MVA.

5.4. Protection and T cell responses in the lytic or latent phase of MHV68 infection after rec-MVA vaccination

Our data support the effectiveness of the rec-MVA vaccines tested to allow for antigen presentation, strong effector and memory T cell responses after primary immunization and enhanced T cell responses upon boost vaccination. Although the vaccines were designed mainly for therapeutic treatment, it was of interest to know if they are able to generate prophylactic T cell immunity. Previously, vaccination of peptide pulsed DCs, attenuated MHV68 virus and plasmid vectors have been utilize to enhance MHV68-specific T cell

immunity, which was able to control lytic but not latent infection (Liu, Usherwood *et al*. 1999, Tibbetts, Loh *et al*. 2003, Hoegh-Petersen, Thomsen *et al*. 2009, Jia, Freeman *et al*. 2010). As recombinant MHV68 is already known to allow for antigen-specific immune responses (El-Gogo, Susanne *et al*. 2007) we assumed that a strategy using rec-MVA encoding MHV68 antigens should be more effective than previous studies.

After MHV68 infection, CD8+ T cell responses to $ORF6_{487}$ epitope were prominent during acute infection but declined at 10 dpi, while $ORF61_{524}$-specific T cell responses continued during both acute and latent infection (more than 14dpi) (Liu, Flano *et al*. 1999, Obar, Fuse *et al*. 2006). MHV68 establishes latency in spleen, where B lymphocytes have been identified as a major antigen source of the $ORF61_{524}$ epitope (Stevenson, Belz *et al*. 1999). Previously, post exposure vaccination with recombinant vaccinia viruses (VACV-$ORF6_{487}$ and VACV-$Orf61_{524}$) has shown a transient viral replication control in lungs but had no impact on latency establishment (Stevenson, Cardin *et al*. 1999). Whereas, we have observed at 7 days of MHV68 challenge (lytic infection) after prime-boost immunization with rec-ORF6-MVA and rec-ORF61-MVA that effector CD8+ and CD4+ T cells were strongly induced.

Similarly, both vaccines maintained strong CD8+ T cells responses during the latent phase of MHV68 infection. Whereas, both ORF6- and ORF61 specific CD4+ T cell responses were low (absolute no. of CD4+IFNγ+ T cells were also reduced) compared to mice having no pre-immunization with rec-MVA. Including B5-specific CD4+ T cells response (used as a rec-MVA immunization control) was also low with the similar no. CD4+IFNγ+ T cells like in PBS control group but lower than MVA-F6 control group. Notably, there was a low CD4+ T cell response specific to B5, ORF6 and ORF61 in MVA-F6 pre-immunized mice, but absolute no. of B5-specific CD4+IFNγ+ T cells were higher (ORF6- and ORF61-specific were similar) as compare to PBS immunized mice after MHV68 challenge. Interestingly, weight of spleens were also reduced significantly ($P<0.05$) in rec-MVA-immunized mice at latent time point.

Strong CD8+ T cells are known to control MHV68 infection, in contribution of CD4+ T cells which helps mainly to control the latent infection. Our data support this view, since rec-MVA-induced strong T cell responses, where CD4+ T cells producing IFNγ may have been utilized to control the latent infection as the reduced spleen weight reflect the reduced splenomegaly (Nash, Dutia *et al*. 2001) and also reduced frequency of latent virus reactivation in pre-immunized mice with rec-MVA. Given that MVA prime/boost vaccination regimen had a profound impact on T cell responses as determined in both lytic and latent infection phase, the use of rec-ORF6-MVA and rec-ORF61-MVA vaccines seems a promising candidate for further testing strategies for long term latent virus control.

Furthermore, rec-ORF51-MVA induced strong CD4+ T cell responses during the lytic phase after MHV68 challenge, which was reduced more than half in terms of no. of cytokine producing CD4+ T cells (absolute no. of CD4+IFNγ+ also slightly reduced) during latent infection in rec-ORF51-MVA pre-immunized mice after MHV68 challenge as compare to PBS and MVA-F6 control group. Whereas, th B5-specific CD4+ T cell response was also reduced (absolute no. of CD4+ IFNγ+ also slightly reduced) compared to MVA-F6 control group. Although ORF51 is a well characterized MHV68 antigen that elicits CD4+ T cell responses which are maintained throughout long term infection (Freeman, Burkum *et al*. 2011). Our data indicate that ORF51-specific CD4+ T cells participate in the control of latent viral infection as splenomegaly as well as the number of latently infected cells in spleen were significantly ($P<0.05$) reduced following the acute MHV68 infection in rec-ORF51-MVA pre-immunized mice. Although, it might be possible that ORF51 has also CD8+ T cell response which contributed to reduce the latent viral load strongly and it would be interesting to identify the MHC calss-I restricted epitope for this antigen. ORF51 encodes for MHV68 gp150 which was used as model antigen for vaccine generation because it induces neutralizing antibody responses (Stewart, Micali *et al*. 1999, Ruiss, Ohno *et al*. 2012). This feature additionally supports the use of gp150 as an antigen for long term viral control.

Moreover, M2 as a latent antigen has shown transient reduction of early latency not later than day 14 upon M2 DNA vaccination in Balb/c mice (Usherwood, Ward *et al*. 2001). Similarly, in C57BL/6 mice, we found that rec-M2-MVA was able to induce CD4+ T cell responses during lytic infection, whereas at latent time point showed a reduced response similar to the one found in mice which were pre-immunized with PBS and MVA-F6. In line with the above findings, we detected a severely increased splenomegaly (not significant) and a high latent virus reactivation frequency which was almost similar to non-immunized mice indicating that rec-M2-MVA was not able to control latent virus infection. M2 is an early and lately expressed antigen that is mainly associated with latent infection (Virgin, Presti *et al*. 1999, Usherwood, Roy *et al*. 2000). M2 protein is relevant for the establishment of and reactivation from viral latency, modulate the B cells by inhibiting apoptosis and also downregulate the MHC calss-II molecule presentation (Jacoby, Virgin *et al*. 2002, Herskowitz, Jacoby *et al*. 2005). An effect on T cell immunity could be considered during lytic infection, if immunization with rec-M2-MVA would result in reduced lung titers. Otherwise, we might need to consider later time points of MHV68 infection e.g. 60dpi to analyze the role of rec-M2-MVA for latent virus control, since rec-M2-MVA was not effective in our experimental settings at 17dpi.

6. Conclusion

The *en passant* mutagenesis of recombinant MVA-BAC has been proved to be a rapid and highly efficient system to generate recMVA. The selection marker (anitibiotic) gene was removed from rec-MVA-BAC. Furthermore, BAC-cassette was efficiently deleted from self-excising MVA-BAC. All rec-MVA generated in this study had no sign of genetic instability after removal of the BAC cassette or shown growth deficiencies compared to the parental MVA. Each recombinant antigen inserted in the dVI region of the MVA genome and driven by the modified vaccinia virus PH5 promoter was expressed at high levels as determined transcriptional and translational. Additionally, genomic sequencing confirmed the insertion of recombinant antigens in MVA dVI region in the right orientation and excluded any mutations. Taken together, our data has been proven MVA-BAC mutagenesis as a reliable and promising platform for generation of recombinant MVA vaccines.

In order to test the vaccines, we utilized in vitro T cell activation assays and performed immunization experiments at different phases after vaccination and after challenge infections with MHV68. All rec-ORF6-MVA, rec-ORF61-MVA, rec-ORF51-MVA and rec-M2-MVA were able to present MHV68 antigens efficiently as demonstrated by specific recognition by CD8+ or CD4+ T cell lines. The rec-MVA were also able to generate efficient immune reponses determined for the acute and memory phase after primary vaccination as well as upon boost vaccinations. We conclude that rec-ORF6-MVA, rec-ORF61-MVA, rec-ORF51-MVA and rec-M2-MVA are efficient in the induction, reactivation and expansion of T cell immunity.

We further proved the protective efficacy of rec-MVAs after MHV68 challenge in pre-immunized mice. Next to enhanced T cell responses in the lytic phase of MHV68 infection for all rec-MVA, rec-ORF6-MVA, rec-ORF61-MVA and rec-ORF51-MVA reduced splenomegaly and the latent virus reactivation frequency in the latent phase of MHV68 infection. In contrast to rec-M2-MVA, we conclude that rec-ORF6-MVA, rec-ORF61-MVA and rec-ORF51-MVA have strong prophylactic efficiency supporting further testing in vaccination strategies aiming for T cell-based therapy e.g. in combination with adoptive T cell transfer.

7. References

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"And, when you want something, the entire universe conspires in helping you to achieve it."

Paulo Coelho, The Alchemist

Eidesstattliche Versicherung

Ich, Frau Baila Samreen, versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Duesseldorf, 06.02.2017 — Baila Samreen

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