Characterization of a Recombinant Modified Vaccinia Virus Ankara Encoding a Novel Synthetic Immunogen of Human Cytomegalovirus

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LIST OF ABBREVIATIONS

aa	amino acids
AIDS	acquired immunodeficiency syndrome
ALP	alkaline phosphatase
ALVAC®	canarypox virus vector
APC	allophycocyanin
APCs	antigen-presenting cells
BHK	baby hamster kidney cells
bp	base pairs
BSA	bovine serum albumin
C	control
CD	cluster of differentiation
cDNA	complementary DNA
CEF	chicken embryo fibroblasts
CMV	cytomegalovirus
CpG	cytosine-phosphate-guanine
CVA	Chorioallantoic Vaccinia Virus Ankara
(D) MEM	VLE (Dulbecco's) Modified Eagle Medium
DCs	dendritic cells
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
e.g.	exempli gratia (lat.)
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
ER	endoplasmic reticulum
ERAP	ER-amino peptidase
ERp57	ER-protein 57
et al.	et alii (lat.)
FACS	fluorescence activated cell sorting

FCS	fetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
g	gravidity
GAPDH	glycerinaldehyd-3-phosphat-dehydrogenase
GMP	good manufacturing practice
gp85	glycoprotein 85 of EBV
Gy	gray
HaCaT	human keratinocyte cell line
HCMV	Human Cytomegalovirus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HRP	horseradish peroxidase
HSC (T)	hematopoietic stem cells (transplantation)
IE-1	immediate early 1 protein
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
kb	kilo base pairs
kDa	kilodalton
KIR	killer-cell immunoglobulin-like receptor
LCL	lymphoblastoid cell lines
LL8	murine fibroblasts expressing human CD40 ligand
M	molarity
MWM	molecular weight marker
MCMV	murine cytomegalovirus
MERS-CoV	Middle East respiratory syndrome coronavirus
MF59	oil-in-water adjuvants
MHC	major histocompatibility complex
MIIC	MHC class II compartment

MOI	multiplicity of infection
MVA	Modified Vaccinia Virus Ankara
NEAS	non essential amino acids
NK cell	natural killer cell
NLS	nuclear localization signal
nM	nanomole
NYVAC	highly attenuated Vaccinia Virus strain (vP866)
p.i.	post infectionem (lat.)
P/S	penicillin/streptomycin
PBMC	peripheral blood mononuclear cells
$PBMC_{di}$	PBMC after i days, where $i \in \{0,10\}$
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PmH5	Modified Vaccinia Virus promoter H5
p-NPP	para-nitrophenylphosphate
Pos.	positive control
pp65	phosphoprotein 65
rad	radian
RPMI-1640	cell culture medium: Roswell Park Memorial Institute
SDS	sodium dodecyl sulfate
Se	selenium
SO (T)	solid-organ (transplantation)
STAT2	signal transducer and activator of transcription 2
Tab.	table
TAP	transporter associated with antigen processing
TCR	T cell receptor
TEMED	tetramethylethylenediamine
$T_H\ i$	type i T helper cell, where $i \in \{1,2,17\}$
T_{FH}	T follicular helper cell
T_{reg}	T regulatory cell

TNF	tumor necrosis factor
U	units
UL	unique long
VACV	vaccinia viruses
VLP	virus-like particle
w/o	without
WR	Western Reserve

I. INTRODUCTION

For a long time many research groups have spent much effort in gaining control over human cytomegalovirus (HCMV) disease. HCMV infection is usually asymptomatic and without severe problems in healthy individuals. However, in immunocompromised and immunosuppressed hosts (e.g. infected newborns, HIV-patients, and transplant recipients) HCMV may cause lifelong problems and require expensive treatment with harmful secondary effects.

A large number of research groups focused on the development of a vaccine to successfully prevent HCMV diseases by following different approaches (e.g. live-attenuated HCMV vaccines, DNA vaccines, virus-like particle vaccines (VLP), peptide vaccines, and different viral vector vaccines). Although there are some HCMV vaccine candidates that already entered preclinical studies and clinical trials, not a single efficient vaccine has been generated and licensed until now.

Within the viral vector vaccines, Modified Vaccinia Virus Ankara (MVA) is one of the most promising candidates due to its replication deficiency in human cells but efficient expression of recombinant proteins encoded by heterologous genes. In addition, MVA induces both cellular and humoral immune responses. Thus, MVA is meeting another important prerequisite for a satisfying vaccine.

This study evaluates an HCMV vaccine candidate (MVA-pp65/IE-1) based on MVA that has recently been constructed in our laboratory. MVA-pp65/IE-1 encoding a fusion gene of the full-length HCMV pp65 and a modified IE-1 cDNA is characterized genetically and immunologically. Recombinant MVA-infected CD40 B cells are established as valuable tool for immunological characterization of recombinant HCMV-MVAs and used to show successful propagation and functionality of HCMV-specific T cells within PBMC by MVA-pp65/IE-1.

II. LITERATURE REVIEW

1. Modified Vaccinia Virus Ankara (MVA)

The host-range restricted Modified Vaccinia Virus Ankara (MVA) is an attenuated strain of vaccinia virus that emerged after more than 516 strict passages of Chorioallantoic Vaccinia Virus Ankara (CVA) in primary chicken embryo fibroblasts (CEF) (MAYR & MUNZ, 1964; MAYR et al., 1975). MVA lost approximately 15% of CVA's total genome including the six major deletion sites (Fig. 4) as well as numerous small deletions and mutations (MEYER et al., 1991; ANTOINE et al., 1998). As a result, MVA and CVA have distinct differences in cell culture and infectivity (MAYR et al., 1978). Although the genome between parental CVA and MVA changed substantially, the genome of MVA was considered as stable after examination of different passages of MVA (MAYR et al., 1978). Despite MVA's inability to replicate in mammalian cells, it still retains infectivity of them (MEYER et al., 1991; CARROLL & MOSS, 1997; BLANCHARD et al., 1998; DREXLER et al., 1998).

1.1. MVA as vaccine platform

Vector and vaccine production is convenient, including established protocols for the generation of recombinant vaccinia viruses (BRODER & EARL, 1999).

Vaccinia viruses (VACV) have been established as a valuable tool for vaccination since 1982, for its ability to express foreign genes (MACKETT et al., 1982; PANICALI & PAOLETTI, 1982). Further advantages of VACV are their lack of persistence, their large DNA capacity, their replication cycle which completely takes place outside the cellular nucleus, and their high immunogenicity as vaccines (MOSS, 1996). Moreover, all orthopoxviruses including VACV have many virulence factors that interact with components of the innate immune system. Though, recombinant VACV are able to induce a strong adaptive immune response, both the humoral and cellular, against heterologous genes they encode (BENNINK et al., 1984; SMITH et al., 2013).

Despite of the multitude of advantages, VACV may cause life-threatening complications (postvaccinal encephalitis, vaccinia necrosum, and eczema

vaccinatum) and side effects (fever, pruritus, lymphadenopathy, muscle aches, and nausea) which were observed during the eradication campaign against the variola virus (LANE et al., 1969; KENNEDY et al., 2009).

In order to eliminate these complications and side effects, efforts were made to develop VACV with reduced virulence. Approaches included the insertion of lymphokine, the deletion of genes associated with virulence, and the development of VACV strains that were attenuated by passaging, such as NYVAC or MVA, which both partly lost their virulence (MOSS, 1991, 1996). Since the 1990s, MVA has been shown to be a particularly useful vaccine platform (SUTTER & MOSS, 1992). While the replication of NYVAC in human cells is already blocked at an early stage (TARTAGLIA et al., 1992; PAOLETTI, 1996), MVA's early and late gene expression is not impaired; instead virion assembly is blocked (SUTTER & MOSS, 1992). Hence, MVA offers an advantage over NYVAC as a vaccine platform.

The safety of MVA has already been tested in over 100,000 individuals during a smallpox vaccination program in Bavaria with high acceptance rates and negligible side effects (STICKL et al., 1974; MAYR et al., 1978). Since 2013, MVA is a licensed smallpox vaccine in Europe and Canada (EUROPEAN MEDICINES AGENCY, 2013). MVA was tested in immunosuppressed monkeys without severe side effects, providing evidence that immunocompromised individuals can be vaccinated with recombinant MVA vaccines to achieve protection against various diseases (STITTELAAR et al., 2001). It has been over 20 years since the first recombinant MVA vaccines were observed to successfully induce adaptive immune responses in the form of CD8 T cells and antibodies, and provide protection against viral challenge infections in animal models (SUTTER et al., 1994; HIRSCH et al., 1996).

Compared to replication competent VACV, MVA is able to trigger immune responses due to different mechanisms. MVA lacks several functional viral receptors for host cytokines including CC chemokines, IFN-α/β, IFN-γ, and TNF that are involved in viral-host interaction and immune modulation (BLANCHARD et al., 1998). Furthermore, it has been shown that only MVA, not replication competent VACV, is able to induce early recruitment of leukocytes and the production of interferon and other chemokines (e.g. CCL2) (WAIBLER et al.,

2007; LEHMANN et al., 2009). Moreover, MVA was equally or better able to induce the immune system against their recombinant foreign gene sequences, whereas immune responses against MVA's own antigens were lower compared to replication competent VACV Western Reserve (WR) (RAMIREZ et al., 2000).

Overall, MVA is a safe and immunogenic vector vaccine platform that efficiently produces recombinant proteins and stimulates the immune system without requiring any further adjuvants (KREIJTZ et al., 2013).

1.2. MVA in preclinical studies and clinical use

In recent years, the MVA vector has become increasingly important for the development of vaccines against various emerging infectious diseases (e.g. influenza, West Nile, chikungunya), diseases caused by intracellular pathogens that are very difficult to control (e.g. AIDS, tuberculosis, malaria), and multiple tumors (e.g. cervical cancer, melanoma) (GILBERT, 2013; KREIJTZ et al., 2013; VOLZ & SUTTER, 2013).

Recombinant MVA vaccines against various infectious agents have been successfully evaluated in different animal models for being safe and immunogenic (YUE et al., 2008; KREIJTZ et al., 2010; VAN DEN DOEL et al., 2014). Moreover, different recombinant MVA vaccine candidates provided protection against otherwise lethal challenges in mice (KREIJTZ et al., 2007; KREIJTZ et al., 2009; VAN DEN DOEL et al., 2014).

Tumor therapy is another important application of MVA. First, it has been shown that tumors associated with human papillomavirus were no longer able to grow in mice after MVA vaccination (VALDEZ GRAHAM et al., 2000) and in a clinical phase I-trial the growth of such tumors was transiently stabilized (ROCHLITZ et al., 2003). In both studies no severe side effects were detectable when MVA was used. Second, when comparing MVA to the replication competent VACV WR in melanoma cells, only MVA was able to induce an effectual immune response of bystander dendritic cells (DCs) (GREINER et al., 2006). Third, clinical trials showed that vaccination with recombinant MVA encoding for papillomavirus antigens resulted in immunogenicity detected by measuring specific antibodies and cytotoxic responses against papilloma-transformed cells (ALBARRAN et al., 2007). Recently, a phase III clinical trial of the same recombinant MVA encoding

papilloma antigens showed promising results in containment of mucosal lesions caused by papilloma virus (ROSALES et al., 2014).

Due to established generation procedures, recombinant MVA vaccines can be targeted against rapidly evolving or emerging infectious diseases, e.g. influenza or the Middle East respiratory syndrome coronavirus (MERS-CoV).

MVA-NP-M1, a promising recombinant MVA vaccine candidate against influenza, was successfully evaluated in three different clinical trials. First, the vaccine was confirmed as safe and immunogenic (BERTHOUD et al., 2011). In a second study, volunteers were challenged with influenza A/Wisconsin/67/2005 after immunization using MVA-NP-M1, which showed that virus shedding and signs of illness were reduced (LILLIE et al., 2012). Further, MVA-NP-M1 was tested in patients over age 50 to address those people that would benefit most from the development of a vaccine against influenza. In all trials, the MVA vaccine candidate showed no severe side effects and activation of T cells could be observed (ANTROBUS et al., 2012).

It has been verified that MVA can be used as vector vaccine platform against MERS-CoV, a viral disease that was first described in 2012 (ZAKI et al., 2012). In 2013, a promising vaccine candidate based on MVA (MVA-MERS-S) was characterized *in vivo* and was proven to provide protection against MERS-CoV challenge in mice in 2015 (SONG et al., 2013; VOLZ et al., 2015). Most recently, it was even possible to show that the excretion of infectious viruses was significantly reduced after immunization of dromedaries with MVA-MERS-S followed by MERS-CoV challenge (HAAGMANS et al., 2016). Dromedaries are believed to play a role as a virus reservoir and transmission vector (REUSKEN et al., 2015), hence their immunization might curb epidemics and prevent infection of humans.

As these examples show, recombinant MVA viruses have been successfully tested in various preclinical and clinical studies as vaccines against various diseases. This progress encourages further investigation and usage of multiple MVA-based vaccines against infectious diseases that are emerging or have been difficult to control through other means.

2. Human Cytomegalovirus (HCMV)

Human Cytomegalovirus (HCMV) belongs to the subfamily of β-Herpesvirinae and has the largest genome among all herpesviruses, roughly 230 kb encoding approximately 165 genes (DAVISON et al., 2003). Among the human population in Europe, the seroprevalence of HCMV ranges between 30 and 90% increasing with age and differing in geography (LUDWIG & HENGEL, 2009). Differences in prevalence rates of HCMV throughout the world depend on socioeconomic as well as geographical factors (CANNON et al., 2010).

The HCMV virion consists of a double-stranded linear DNA genome in an icosahedral nucleocapsid surrounded by the proteinaceous tegument (Fig. 1). The tegument compartment contains the majority of the virion proteins, including the lower matrix phosphoprotein 65 (pp65), a major target of T cell responses (SYLWESTER et al., 2005).

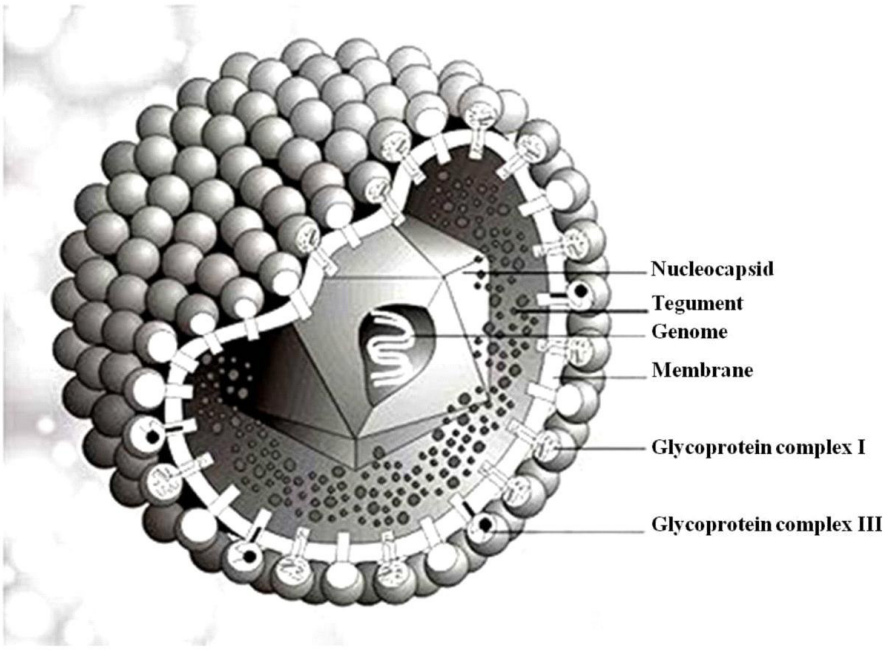


Fig. 1: Structure of HCMV virion. Image obtained from https://siinfekl.files.wordpress.com/2014/12/hcmv-virion.jpg by courtesy of Dr. Marko Reschke, Berlin, Germany.

The DNA core and tegument are enclosed by a lipid envelope that contains several different viral glycoproteins (gB, gH, gL, gM, gN, gO) that form complexes known as glycoprotein complex I, II, and III (Fig. 1) which are involved in virion maturation, cell attachment, and penetration (MOCARSKI et al., 2013). The glycoprotein complexes display major targets of antibody responses. For

example, neutralizing antibodies during HCMV infection are directed against gB (BRITT et al., 1990) and a pentameric complex consisting of gH, gL, and three additional glycoproteins (FOUTS et al., 2012; FREED et al., 2013). HCMV is highly species-specific and can be transmitted not only through various body fluids, most commonly saliva and urine, but also through solid-organ transplantation (SOT) or hematopoietic stem cell transplantation (HSCT) (SIA & PATEL, 2000).

A cascade of immediate-early, early, and late gene expression sequentially occurs during HCMV infection (STINSKI, 1978). The immediate-early 1 (IE-1) protein has been recognized as another major target of T cell responses against HCMV (BORYSIEWICZ et al., 1988; SYLWESTER et al., 2005). HCMV requires gB and gH/gL to enter fibroblasts and the gH/gL-pentamer complex composed of gH, gL, UL128, UL130, and UL131A for penetration of epithelial/endothelial cells (WUSSOW et al., 2014).

Primary HCMV infection in healthy individuals is usually asymptomatic and leads to a lifelong latency without severe problems, as long as the immune system is unaffected. However, primary infection or reactivation of HCMV may cause severe to life-threatening problems in newborns and immunocompromised hosts, such as mental retardation, hearing loss, and visual impairment in newborns (PASS et al., 1980; CONBOY et al., 1987), retinitis in HIV patients (JABS et al., 1989), as well as hepatitis, pneumonia, and gastrointestinal diseases in transplant recipients (HUMAR & MICHAELS, 2006). Furthermore, HCMV infection strongly influences secondary infection outbreaks and mortality rates after transplantation (BOECKH & LJUNGMAN, 2009). HCMV is “the leading non-genetic cause of congenital malformations in developed countries” (HAMILTON et al., 2014) and the leading viral pathogen in transplantation. Thus, it is highly relevant to focus on its treatment and prevention.

2.1. Therapeutic strategies

Different therapeutic strategies are pursued to control HCMV diseases, including medication with antiviral drugs, passive immunization with immunoglobulins, or adoptive transfer of cytotoxic-specific T cells (CROUGH & KHANNA, 2009).

2.1.1. Antiviral drugs

Four antiviral drugs (Ganciclovir, Valganciclovir, Foscarnet, and Cidofovir) are currently approved for HCMV treatment and prevention; all of them, however, may cause major toxicity. Thus, their administration requires limitation and is only advised in patients that are of high risk to develop life threatening HCMV diseases (MOCARSKI et al., 2013). Treatment with Ganciclovir during the first 100 days after transplantation had a positive influence on the survival rate of transplant recipients. However, an increase of late HCMV disease was observed (GOODRICH et al., 1991; LI et al., 1994). Additionally, mutations in HCMV's phosphotransferase-gene (UL97) and polymerase gene (UL54) may give rise to resistance to each of these antiviral agents (LURAIN & CHOU, 2010).

Therefore, other drugs like Maribavir have been currently developed and evaluated in clinical trials (MARTY et al., 2011; WINSTON et al., 2012), but none reached market authorization, yet. Although Maribavir was not completely successful in preventing HCMV disease in these trials, it was effectively used in one heart transplant recipient to gain control of otherwise drug-resistant cytomegalovirus colitis (ZEGRI REIRIZ et al., 2015).

2.1.2. Immunoglobulins

In some clinical settings, SOT recipients received HCMV-specific hyperimmune globulin, which correlated with increased survival rates (FALAGAS et al., 1997) and reduced diseases associated with HCMV (SNYDMAN et al., 1987).

In contrast, benefit of globulin application in the case of HSCT recipients is less clear. Some analyses claimed beneficial outcomes (MESSORI et al., 1994; SCHOPPEL et al., 1998), whereas others demonstrated no or even a negative correlation between immunoglobulin administration and outcome (MUNOZ et al., 2001; LUDWIG et al., 2006). Even for pregnant women, where the role of HCMV-specific antibodies is regarded as crucial, no decrease in transmission rates of HCMV to the child after administration of globulins could be attested (REVELLO et al., 2014).

2.1.3. Adoptive immunotherapy of HCMV

The transfer of cytotoxic-specific T cells is widely acknowledged as beneficial. The role of T cells is considered very important in the control of HCMV disease, since its incidence is inversely correlated to the reconstitution of cellular immunity after allogeneic bone marrow transplantation (QUINNAN et al., 1982; REUSSER et al., 1991). In complementary studies, immunosuppressed mice showed protection against MCMV challenge after transfer of MCMV-specific T cells (REDDEHASE et al., 1985; MUTTER et al., 1988). In line with these results, stem cell donor-derived HCMV-specific T cells were successfully generated and transferred to transplant patients to prevent HCMV disease (RIDDELL et al., 1992; WALTER et al., 1995).

Until now, there are two different approaches for adoptive transfer of cytotoxic-specific T cells: *in vitro* stimulation or direct enrichment of HCMV-specific T cells (CROUGH & KHANNA, 2009). HCMV-specific T cells can be generated by infecting autologous fibroblasts with HCMV viruses (e.g. AD169) for stimulation of PBMC (RIDDELL et al., 1992), by infection of PBMC with recombinant viruses encoding HCMV antigens (RIST et al., 2005), or by co-cultivation with HCMV-antigen-pulsed DCs (PEGGS et al., 2001; MICKLETHWAITE et al., 2007). The enrichment of HCMV-specific T cells in PBMC can be performed by MHC/peptide tetramer staining followed by direct infusion of these cells (COBBOLD et al., 2005).

2.2. Prevention

It is often neglected that basic precautions can be effective in preventing transmission of HCMV infection. Hygiene (e.g. hand washing) and proper protective equipment (e.g. gloves, condoms) decreases HCMV delivery by saliva and sexual contact (ROBAIN et al., 1998; ADLER et al., 2004). However, since the incidence of infection is high and infectivity of carriers is difficult to predict, the development of a protective vaccine remains an urgent necessity.

In particular, vaccination of HCMV-negative women before conception is likely to reduce or prevent infection of newborns during pregnancy. Moreover, recipients of both single organs (SO) and hematopoietic stem cells (HSC) would benefit from a vaccine against HCMV. In patients with HCMV-positive serostatus prior to stem cell transplantation, the risk of HCMV disease is higher when the donor is

seronegative (MOCARSKI et al., 2013) because in this case HCMV-specific immunity is not co-transferred during stem cell transplantation. Thus, it might be most beneficial to vaccinate the donor before transplantation. A seronegative patient receiving an organ from a seropositive donor is at the highest risk to develop HCMV disease during immunosuppressive therapy (MOCARSKI et al., 2013). Here, the vaccination of the recipient before transplantation would be most favorable.

A more extensive approach would be the immunization of the whole population that might lead to decreases in HCMV infections and reactivations during transplantations and pregnancies due to the minimization of HCMV seroprevalence (KRAUSE et al., 2013).

2.2.1. Vaccination to prevent HCMV disease

Research on the development of a vaccine against HCMV has been conducted for more than 40 years. Many groups are working on this subject by following different kinds of strategies. The type of vaccines that have been generated to prevent HCMV disease can be split into two main groups: 1) live-attenuated HCMV vaccines derived from different HCMV strains and 2) vaccines that include only immunogenic parts of the HCMV virus. The latter can be grouped into DNA vaccines (WLOCH et al., 2008), virus-like particle (VLP) vaccines (PEPPERL et al., 2000), peptide vaccines (ZHONG et al., 2008), and different viral vector vaccines (WANG et al., 2004; REAP et al., 2007; WILSON et al., 2008). Although some vaccine candidates are in clinical trials, none of them completely prevent HCMV disease.

The first vaccines were based on attenuated HCMV viruses which presented different antigens that induced antibodies and cytotoxic T cells. The attenuated HCMV viruses AD169 and Towne 125 strain were tested in clinical trials for their safety and immunogenicity (ELEK & STERN, 1974; PLOTKIN et al., 1976). Both viruses induced neutralizing antibodies when being applied in different infection routes (subcutaneous and intranasal). Unfortunately, antibody responses dropped over time and complete protection from HCMV infection was not achieved. In consequence, Towne 125 strain was modified to increase its immunogenicity by designing a chimeric virus composed of Towne 125 and the unattenuated HCMV strain Toledo which is not yet a suitable vaccine

(HEINEMAN et al., 2006). A potential reason why the Towne 125 strain failed in neutralizing virus-infected epithelial cells (CUI et al., 2008) are mutations in the viral UL128-131 locus that probably appeared during passaging on fibroblasts (PRICHARD et al., 2001; DARGAN et al., 2010). This locus forms the pentameric complex together with the glycoproteins gH and gL and plays a central role during HCMV infection of epithelial cells (RYCKMAN et al., 2008; LOUGHNEY et al., 2015).

Vaccine development is not only focused on attenuated HCMV live viruses, but also on heterologous vaccines based on immunogenic HCMV antigens. The first subunit vaccine against HCMV consisted of the HCMV gB and MF59, an oil-in-water adjuvant. This vaccine was able to induce only short term gB-specific antibodies in HCMV seronegative donors (FREY et al., 1999; PASS et al., 1999) that failed in neutralizing virus-infected epithelial cells. One reason might be that these antibodies cannot prevent viral entry to endothelial and epithelial cells (CUI et al., 2008; GERNA et al., 2008; MACAGNO et al., 2010). In 2012, Fouts et al. observed that antibodies against the pentamer-complex and not antibodies against gB are an important target for neutralizing virus-infected epithelial cells (FOUTS et al., 2012).

Moreover, clinical trials using DNA vaccines showed poor immunogenicity and could not prevent HCMV disease (DONNELLY et al., 1997). However, an increase in immunogenicity was partly achieved by combining DNA vaccines with other vector platforms such as MVA (GIL et al., 2013).

Peptide based vaccines have been tested in a preclinical study using transgenic mice. In this examination the peptides themselves - without any adjuvants - were able to induce immune responses against HCMV (LA ROSA et al., 2002). In a human clinical trial phase Ib, adjuvant was necessary to obtain a response to peptide vaccine, but this response was limited to HCMV-positive probands (LA ROSA et al., 2012).

In contrast to the aforementioned vaccines that did not successfully prevent HCMV disease or were not shown to be sufficiently immunogenic, viral vector-based vaccines appear to be a more promising approach. For example, two alphavirus replicon vaccines encoding either a soluble version of the

HCMV-gB or a fusion of the complete pp65 and IE-1 gene sequences were tested simultaneously in a clinical trial phase I that activated neutralizing antibodies, CD4, and CD8 T cells (BERNSTEIN et al., 2009). However, a vaccine based on canary pox (ALVAC) failed to induce neutralizing activities and HCMV-specific IgGs. This was not surprising, as they again encoded for the HCMV gB protein that also failed to prevent HCMV disease in all previous vaccine attempts (ADLER et al., 1999). In contrast, a substantial induction of cell-mediated responses was detectable in a study using the ALVAC vector encoding for pp65 instead of gB (BERENCSI et al., 2001).

Another viral vector-based HCMV vaccine approach uses MVA as a vaccine platform. Both vaccine candidates encoding either pp65 or exon 4 of IE-1 (WANG et al., 2004) and a further HCMV-MVA encoding pp65 and exon 4 of IE-1 separately, have been successfully tested in preclinical studies (WANG et al., 2007).

As these experiences show, the development of an HCMV vaccine appeared to be difficult and suffered from drawbacks. However, recent progress in research, in particular regarding HCMV immunity, has improved the chances to reach this goal in the future.

3. The immune system

The human immune system is composed of various cellular and molecular constituents that are able to defend the human body against invading pathogens such as viruses, bacteria, parasites, and fungi. The skin and mucous membranes form a first barrier for pathogens. If these physical barriers are bypassed, the innate immunity, consisting of various effector cells (e.g. macrophages, NK cells) and molecules, is activated immediately and combats the pathogens. In addition, the adaptive immune system is able to recognize and contain individual pathogens more specifically by either a humoral or cellular pathway. B cells play a central role in the humoral immune system and directly detect pathogenic particles. After contact with antigen, B cells differentiate to plasma cells that secrete specific antibodies into blood and body fluids, where they circulate until they interact with specific antigenic structures (epitopes) of pathogens. Thereafter, these antigen-antibody complexes are cleared by other immune cells,

e.g. macrophages. In contrast, T cells – one major component of the cellular immune system – recognize infected cells that present peptides from the pathogen's proteins on specific cellular surface molecules. The first production of specific B and T cell responses usually takes 1-2 weeks after a pathogen enters the human body. After that, clonally expanded memory cells are maintained in the body, which ensures that the immune response to reinfection or reactivation is even faster (MURPHY et al., 2008).

3.1. T cells

T cells originate from pluripotent HSC of the bone marrow. T cell precursors move to the thymus where they mature into two distinct T cell lineages: α:β or γ:δ T cells. A small fraction mature into the latter type of T cells whose purpose is not fully understood. However, recent research illustrated that γ:δ T cells recognize phospholipids, soluble proteins, as well as smaller peptides (BORN et al., 2013). The majority of T cells end up with a T cell receptor (TCR) consisting of the two chains α and β that are encoded at respective gene clusters composed of different gene segments (V, D, J). These are rearranged and joined during T cell maturation, resulting in a large variety of possible TCRs (DAVIS & BJORKMAN, 1988) that recognize a distinct epitope sequence on antigens. A mature T cell generally carries one TCR, which defines its specificity, and the mature T cell repertoire contains millions of specificities.

T cells can be further distinguished by the appearance of different surface co-receptors, either cluster of differentiation 8 (CD8) or 4 (CD4). The major function of cytotoxic CD8 T cells is to mediate cell death of infected cells by secreting granzymes, perforin, and cytokines (e.g. IFN-γ, TNF) (KAECH & CUI, 2012). Native CD4 T cells mostly fulfill auxiliary functions for other immune cells and can be differentiated into five effector subsets (T_H1, T_H2, T_H17, $T_{FH,}$ and T_{reg}) that vary in the presence of different transcription factors and the proportions of cytokines they produce including IL-4, IL-5, IL-10, and IFN-γ (GEGINAT et al., 2014).

Only a minor portion of premature T cells become MHC-restricted and self-tolerant mature T cells, whereas the majority are deleted in the thymus during maturation, because they do not fulfill all necessary requirements (e.g. appropriate receptor specificities) (MURPHY et al., 2008).

3.2. Antigen presentation

Cells present small protein fragments (peptides) on their cellular surface by loading them onto glycoprotein molecules known as major histocompatibility complex (MHC) class I or II. Almost every human cell, except for human blood cells, present peptide-loaded MHC class I molecules on their cellular surface to CD8 T cells. Healthy cells only load self-peptides on MHC class I molecules, while infected cells additionally present peptides derived from their internal pathogen. In contrast, MHC class II molecules interact with CD4 T cells. They are exclusively present on specialized immune cells, named antigen presenting cells (APCs), which include B cells, DCs, endothelia cells, macrophages, and monocytes (GERMAIN, 1994).

3.2.1. Structure of MHC class I and II molecules

MHC class I molecules consist of one α-chain composed of 3 domains (α_1-α_3) and a β_2-microglobulin (Fig. 2). The α-chain is encoded by one of three major genes named human leucocyte antigen (HLA-A, HLA-B, and HLA-C) that are located on chromosome six in humans. Polymorphism and allelic variations of these genes result in a broad range of different α-chains. The peptide-binding cleft of MHC class I molecules is formed by the α_1 and α_2 domain and allows for the binding of peptides that are generally 8-11 amino acids (aa) long, most often 9 aa (Fig. 2) (MURPHY et al., 2008).

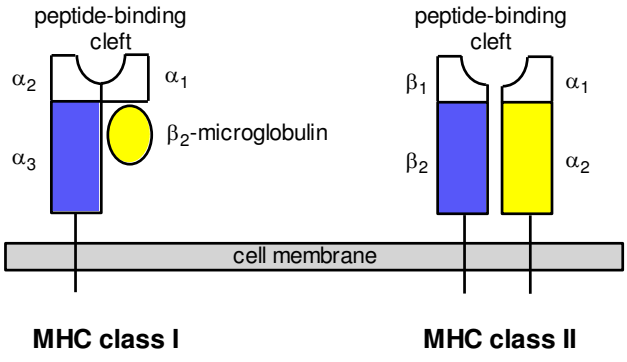


Fig. 2: Structure of MHC class I and MHC class II molecules. Both molecules consists of an α and β domain. MHC class I molecules have three covalently bound parts of the α chain (α_1-α_3), one transmembrane region with cytosolic region and an β_2-microglobulin that are noncovalently bound. Both chains (α and β) of MHC class II molecules have their own transmembrane region with cytosolic region. Contrary to MHC-I, the α_1 and β_1 subdomains of MHC-II are noncovalently linked to each other (Modified according to Murphy, 2008).

MHC class II molecules consist of one α and one β chain that are both anchored in the cell membrane (GERMAIN, 1994). Similar to MHC class I molecules, MHC class II molecules are encoded by polymorphic genes (in humans HLA-DR, HLA-DQ, and HLA-DP) (NEEFJES et al., 2011). Here, the α_1 and β_1 domains form the peptide-binding cleft which differs slightly to its MHC class I counterpart (Fig. 2) by allowing longer aa-sequences (13-17 aa) to be loaded and presented (MURPHY et al., 2008).

3.2.2. Peptide loading on MHC class I and MHC class II molecules

In healthy cells, proteins are constantly being cleaved into small peptides by the proteasome, a protease complex in the cytoplasm. The evolving peptides reach the endoplasmic reticulum (ER) by a heterodimeric transporter associated with antigen processing (TAP) composed of two subunits (TAP-1 and TAP-2) (CRESSWELL, 2000). Peptides remaining in the cytoplasm are degraded within seconds if they are not bound to TAP-1 or -2 (REITS et al., 2003).

Inside the ER, the partly-folded MHC class I molecules are stabilized by different chaperons (calreticulin, ERp57, and tapasin). Together with TAP, they form the peptide-loading complex that is essential for peptide-loading of MHC class I molecules (CRESSWELL et al., 1999). Without peptides, MHC molecules are not stable and remain in their partly-folded state inside of the ER (CRESSWELL, 2000). Only the complete peptide/MHC class I complex is transported to the plasma membrane and recognized by epitope-specific CD8 T cells. Peptides not suited for the peptide-binding cleft of MHC class I molecules fail to bind (MURPHY et al., 2008). These peptides are either cut by ER amino peptidases (ERAP1 and ERAP2) to enable binding to MHC class I molecules or released into the cytoplasm, where they are degraded (SAVEANU et al., 2005).

Peptide loading of MHC class II molecules occurs in the MHC class II compartment (MIIC). Previously, the dimerization of the α and β chain takes place in the ER and is stabilized by the chaperon invariant chain (Ii) that blocks the MHC class II-binding groove for antigenic peptides (NEEFJES et al., 2011). The Ii-MHC class II complex enters a late endosome to form the MIIC (BRYANT & PLOEGH, 2004). Within the MIIC, the invariant chain is cleaved by cysteine proteases until only a short peptide, class II-associated Ii peptide (CLIP), remains inside the binding groove of the MHC class II molecule (HONEY & RUDENSKY,

2003). In humans, MHC class II molecules interact with the chaperone-like HLA-DM (in B cells additionally with HLA-DO) that support the replacement of CLIP by appropriate peptides within the MIIC (BROCKE et al., 2002). The complete peptide/MHC class II complex is displayed at the cellular membrane and recognized by peptide-specific CD4 T cells (NEEFJES et al., 2011).

MHC class I molecules usually embed peptides of various antigens that are present in the cytoplasm including intracellular pathogens, while MHC class II molecules are mainly loaded with exogenous antigens such as specific pathogens (e.g. bacteria, parasites) that are taken up and reach the MIIC through vesicular transport (MURPHY et al., 2008). Apart from this, APCs are capable of loading pathogenic peptides on their MHC class I molecules which is known as cross-presentation (JOFFRE et al., 2012). Furthermore, MHC class II molecules load not only peptides from pathogens but also endogenous antigens including cellular peptides (GROMME et al., 1999; CROTZER & BLUM, 2010).

3.3. HCMV and the cellular immune response

During transplantation, HCMV disease usually occurs once the cellular immune response is suppressed. Thus, it is very likely that parts of the cellular immune system play a crucial role in controlling HCMV.

This goes in parallel with observations seen in bone marrow transplants where the recovery of cytotoxic T cells was strongly correlated with the prevention of HCMV diseases (REUSSER et al., 1991; BARRON et al., 2009). In addition, adoptive transfer of HCMV-specific T cells prevented transplant patients from HCMV disease (RIDDELL et al., 1992; WALTER et al., 1995). Moreover, in renal transplant recipients, a large increase in numbers of HCMV-specific T cells was detectable only in those that were able to control HCMV disease (RADHA et al., 2005).

Studies using the murine animal model provide experimental *in vivo* proof of an important role of CD8 T cells concerning CMV infection (REDDEHASE et al., 1985; POLIC et al., 1998). Furthermore, HCMV diseases in immunosuppressed transplant recipients were more frequent in cases where the reconstitution of CD8 T cells was either undetectable or delayed (REUSSER et al., 1991; BOECKH et al., 2003).

An important role of CD4 T cells is also implied by a majority of studies. The murine model initially suggested that CD4 T cells are not crucial, since CD8 T cells alone were able to inhibit severe CMV infections (REDDEHASE et al., 1987; REDDEHASE et al., 1988). In contrast, more recent studies argued that the presence of CD4 T cells and NK cells had a positive impact in the murine CMV model (POLIC et al., 1998). Furthermore, adoptively transferred CD8 T cells only remained detectable in transplant recipients if CD4 T cells were present (WALTER et al., 1995). In addition, recovery of renal transplant recipients from HCMV disease was only achievable in the presence of functional CD4 T cells (GAMADIA et al., 2003).

In murine animal models, NK cells were shown to be necessary to control CMV infection (TAY et al., 1999). This goes in parallel with the observation of a more severe HCMV disease in a patient with NK cell deficiency (BIRON et al., 1989).

These considerations are not unexpected as various different components of the innate immune system and the adaptive one interact with each other to successfully control pathogenic agents; nevertheless, developing immunization should address CD4 and CD8 T cell responses that control HCMV diseases.

3.4. HCMV antigen presentation with MVA as vector vaccine

A correct presentation of HCMV antigens by recombinant MVA-infected host T cells to the immune system requires processing of HCMV antigens in the cytoplasm before loading them onto MHC molecules. The recognition of HCMV-infected cells by epitope-specific cytotoxic T cells is dependent on the allelic variants of epitope-presenting MHC molecules. Thereby, different HLA-A, -B, and -C molecules present different range of epitopes of HCMV to their corresponding cytotoxic T cells.

For a long time it has been known that recombinant MVAs are able to induce protection against infections by both cellular and humoral immune responses (SUTTER et al., 1994). During MVA infection, cross-presentation by APCs is most important for the activation of cytotoxic T cells (GASTEIGER et al., 2007; PASCUTTI et al., 2011). To enable correct cross-presentation, a long-lasting antigen is proposed to be the best precondition (GASTEIGER et al., 2007). A recent study showed that foreign antigens of recombinant MVA are addressing

CD4 T cells by displaying peptides via the endogenous MHC class II pathway (THIELE et al., 2015).

Taken this together, MVA is a promising vector vaccine platform as it ensures a very efficient presentation of foreign antigens and is able to activate both major subsets of the adaptive cellular pathway, CD4 T cell and CD8 ones.

4. Objectives

Since several decades many different research groups focused their efforts on the generation of a vaccine to prevent HCMV disease. However, there is not even a single licensed vaccine against HCMV available, yet.

For this purpose, an HCMV vaccine candidate (MVA-pp65/IE-1) based on the very safe and efficient MVA backbone with several novel features had recently been constructed in our laboratory.

This study describes the genetical analysis of MVA-pp65/IE-1 in comparison to MVA-pp65 and MVA-IE-1. Moreover, the data confirms that HCMV-MVA-infected CD40 B cells are a valuable tool for studying the functional properties of HCMV-encoding MVAs.

Lastly, this study includes the immunological characterization of MVA-pp65/IE-1 in relation to MVA-pp65 and MVA-IE-1 by evaluation of the expansion and functionality of HCMV-specific T cells using CD40 B cells.

III. MATERIAL AND METHODS

1. Oligonucleotide primers

Table 1 lists the binding sites of oligonucleotide primers for Human herpesvirus 4 complete wild-type genome (EBV, GenBank: NC_007605.1), Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank: NM_001256799.2), and complete genome of vaccinia virus strain Acambis 3000 Modified Virus Ankara (MVA) (GenBank: AY603355).

Oligonucleotide primers	Nucleotide sequence	Oligonucleotide binding site	PCR product size
gp85c	5'-TGG TCA GCA GCA GAT AGT GAA CG-3'	128,761-128,783	461 bp
gp85d	5'-TGT GGA TGG GTT TCT TGG GC-3'	129,202-129,221	
GAPDH-F3	5'-GAC ATC AAG AAG GTG GTG AAG CAG-3'	991-1,014	152 bp
GAPDH-B4	5'-AAG TGG TCG TTG AGG GCA ATG-3'	1,122-1,142	
MVA-Del 1-F	5'-CTT TCG CAG CAT AAG TAG TAT GTC-3'	3,896-3,919	291 bp
MVA-Del 1-R	5'-CAT TAC CGC TTC ATT CTT ATA TTC-3'	4,163-4,186	
MVA-Del 2-F	5'-GGG TAA AAT TGT AGC ATC ATA TAC C-3'	14,795-14,819	354 bp
MVA-Del 2-R	5'-AAA GCT TTC TCT CTA GCA AAG ATG-3'	15,125-15,148	
MVA-Del 3-F	5'-GAT GAG TGT AGA TGC TGT TAT TTT G-3'	143,359-143,383	446 bp
MVA-Del 3-R	5'-GCA GCT AAA AGA ATA ATG GAA TTG-3'	143,781-143,804	
MVA-Del 4-F	5'-AGA TAG TGG AAG ATA CAA CTG TTA CG-3'	160,856-160,881	502 bp
MVA-Del 4-R	5'-TCT CTA TCG GTG AGA TAC AAA TAC C-3'	161,333-161,357	
MVA-Del 5-F	5'-CGT GTA TAA CAT CTT TGA TAG AAT CAG-3'	13,685-13,711	603 bp
MVA-Del 5-R	5'-AAC ATA GCG GTG TAC TAA TTG ATT T-3'	14,263-14,287	
MVA-Del 6-F	5'-CGT CAT CGA TAA CTG TAG TCT TG-3'	116,788-116,810	702 bp
MVA-Del 6-R	5'-TAC CCT TCG AAT AAA TAA AGA CG-3'	117,467-117,489	

Tab. 1: Oligonucleotide primers.

2. Antibodies

Antibodies used for flow cytometry			
Specificity	**Conjugate**	**Clone**	**Company**
CD19	FITC	HIB19	BioLegend, San Diego, USA
CD8a	APC	RPA-T8	BioLegend, San Diego, USA
KIR2DL2/3	FITC	CH-L	BD Biosciences, Heidelberg, Germany

Antibodies used for Western blot		
Specificity	**Dilution**	**Company**
mouse anti-CMV pp72 IEA	1:500	Ray Biotech, Inc., Norcross, USA
mouse-anti-CMV pp65	1:200	Santa Cruz Biotechnology, Inc., Texas, USA
mouse anti-GAPDH	1:10,000	Millipore, Darmstadt, Germany
rat anti-VALV C7	1:200	Hybridoma culture supernatants (BACKES et al., 2010)
anti-mouse MFP488 (A1001)	1:200	Mobitec, Berkheim, Germany
anti-rat HRP	1:20,000	BioLegend, San Diego, USA

Antibodies used for Immunostaining		
Specificity	**Dilution**	**Company**
rabbit anti-VACV	1:2,000	Acris GmbH, Arnbruck, Germany
goat anti-rabbit	1:5,000	Jackson Immuno Research, West Grove, USA

Tab. 2: Antibodies.

3. Peptides

The HCMV-specific peptides (JPT, Berlin, Germany) used in this study are listed in Table 3, had >70% purity, and were dissolved in 100% dimethyl sulfoxide (DMSO).

Antigen	Amino acids sequence (short name)	HLA restriction	Amino acids position	References
HCMV IE-1	**VLE**ETSVML (VLE)	HLA-A*02:01	316-324	(KHAN et al., 2002)
	ILEETSVML (ILE)	HLA-A*02:01	316-324	
	CRVLCCYVL (CRV)	HLA-C*07:02	309-317	(AMERES et al., 2013)
	ELKRKMMYM (ELK)	HLA-B*08:01	199-207	(ELKINGTON et al., 2003)
	TMYGGISLL (TMY)	HLA-A*02:01	297-304	(GALLEZ-HAWKINS et al.,
	KEVNSQLSL (KEV)	HLA-B*40:01	42-50	(KHAN et al., 2007)
	RIKEHMLK (RIK)	HLA-A*03:01	99-107	(AMERES et al., 2013)
HCMV pp65	**NLV**PMVATV (NLV)	HLA-A*02:01	495-503	(DIAMOND et al., 1997)
	TPRVTGGGAM (TPR)	HLA-B*07:02	417-426	(WILLS et al., 1996)
	RPHERNGFTVL (RPH)	HLA-B*07:02	265-275	(LONGMATE et al., 2001)
	IPSINVHHY (IPS)	HLA-B*35:01	123-131	(GAVIN et al., 1993)

Tab. 3: Peptides.

4. Peptide/human leucocyte antigen (HLA) multimers

Antigen	Peptide	HLA restriction	Type of multimer	Used in donor
HCMV pp65	**NLV**	A*02:01	dextramer-PE	A
HCMV pp65	**NLV**	A*02:01	pentamer unlabeled	C-E
HCMV IE-1	**VLE**	A*02:01	pentamer unlabeled	A, C-E
HCMV IE-1	**ELK**	B*08:01	pentamer unlabeled	A
HCMV pp65	**IPS**	B*35:01	pentamer unlabeled	E
HCMV IE-1	**CRV**	C*07:02	streptamer	B
HCMV pp65	**TPR**	B*07:02	pentamer unlabeled	B
HCMV pp65	**RPH**	B*07:02	pentamer unlabeled	B

Tab. 4: Peptide/HLA multimers.

Companies:

Pentamers (n=5) unlabled and Pro5 Fluorotag R-PE (PROIMMUNE, Oxford, England); Dextramer-PE (>5) (Immudex, Kopenhagen, Denmark); Streptamer (n=4) (Fabian Schlott/ Dirk Busch/ Michael Neuenhahn, Institute for Medical Microbiology, Immunology and Hygiene, TU München).

5. Cells

5.1. Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from human buffy coats (Institute of Transfusion Medicine, University of Ulm, Germany). CD40-stimulated B cells and polyclonal T cell lines were generated from PBMC in this study (Sections III.5.5 and III.5.6). Murine fibroblasts expressing human CD40 ligand (LL8) were used to keep CD40 B cells in culture. LL8 cells, PBMC, polyclonal T cells, and CD40-stimulated B cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin/ 100 µg/ml streptomycin (P/S), and 100 nM sodium selenite (Se) (“standard

medium").

Chicken embryo fibroblasts (CEF) were freshly prepared from pathogen-free chicken eggs (Charles River SPAFAS or VALO Biomedia) and incubated for 11 days. CEF and baby hamster kidney cells (BHK) were maintained in Minimum Essential Medium Eagle (MEM) supplemented with 10% FCS, P/S, and 1% non-essential amino acids (NEAS).

Human keratinocyte cell line (HaCaT) was maintained in VLE Dulbecco's MEM (DMEM) supplemented with 10% FCS, P/S, and 2% 1 M HEPES.

Cells were cultivated in a humidified incubator at 37°C under 5% CO_2. During infection, all cells were kept in medium with 2% FCS. Dense cell culture flasks were split 1-2 times per week. Trypsin-EDTA was added to detach the cells.

5.2. Cell counting

Cells were diluted (1:1 or 1:10) with Trypan blue solution containing of 0.1% trypan blue and 0.2% sodium azide in sodium phosphate buffer for live/death staining before counting with a Neubauer Chamber.

5.3. Cryopreservation of cells

PBMC (donor A-E), polyclonal T cells (donor A+B), and CD40-stimulated B cells (donor A+B) were resuspended in cold RPMI-1640/FCS/DMSO mixed at a ratio of 5:4:1. The polyclonal T cells (donor C-E) and CD40-stimulated B cells (donor C-E) were resuspended in cold FCS/DMSO at a ratio of 9:1 immediately before transfer to -80°C. Long-term storage was in the gas phase over liquid nitrogen.

5.4. Isolation of peripheral blood mononuclear cells

PBMC were purified from buffy coats of five CMV positive healthy HLA class I typed donors (donor A, A1/A2 B8/B60; donor B, A3/A28 B7/B63; donor C, A2/A2 B44/B60; donor D, A2/A3 B47/B60; donor E, A2/A11 B35/B44) by density gradient centrifugation using Ficoll/Hypaque (Biochrom). The total volume of each buffy coat was divided equally into 2 50 ml falcon tubes, filled up to 40 ml with phosphate buffered saline (PBS) and mixed. The buffy coat/PBS mixture was gently underlaid with 10-12 ml Ficoll and centrifuged at 1,200 × g for 25 min at room temperature. Afterwards, the PBMC layer was carefully harvested, washed 4-5 times by adding 50 ml PBS and centrifuged at 350-400 × g. Finally,

PBMC were counted and partly used for generation of permanent CD40-stimulated B cells. The remaining PBMC were cryopreserved for later use.

5.4.1. HCMV IgG serostatus in blood donors

HCMV serostatus was determined from diluted plasma by HCMV-ELISA kit (EnzygnostR Anti-CMV/IgG, Siemens) following the supplier's instructions.

5.5. Generation of CD40-stimulated B cells

CD40-stimulated B cells were generated from PBMC according to Wiesner et al, 2008.

Firstly, LL8 cells were irradiated with 180 Gy and plated either in 24-well or 96-well flat bottom plates. After 1 day, PBMC were added on LL8 cells in different concentrations (2.5, 5, 10, 20 $\times$ 10^4 cells per well of a 96-well plate or 12.5, 25, 50, 100 $\times$ 10^4 cells per well of a 24-well plate). Standard cell culture medium was supplemented with IL-4 (2 ng/ml). Reactivation of T cells and NK cells was prevented by adding 1 µg/ml cyclosporine A throughout the first four weeks of cultivation. B cells were seeded on newly irradiated LL8 cells once per week until stably proliferating CD40 B cells were obtained on 12-well plates for each donor. The lowest initial concentration of PBMC leading to stable CD40 B cell cultures were chosen for further cultivation. Cells were cultured in 12-well plates, split 1-2 times per week, and seeded on recently irradiated LL8 cells until they were used for later experiments.

5.6. Generation of HCMV-specific polyclonal T cell lines

Polyclonal peptide-stimulated T cell cultures were generated from cryopreserved PBMC of HCMV seropositive donors. For this purpose, about 15 $\times$ 10^6 PBMC were cultured together with one of the following HCMV-specific peptides (5 µg/ml): NLVPMVATV, pp65 aa 495-503, HLA-A*02:01-restricted (abbreviated NLV) (DIAMOND et al., 1997); VLEETSVML, IE-1 aa 316-324, HLA-A*02:01-restricted (abbreviated VLE) (KHAN et al., 2002); ILEETSVML, IE-1 aa 316-324, HLA-A*02:01-restricted (abbreviated ILE), CRVLCCYVL, IE-1 aa 309-317, HLA-B*07:02-restricted (abbreviated CRV) (AMERES et al., 2013), and RPHERNGFTVL, pp65 aa 265-275, HLA-B*07:02-restricted (abbreviated RPH) (LONGMATE et al., 2001), in a final volume of 5 ml for 1 h at 37°C.

Afterwards, cells were washed 3 times with PBS and centrifuged at 350 × g for 10 min each. Thereafter, PBMC were resuspended to a final concentration of 2.5×10^6 cells/ml in standard medium supplemented with IL-2 (25 U/ml). Then, cells were incubated for 10 days at 37°C. At days 4 and 7, 50% of the cellular supernatant was replaced by fresh medium supplemented with IL-2 (25 U/ml). After 10 days all cells were harvested, counted, and stored in aliquots in gas phase over liquid nitrogen until they were used in further experiments.

6. Viruses

6.1. Viruses used in this study

All HCMV-MVAs had been generated by homologous recombination before the start of this study.

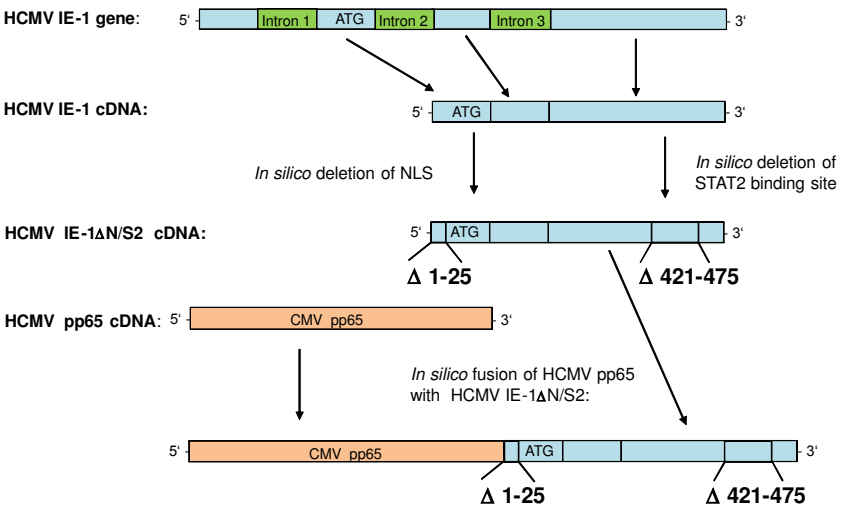


Fig. 3: *In silico* construction of the HCMV pp65/IE-1 gene. Gene sequences of pp65 and IE-1 originate from HCMV strain TB40E. Modified IE-1 (IE-1ΔN/S2) lacks the nuclear localization signal (NLS) and the STAT2 binding site. A fusion gene of IE-1ΔN/S2 and pp65 was synthesized *de novo* for cloning into MVA (personal information by M. H. Lehmann).

MVA-pp65/IE-1 was generated based on MVA (clonal isolate F6) as described in (MEYER et al., 1991). The full length pp65 gene (HCMV strain TB40E) and a truncated version of the IE-1 gene (HCMV strain TB40E) lacking the nuclear localization signal (NLS; aa, 1-25) and the STAT2 binding site (aa, 421-475), were constructed *in silico* as a fusion gene (Fig. 3), gene synthesized, cloned into plasmid pIIIH5redK1L, included into deletion site III of the MVA genome by homologous recombination and encoded under transcriptional control of the Modified Vaccinia virus promoter PmH5 (WYATT et al., 1996). Marker gene

deletion occurred during plaque passaging. Those virus plaques, missing the marker gene, were selectively picked and separately amplified (Fig. 4). MVA clonal isolate F6 (MEYER et al., 1991) was used as non-recombinant control virus. The MVA-IE-1 and MVA-pp65 viruses were generated based on MVA-II new (STAIB et al., 2003). Either the full length pp65 gene or the full length IE-1 gene of the HCMV strain AD169 was inserted into deletion site III of the MVA genome by homologous recombination and put under the control of the PmH5 and the VACV E3L promoter, respectively.

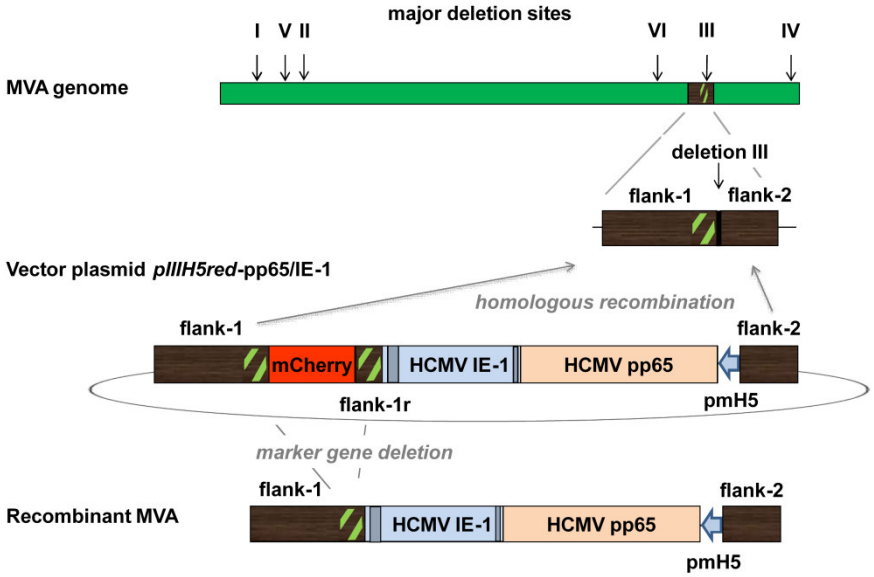


Fig. 4: Scheme of recombinant MVA-pp65/IE-1 generation. Locations of the six major deletion sites (I-VI) are indicated. The HCMV-pp65/IE-1 fusion gene was introduced into deletion III of MVA using plasmid pIIIH5red-pp65/IE-1. Deletion III flanking nucleotide sequences of MVA-genome (flank-1, flank-2). Flank-1 repeat sequence (flank-1r).

6.2. Virus handling

Viruses were long term stored at -80°C. Before using viruses in assays, they were thawed on ice and always sonicated 3 times for 1 min each.

6.3. Amplification/Purification

Amplification of viruses was performed on CEF cell monolayers. Cells were infected with virus and incubated for 3 to 5 days at 37°C. Flasks were frozen when widespread cytopathic effects were observed.

Cells were subjected to 3 freeze-thaw cycles and centrifuged at 36,057 × g for 3 h at 4°C (Avanti J-26XP, Beckman Coulter). Pellets were carefully resuspended in a total volume of 20 ml virus buffer (10 mM Tris-HCl; pH 9.0). Thereafter, this suspension was sonicated 3 times for 15 sec and centrifuged at 290 × g for 5 min at 4°C. Then, the supernatants were collected and pellets were resuspended in 5 ml virus buffer (10 mM Tris-HCl; pH 9.0). These steps were repeated 3 times.

Next, the supernatant was purified by 36% sucrose gradient centrifugation. For this purpose, autoclaved plastic tubes were filled with 15 ml sucrose and 40 ml supernatant was carefully added to the buffer before centrifugation (Optima™ LE-80K Ultracentrifuge, Beckman Coulter) at 40,500 × g for 90 min at 4°C.

Finally, the pellets were resuspended in virus buffer (10 mM Tris-HCl, pH 9.0) in a total volume of 1.5 to 3 ml depending on pellet size and stored in -80°C.

6.4. Titration/Immunostaining

Virus titration was performed on confluent CEF monolayers in 6-well plates. Tenfold serial virus dilutions (10^{-1}-10^{-9}) were added in duplicates to CEF cells and incubated for 2 h at 37°C. After washing with 2 ml MEM/P/S/NEAS, MEM/2%FCS/P/S/NEAS was added. Then, plates were incubated for 48 h at 37°C. Fixation and permeabilization of cells was performed with ice-cold acetone-methanol (1:2) at room temperature for 5 min. Unspecific binding was blocked by PBS/3%FCS overnight at 4°C.

Rabbit anti-VACV (Acris GmbH) was added at the dilution 1:2,000 in PBS/3%FCS for 1 h at room temperature. Plates were shaken during incubation to ensure antibody distribution. Plates were washed 2 times with PBS. Secondary goat anti-rabbit (Jackson Immuno Research) was added at the dilution 1:5,000 for 1 h in PBS/3%FCS. Cells were washed 2 times with PBS and stained with 0.5 ml True Blue™ Peroxidase (KPL) per well. Finally, plaques were counted after approximately 20 min.

6.5. Multi-step growth kinetic assays of recombinant HCMV-MVAs

CEF and HaCaT monolayers were infected with MVA, MVA-IE-1, MVA-pp65, and MVA-pp65/IE-1 at a multiplicity of infection (MOI) of 0.01. After 30 min of incubation at 4°C cells were washed with MEM/P/S/NEAS medium and thereafter 2 ml MEM/2%FCS/P/S/NEAS or DMEM/2%FCS/P/S/HEPES were added, respectively. Cells were incubated at 37°C and frozen at different points in time post infection (0, 4, 12, 24, 48, or 72 h). Cells and supernatants were subjected to 3 freeze-thaw cycles, resuspended and 750 µl per well was harvested for virus plaque assay. Virus-titration was performed on 6-well CEF monolayers. Samples from HaCaT and CEF cell monolayers were diluted at 10^{-6} and 10^{-8}, respectively. Virus titration was performed as described in Section III.6.4.

7. Polymerase chain reaction (PCR)

7.1. MVA 6-deletion PCR

BHK were infected with MVA at an MOI of 5 and incubated for 24 h. Cells and supernatants were harvested, centrifugated at 290 × g for 5 min, and resuspended in a remaining volume of 250 µl. Genomic viral DNA was purified using QIAamp DNA Mini Kit (Qiagen) following the company's protocol. In order to yield a higher concentration of DNA, the samples were digested for 3 h with proteinase K in the first step of purification.

Polymerase chain reaction (PCR) was performed using 50 ng DNA (1 µl) and PCR-mixture of 24 µl composed of:

19.8 µl distilled water
2.5 µl buffer (10×)
0.5 µl forward oligonucleotide primer
0.5 µl reverse oligonucleotide primer
0.5 µl dNTP's
0.2 µl Dynazyme II

MVA-Del1-6 oligonucleotide primers were used, as listed in Tab. 1. The following PCR-protocol was applied for 6-deletion PCR using peqSTAR 2× thermocycler (PEQLAB Biotechnology GmbH). Initial denaturation was performed for 3 min at

95°C followed by 30 cycles of denaturation for 30 sec at 95°C, primer-hybridization for 45 sec at 57°C, and elongation for 1 min and 15 sec at 72°C. Final elongation step was conducted for 5 min at 72°C.

7.2. CD40 B cells dual PCR

CD40-stimulated B cells were pelleted, supernatants were removed and cells were resuspended in 200 µl PBS. Samples were treated with proteinase K for 3 h and DNA was extracted using QIAmp DNA Mini Kit (Qiagen) by following the manufacturer's protocol. PCR was performed using gp85c and gp85d as oligonucleotide primers suitable for amplification of BXLF2 gene. Additionally, GAPDH-F3 and GAPDH-B4 oligonucleotide primers were added to amplify cellular GAPDH. B95.8-infected B lymphoblastoid cell line (kindly provided by Xiaoling Liang, Helmholtz Zentrum München) served as positive control. Dual PCR using peqSTAR 2× thermocycler (PEQLAB Biotechnology GmbH) was performed with initial denaturation for 3 min at 96°C, 30 cycles of denaturation at 96°C, primer-hybridization at 59°C, and elongation at 72°C were conducted for 45 sec each. Final elongation lasted for 5 min at 72°C.

8. Gel electrophoresis

PCR products were size-separated by agarose gel electrophoresis. Gel Red™ was used to visualize nucleic acids. DNA was mixed with loading buffer before it was loaded onto the gel. The composition of buffers is described in Appendix IX.1. Ready to use DNA standard (F-303SD, Thermo Fisher Scientific) was applied as molecular weight marker. Furthermore, gel ran in 1 × TAE buffer and nucleic acid was detected using ChemiDoc™MP, Imaging System (Bio-Rad).

9. Western blot analysis

9.1. Verification of HCMV gene expression in recombinant MVAs

BHK monolayers were infected with MVA, MVA-IE-1, MVA-pp65, or MVA-pp65/IE-1 at an MOI of 5. After 24 h, supernatants were removed and cells were harvested with lysis buffer and kept on ice. Samples were loaded onto 4-20% Criterion TGX stain free gel (Bio-Rad) and protein electrophoresis (Power Ease 500, Invitrogen life technologies) was performed in Tris-Glycine buffer (1×, Bio-Rad) for 90 min at 100 V. Precision Dual Color protein ladder (Bio-Rad,

Fig. 7: red) was used as molecular weight marker. Proteins were transferred onto a 0.2 µm nitrocellulose membrane (Bio-Rad) using Bio-Rad Trans Blot Turbo system. Blot was blocked in Tris-buffered saline and 0.1% Tween20 (TBS/T) supplemented with 5% BSA overnight. Membrane was washed 3 times in TBS/T for 10 min per wash.

Mouse anti-CMV pp72 IEA (1:500, Ray Biotech, Inc.), mouse-anti-CMV pp65 (1:200, Santa Cruz), and mouse anti-GAPDH (1:10,000, Millipore) were added at the indicated dilutions in TBS/T/5% BSA for 1 h. Membrane was washed 3 times in TBS/T for 10 min per wash. Secondary anti-mouse MFP488 (1:200, Mobitec A1001, Fig. 7: blue) was added in TBS/T/5% BSA for 1 h and membrane was washed 3 times for 10 min each time in TBS/T. Rat polyclonal anti-C7L (1:200) (BACKES et al., 2010) was added in TBS/T/2.5% milk for 1 h. Membrane was washed 3 times in TBS/T for 10 min per wash. Secondary anti-rat HRP (BioLegend, Fig. 7: green) was added at a dilution 1:20,000 for 1 h in TBS/T/2.5% milk. Positive signals were visualized using Clarity™ ECL Western Blotting Substrate using a ChemiDoc™MP Imaging System (Bio-Rad) and analyzed with Image Lab 5.0 software (Bio-Rad).

9.2. Verification of HCMV gene expression in fifth passage of MVA-pp65/IE-1

BHK monolayer was infected with MVA-pp65/IE-1 at an MOI of 0.01. After 60 h, cells and supernatant were harvested and virus titer was determined as described in Section 6.4. First passage was diluted 1:1,000 before infecting the next BHK monolayer to obtain roughly the same MOI of 0.01. Passages 2 to 5 were performed similarly.

The titer of the fifth passage of MVA-pp65/IE-1 was verified (Section III.6.4). To obtain single plaques the fifth passage of MVA-pp65/IE-1 was seeded in 10-fold serial dilutions on BHK monolayers in 96-well plates. After 48 h, 30 single plaques were picked and amplified on BHK monolayers for 4 days before Western blot was performed.

Supernatants were removed, cells were resuspended in lysis buffer and kept on ice until usage. Samples were loaded onto a 10% SDS-page gel and protein electrophoresis (Power Ease 500, Invitrogen life technologies) was performed in

Tris-Glycine buffer for 90 min at 100 V. Precision Dual Color protein ladder (Bio-Rad) was used as molecular weight marker. Proteins were transferred onto a 0.2 µm nitrocellulose membrane (GE Healthcare) using Bio-Rad Trans Blot Turbo system. The membrane was incubated in Tris-buffered saline and 0.1% Tween20 (TBS/T) supplemented with 5% BSA overnight to block unspecific binding of antibodies. Membrane was washed 3 times in TBS/T for 10 min per wash.

Mouse anti-CMV pp72 IEA (1:500, Ray Biotech, Inc.) and mouse anti-GAPDH (1:10,000, Millipore) were added in TBS/T/5% BSA for 1 h. The membrane was washed 3 times in TBS/T for 10 min each. Secondary anti-mouse MFP488 (Mobitec A1001) was added (1:200) in TBS/T/5% BSA for 1 h. Afterwards, membrane was washed 3 times in TBS/T for 10 min. Positive signals were visualized using a ChemiDoc™MP Imaging System (Bio-Rad) and analyzed with Image Lab 5.0 software (Bio-Rad).

10. Immunological assays

10.1. Propagation of HCMV-specific CD8 T cells by stimulation with MVA-infected cells

CD40 B cells from established cultures were harvested, centrifuged at 350 × g for 10 min and resuspended in standard medium. Then, cells were irradiated (54 Gy), infected with MVA, MVA-IE-1, MVA-pp65, or MVA-pp65/IE-1 at an MOI of 0.2 and incubated for 2 h at 37°C. PBMC and MVA-infected CD40 B cells were cultured together in 2 ml at a rate of 5:1. The total number of PBMC varied between 8-40 × 10^6 cells. At day 4 and day 7, 50% fresh medium (RPMI-1640/10%FCS/P/S/Se) supplemented with IL-4 (25 U/ml) was added to the cells. After 10 days, cells were harvested, counted, and stored in aliquots in gas phase over liquid nitrogen until used in further analyses.

10.2. Activation of IFN-γ-producing HCMV-specific polyclonal T cells after incubation with MVA-infected CD40 B cells

Autologous CD40 B cells were infected with MVA, MVA-IE-1, MVA-pp65, or MVA-pp65/IE-1 at an MOI of 0.5 and incubated for 2 h at 37°C. CD40 B cells cultured together with one of the following HCMV-specific peptides (5 µg/ml),

NLV; VLE; ILE; CRV; or RPH for 1 h served as positive controls. After peptide loading, B cells were washed once with 50 ml PBS. The polyclonal T cells were thawed, resuspended in pre-warmed standard medium, and counted. 2.5×10^4 CD40 B cells and 5×10^4 polyclonal T cells per well of a V-bottom 96-well plate were co-cultured in triplicates for 16-18 h at 37°C. Plates were centrifuged at 350 × g for 10 min and 50 µl supernatant per well was used for IFN-γ detection. IFN-γ secretion was measured with human-IFN-γ ELISA kit (MABTECH) following the manufacturer's instructions.

Measurement of optical density at 405 nm was conducted in an ELISA reader (Universal Microplate Reader EL-800, BIO-TEK Instruments) and analyzed using KC4 software (TreeStar Inc.).

11. Fluorescence activated cell sorting (FACS)

11.1. Preparation of samples for flow cytometry analysis

$PBMC_{d0}$ (6×10^5 cells/peptide) and $PBMC_{d10}$ (3×10^5 cells/peptide), after 10 days propagation of HCMV-specific T cells, were stained in a 2 or 3 step process, depending on the HLA/peptide multimer that was applied.

Cells were washed with 900-950 µl PBS/2%FCS between each incubation step and centrifuged for 3 min at 1,000 × g. Cells were stained with different HLA/peptide multimers (Tab. 4) in 25 µl PBS/2%FCS for 15 min at room temperature. Afterwards, cells were stained with APC-conjugated anti-CD8 antibody, FITC-conjugated anti-CD19 antibody (donor A, donor C-E), and additionally with PE-conjugated Fluorotag (Proimmune), if HLA/peptide multimers (Proimmune) had been applied in the first step. Donor B was stained with FITC-conjugated anti-CD158b (KIR2DL2/3) instead of FITC-conjugated anti-CD19 antibody (Fig. 14).

Cells were analyzed on a FACS Calibur (BD Biosciences). Cells were gated based on forward/side scatter and an additional lymphocyte gate was included. $0.5\text{-}1 \times 10^5$ lymphocyte-gated events were counted per sample. Data was analyzed with CellQuest™ Pro Software (BD Biosciences).

11.2. Calculation of HCMV-specific T cell propagation and specific expansion

Propagation (P) and specific expansion (E) of HCMV-specific T cells within the PBMC population were calculated out of the analyzed flow cytometer data using the following formulae:

Propagation (P):

$$P = \frac{(AxB)_{d10} - (CxD)_{d10}}{(AxB)_{d0} - (CxD)_{d0}}$$

A = proportion of multimer positive cells within CD8 T cells

B = proportion of CD8 T cells within the total number of PBMC

C = proportion of multimer positive cells within CD8 T cells in negative control (staining without MHC class I/peptide complex)

D = proportion of CD8 T cells within the total number of PBMC in negative control (staining without MHC class I/peptide complex)

$d10$ = PBMC at day 10 after propagation of HCMV-specific T cells

$d0$ = PBMC at day 0

Specific expansion (E):

$$E = P\ x\ \frac{PBMC_{d10}}{PBMC_{d0}}$$

$PBMC_{di}$ = total number of PBMC after i days, where $i \in \{0,10\}$.

12. Propagation of IFN-γ producing HCMV IE-1-specific T cells

12.1. Cell suspension analyzed with ELISPOT assay

ELISPOT plates (Multi Screen HTS Filter plates, Millipore) were pre-coated with anti-IFN-γ antibody (mAb 1-D1K) at a dilution of 15 µg/ml (50 µl per well) and incubated overnight at 4°C. Then, plates were washed 5 times with PBS/0.05% Tween20 and blocked with 200 µl standard medium per well for at least 30 min at room temperature. $PBMC_{d0}$ (1×10^5 cells/well) were incubated in coated ELISPOT wells with HCMV-specific peptides: VLE; ILE; KEVNSQLSL, IE-1 aa 42-50, HLA-B*40:01-restricted (abbreviated KEV); RIK aa 99-107, HLA-A*03:01-restricted (abbreviated RIK); and TMYGGISL, IE-1 aa 297-304, HLA-A*02:01-restricted (abbreviated TMY) in triplicates for 16-18 h. $PBMC_{d10}$ were thawed and counted. $PBMC_{d10}$ (2.5×10^4/well) were incubated in triplicates together with CD40 B cells (5×10^4/well) and HCMV-specific peptides (5 µg/ml) on ELISPOT plates for 16-18 h. IFN-γ spots were developed using the human IFN-γ ELISPOT Kit from Mabtech (Nacka Strand, Sweden) following the company's instructions. IFN-γ spots were counted with ImmunoSpot 5.0 Analyzer ProCD (ImmunoSpot, Cellular Technology Ltd. C.T.L.).

12.2. Supernatants analyzed with ELISA

$PBMC_{d10}$ were thawed and counted. 2.5×10^4 $PBMC_{d10}$/well were incubated together with autologous CD40 B cells (5×10^4/well) and different HCMV IE-1 peptides (5 µg/ml): VLE; ILE; KEV (KHAN et al., 2007); RIK (AMERES et al., 2013); and TMY (GALLEZ-HAWKINS et al., 2003) in triplicates for 16-18 h. Supernatants were analyzed with human IFN-γ ELISA Kit from Mabtech. 96-well flat bottom plates (Nunc Maxisorp) were pre-coated with anti-IFN-γ antibody (mAb 1-D1K) in a dilution of 2 µg/ml which stayed on the plates overnight at 4°C. Then, plates were washed 5 times each with PBS/0.05%Tween20 and blocked with 200 µl standard medium (RPMI-1640/10%FCS/P/S/Se) per well for 1 h at room temperature. Cytokine standard was prepared (triplicates of dilutions: 20,000; 4,000; 800; 160; 32 pg/ml). Supernatants and cytokine standards (50 µl/well) were added to the antibody-coated 96-well flat bottom plates and incubated for 2 h at room temperature. After washing 5 times, secondary antibody (7-B6-1-Biotin) was added and incubated for 1 h, followed by 5 times

washing. Streptavidin-alkaline phosphatase (ALP) was added for 1 h and plates were washed 5 times. ALP substrate solution containing 10% diethanolamine, 1 mg/ml para-nitrophenyl-phosphate (p-NPP), and 0.8 mM $MgS0_4$ was added and incubated for 10 min to 2 h as needed. Absorption was measured with a Universal Micro plate Reader (EL800, Bio Tec. Instruments) at 405 nm and analyzed using KC4 software (TreeStar Inc.).

IV. RESULTS

1. Genetic characterization of recombinant HCMV-MVAs

This study focuses on the characterization of our most promising MVA-pp65/IE-1 vaccine candidate. To begin with, a series of experiments was conducted in order to genetically analyze MVA-pp65/IE-1 as well as the other recombinant MVAs that served as comparisons in the following studies.

1.1. Verification of correct insertion of HCMV genes and of MVA backbone integrity

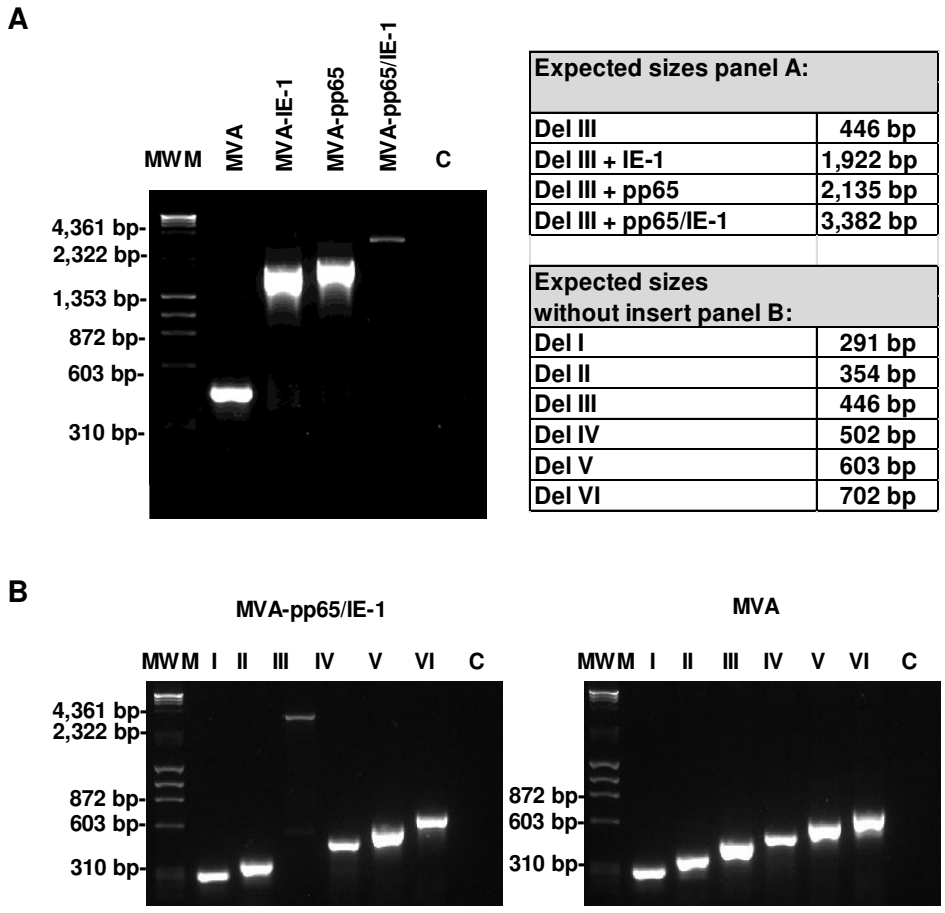
Our lab had established MVA-specific oligonucleotide primer sets (MVA-Del I-VI) that are frequently used in PCR analyses to ensure proper insertion of foreign genes into our recombinant MVA viruses as well as their genetic purity and MVA integrity. Those MVA-Del I-VI oligonucleotide primers had been designed in a way that they flank the six major deletion sites of the non-recombinant MVA genome and amplify one distinct PCR fragment of specific size, each (Fig. 5, “Expected sizes without insert panel B”) (KREMER et al., 2012).

All recombinant HCMV-MVAs used in this study had been generated by homologous recombination of nucleotide sequences encoding HCMV genes into the existing deletion site III (Del III) of the MVA backbone. Thus, specific MVA-Del III oligonucleotide primers were applied to amplify PCR fragments of predetermined molecular weights (Fig 5a, “Expected sizes panel A”) from DNA of MVA by PCR.

The expected molecular weights of the PCR fragments had been calculated by adding the respective nucleotide sequences’ length of IE-1, pp65, and pp65/IE-1 to the sizes of the corresponding PCR fragments shown in Fig. 5a, “Expected sizes without insert panel B”.

Presence of predicted PCR fragments was detectable in all samples except the negative control, in which DNA was replaced by water. Furthermore, the specific non-recombinant MVA PCR band (446 bp) was only detectable in the control sample, which contained DNA from a non-recombinant MVA, and was absent from all tested recombinant HCMV-MVAs (Fig. 5a). These observations

confirmed the correct insertion of HCMV genes into Del III and genetic purity of all recombinant HCMV-MVAs.



Expected sizes panel A:	
Del III	446 bp
Del III + IE-1	1,922 bp
Del III + pp65	2,135 bp
Del III + pp65/IE-1	3,382 bp

Expected sizes without insert panel B:	
Del I	291 bp
Del II	354 bp
Del III	446 bp
Del IV	502 bp
Del V	603 bp
Del VI	702 bp

Fig. 5: PCR assay to check for proper insertion of HCMV genes and MVA identity. PCR from viral DNA was performed using specific oligonucleotide primers for deletion I-VI (Del I - Del VI). (A) HCMV-gene insertion proven by deletion III PCR from viral DNA of MVA, MVA-IE-1, MVA-pp65, and MVA-pp65/IE-1. (B) PCR of MVA-pp65/IE-1 (left panel) and wild-type MVA (right panel) using MVA-Del 1 - MVA-Del 6 oligonucleotide primers. Molecular weight marker (MWM); Control PCR without DNA (C). Del I-VI (I-VI).

Moreover, MVA-pp65/IE-1 candidate vaccine was tested for genetic integrity of the MVA backbone. By adding the corresponding oligonucleotide primer set, six PCRs (Del I-VI) of viral DNA were performed for either MVA-pp65/IE-1- or MVA-infected cells, each. PCR fragments of both viruses were visualized and compared to identify differences in their molecular weight sizes. As expected, all PCR products showed similar results for all PCRs except from Del III PCR (Fig. 5b).

Finally, the genetic stability of MVA-pp65/IE-1 was checked by passaging the virus five times in BHK. Viral DNA was isolated and all six PCRs (Del I-VI) were

repeated. The PCR fragments showed no detectable differences in size compared to the previously conducted PCRs from the first passage of MVA-pp65/IE-1. Moreover, in Del III PCR the specific non-recombinant MVA PCR band (446 bp) was still absent (Fig. 6).

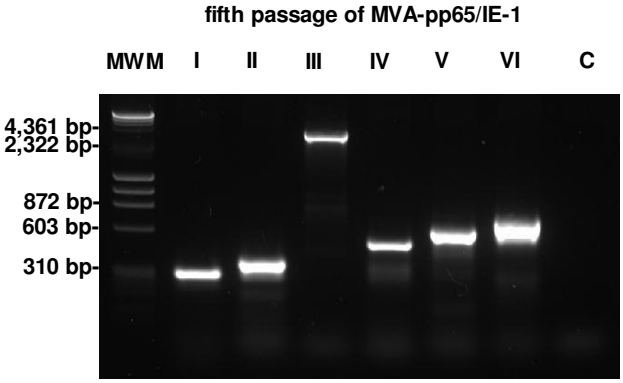


Fig. 6: PCR assay of fifth passage of MVA-pp65/IE-1 to check for stable insertion of HCMV genes and stable MVA identity. PCR from viral DNA after 5 times passaging in BHK was performed using specific oligonucleotide primers for deletion I-VI (I -VI). Molecular weight marker (MWM); Control PCR without DNA (C).

These results confirmed the correct insertion of HCMV genes into Del III for any virus used in this study (Fig. 5a). In addition, integrity, stability, and purity of the first and fifth passage of MVA-pp65/IE-1 was verified (Fig. 5b, left and 6).

1.2. MVA-pp65/IE-1 stably expresses a fusion of the two HCMV antigens

Modifications of the nucleotide sequences encoding for proteins, as e.g. the deletion of some parts, may strongly influence the secondary and tertiary structure of the produced proteins. Hence, all HCMV-MVAs used in this study were verified for protein expression.

1.2.1. Western blot analysis of the first passage of MVA-pp65/IE-1

Western blot experiments were carried out to detect expression of pp65 and IE-1 on BHK infected with recombinant HCMV-MVAs at an MOI of 5. After 24 h, MVA-infected cell lysates were developed by Western blot using anti-IE-1, anti-pp65, anti-C7L, and anti-GAPDH antibodies. Proteins were visualized and VACV protein C7L (18 kDa) and GAPDH (36 kDa) confirmed the presence of viral DNA as well as cellular material in each sample (Fig. 7).

HCMV-specific antibodies detected a specific protein band of 72 kDa (Fig. 7: lane 3) and 65 kDa (Fig. 7: lane 5) in MVA-IE-1 and MVA-pp65 infected cell lysates, respectively. Additionally, three fainter bands of lower weight, between 20 and 50 kDa, were detected in MVA-pp65-infected cell lysates.

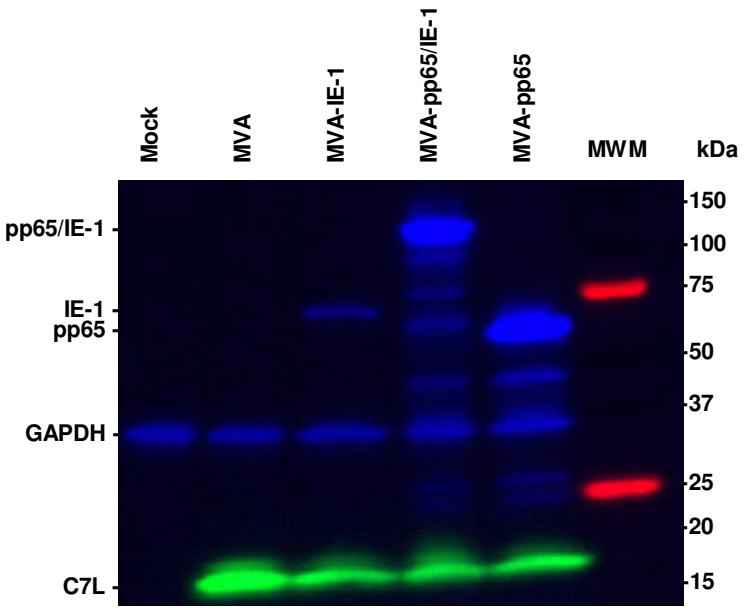


Fig. 7: Verification of HCMV gene expression in recombinant MVA. BHK were infected with viruses as indicated. Cells were harvested 24 h p.i. and protein lysates were analyzed by Western blotting. Multichannel image of fluorescent blot and chemiluminescent blot images is shown. Molecular weight marker (MWM).

Noteworthy, a prominent protein band of approximately 120 kDa was detectable by immunoblot after infecting BHK with MVA-pp65/IE-1 (Fig. 7). Moreover, three fainter bands of smaller size (100 kDa, 80 kDa, 65 kDa) as well as similar bands compared to MVA-pp65 infected cell lysates (between 20 and 50 kDa) were detected.

1.2.2. Western blot analysis of the fifth passage of MVA-pp65/IE-1

The genetic stability of MVA-pp65/IE-1 was examined since earlier studies using MVA as viral vector showed genetic instabilities of recombinant MVA in rare cases (WYATT et al., 1996; WYATT et al., 2009). Hence, MVA-pp65/IE-1 was passaged five times in BHK, thirty single plaques were collected and each plaque was amplified on BHK for 96 h, before Western blots were performed using anti-IE-1, anti-GAPDH, and anti-C7L to visualize protein expression. The results confirmed the stable expression of the HCMV fusion gene by MVA-pp65/IE-1.

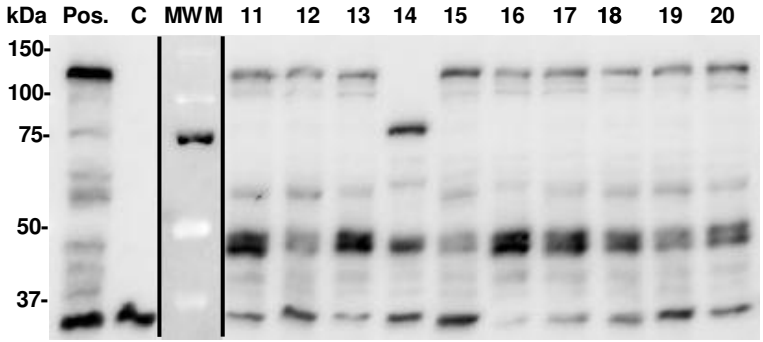


Fig. 8: Verification of stable HCMV gene expression in fifth passage of MVA-pp65/IE-1. BHK were infected with single plaques (1-30) of the fifth passage of MVA-pp65/IE-1. Cells were harvested 96 h p.i. and protein lysates were analyzed by Western blotting. A cell-lysate of BHK infected with the first passage of MVA-pp65/IE-1 for 24h served as positive control (Pos.). Non infected cells (C). Molecular weight marker (MWM). Figure shows one representative Western blot of ten plaques (11 to 20).

The HCMV-specific protein was still present in 90% of the amplified and tested MVA-pp65/IE-1 virus single plaques. However, 3 out of 30 Western blot samples (plaque no. 1, plaque no. 14, and plaque no. 27) showed different results. In these cases, the HCMV fusion protein was missing and a specific 75 kDa protein band appeared instead (Fig. 8).

These data indicate that MVA-pp65/IE-1, although missing 25 C-terminal amino acids and amino acid 421 to 475 of the HCMV IE-1 sequence, is able to efficiently and stably express the pp65/IE-1 fusion protein in BHK.

1.3. Multi-step growth kinetics of recombinant HCMV-MVAs

Before using recombinant MVA in immunotherapy or as a vaccine some important requirements have to be fulfilled.

Among others, these include the verification that the recombinant MVA has not increased its hazard potential and not changed its limited replicative host range. These two conditions are also important to ensure that the recombinant MVAs can still be handled under biosafety level 1 conditions as non-recombinant MVA (ZKBS, 1997).

One very important property of MVA is its inability to productively grow in human cells (MAYR & MUNZ, 1964; SUTTER & MOSS, 1992; CARROLL & MOSS, 1997; BLANCHARD et al., 1998). Therefore, the following experiment examined the ability of MVA-IE-1, MVA-pp65, and MVA-pp65/IE-1 to replicate in human cells by performing multi-step growth analysis in HaCaT, an established

laboratory human cell line. During a total infection period of 3 days, none of the tested recombinant MVA was able to propagate in HaCaT (Fig. 9).

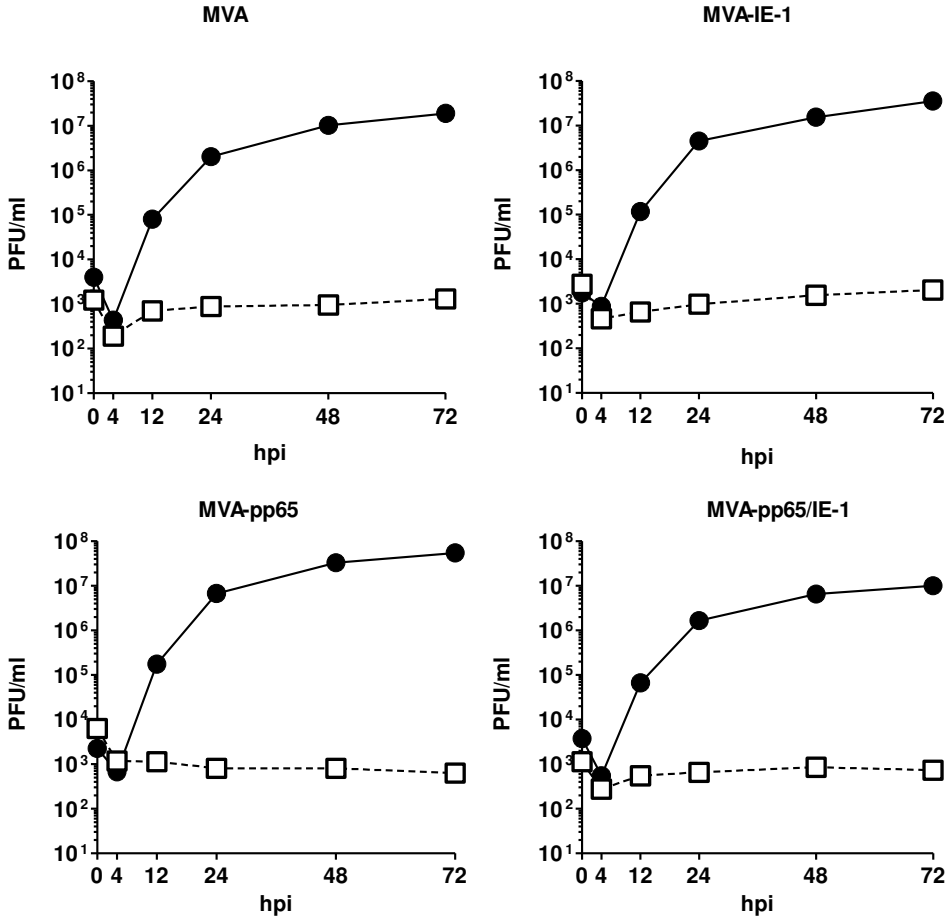


Fig. 9: Multi-step growth kinetics of recombinant MVA. CEF (●) or HaCaT (□) cells were infected with MVA, MVA-IE-1, MVA-pp65 or MVA-pp65/IE-1 at an MOI of 0.01. Samples were collected at times as indicated and titrated on CEF cells. Figures represent mean of two independent experiments. Hours post infection (hpi).

Moreover, the amplification capacity of MVA-pp65/IE-1, MVA-IE-1, and MVA-pp65 was checked in CEF cells. Here, replication of MVA is still possible. After 72 h, a 1,000 to 10,000 fold increase in virus titers was measured. The virus titer of each recombinant MVA was comparable to control MVA, indicating that replication capacity was not reduced.

2. Immunological characterization of MVA-pp65/IE-1

As expected, the previous molecular characterization showed that MVA-pp65/IE-1 productively grew in primary chicken cells, but was not able to replicate in human cells. Moreover, it was genetically stable, pure, and efficiently expressed the desired HCMV genes. In a next step, MVA-pp65/IE-1 was

immunologically characterized.

Thereby, this study focused on the activation and expansion of HCMV-specific T cells, in particular CD8 T cells, because it has been shown that the cellular immune response plays a key role in the prevention of HCMV disease (QUINNAN et al., 1982).

A straightforward approach to study this would be the infection of PBMC with recombinant MVA, followed by analysis of the expansion of HCMV-specific T cells in such a culture. Expecting an extensive literature on such protocols, surprisingly only one group could be identified that was using such an approach (LA ROSA et al., 2006).

Following a modified protocol of La Rosa et al. 2006 for one experiment, we tried to reproduce *in vitro* expansion of HCMV-specific T cells. PBMC of 2 donors, A and B, were incubated with CpG-A (1 µg/ml, Miltenyi) and CpG-B (1 µg/ml, Metabion) for 3 days. Then, CpG-treated PBMC were infected with MVA-pp65/IE-1, MVA-IE-1, MVA-pp65, and MVA at an MOI of 5 for 6 h. After 5 h, MVA-infected PBMC were γ-irradiated with 34 Gy (≙ 3400 rads) and plated as effector cells in a ratio of 1:2 with untreated PBMC as described in the protocol. Cells were checked for vitality at day 4, day 7, and day 10. Unfortunately, neither expansion of HCMV-specific T cells occurred as described by La Rosa et al. nor did many of the PBMC survive the indicated procedure resulting in a 0.5 fold decrease in total cell numbers after 10 days.

Looking for an alternative method to test the potential of recombinant MVA to expand HCMV-specific T cells, we decided to infect an antigen-presenting cell type with the virus and then co-culture it together with the PBMC. We had an established CD40 B cells system available in our lab which offered two advantages for this setting. Firstly, it is possible to easily generate long-term proliferating B cell cultures by CD40 stimulation of PBMC from any donor of interest (WIESNER et al., 2008). Secondly, we already knew that peptide-loaded CD40 B cells are able to easily expand specific T cells from autologous PBMC (ZENTZ et al., 2007; WIESNER et al., 2008).

Thus, we had to ensure that infecting CD40 B cells with recombinant MVA results in antigen presentation to T cells. For this purpose, CD40 B cells were generated

from five suitable HCMV-positive donors as described in Section III.5.5.

In a preliminary experiment, CD40 B cells were infected with the recombinant HCMV-MVAs at a range of different MOIs (0.1-5). Higher MOIs tended to be toxic for CD40 B cells, but a low MOI of 0.1-0.5 led to a satisfactory rate of infection (data not shown). Thus, an MOI of 0.5 was used for activation of IFN-γ-producing HCMV-specific polyclonal T cells after co-incubation with MVA-infected CD40 B cells and an MOI of 0.2 in the following experiments.

2.1. Verification of the absence of EBV infection in CD40 B cells

Epstein-Barr virus (EBV) is present in peripheral B cells of most healthy donors at a frequency of 1 virus in 10^4 to 10^6 B cells (BABCOCK et al., 1998) and may be reactivated and spread in a B cell culture (RICKINSON et al., 1984). Thus, most but not all CD40-stimulated B cell cultures from healthy donors are free of EBV infection (WIESNER et al., 2008).

Before using the generated CD40 B cells in the experiments that follow, we needed to ensure that the cultures were free of EBV, in order to avoid any interference by reactivation of EBV-specific T cells.

Therefore, all CD40 B cells used in this study were routinely verified for the absence of EBV-specific DNA sequences (glycoprotein 85, gp85). A dual PCR was performed where GAPDH and gp85 oligonucleotide primers were applied to detect the presence of human DNA and EBV DNA sequences, respectively. As shown in Fig. 10, PCR fragments specific for GAPDH sequences of 152 bp could be visualized in all PCR samples except the negative control (with water instead of DNA). The gp85-specific PCR band (461 bp) was only detectable in the positive control, DNA from an EBV-infected cell line, and was absent from all tested CD40 B cells. These observations confirmed that none of the used CD40 B cells were infected with EBV.

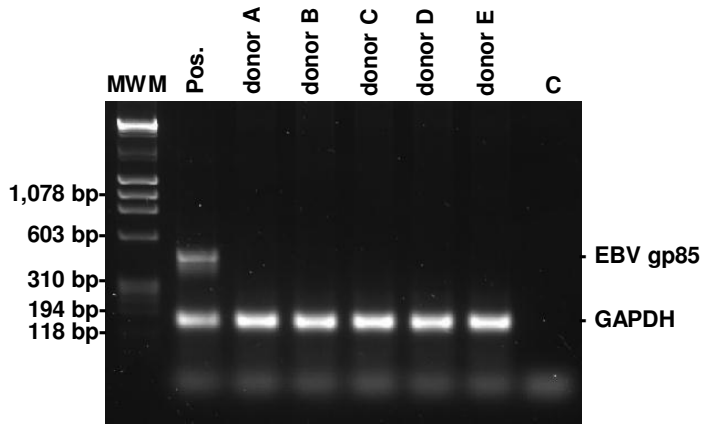


Fig. 10: Verification of the absence of EBV infection in CD40 B cells. B cells were tested for the absence of EBV using dual PCR. Molecular weight marker (MWM). DNA of EBV-infected lymphoblastoid cell line (Pos.). Control without DNA (C).

2.2. Activation of IFN-γ-producing HCMV-specific polyclonal T cells after co-incubation with MVA-infected CD40 B cells

To detect presentation of HCMV antigens on the surface of MVA-infected CD40 B cells, HCMV-specific T cells were required. Since these are present in HCMV-positive donors at considerable high frequency, polyclonal T cell lines containing a high proportion of HCMV-specific T cells can be prepared by a single round of stimulation with HCMV antigen-derived peptides (MOOSMANN et al., 2010).

Autologous polyclonal T cells were generated from PBMC of donors A and B by stimulating them with the peptides VLE, ILE, NLV, CRV, or RPH for 10 days. Thereafter, they served as effector cells to determine whether CD40 B cells are able to present recombinant HCMV antigens delivered by recombinant MVAs. To achieve that, polyclonal T cell lines were co-cultured with MVA-infected B cells and IFN-γ secretion was measured as readout for T cell activation.

Fig. 11 shows the results of this experiment. In order to cover a broader range of epitopes, PBMC of 2 donors who differed in their HLA restriction (donor A, A1/A2 B8/B60; donor B, A3/A28 B7/B63) were selected. Although the T cell lines were only short-term cultivated and specific T cells had not been purified, background reactivity was very low in 4/5 cultures. In one T cell culture (CRV), there was a higher background, but it was still an order of magnitude below recognition of HCMV antigens. Hence, the interpretation of the results was not impaired.

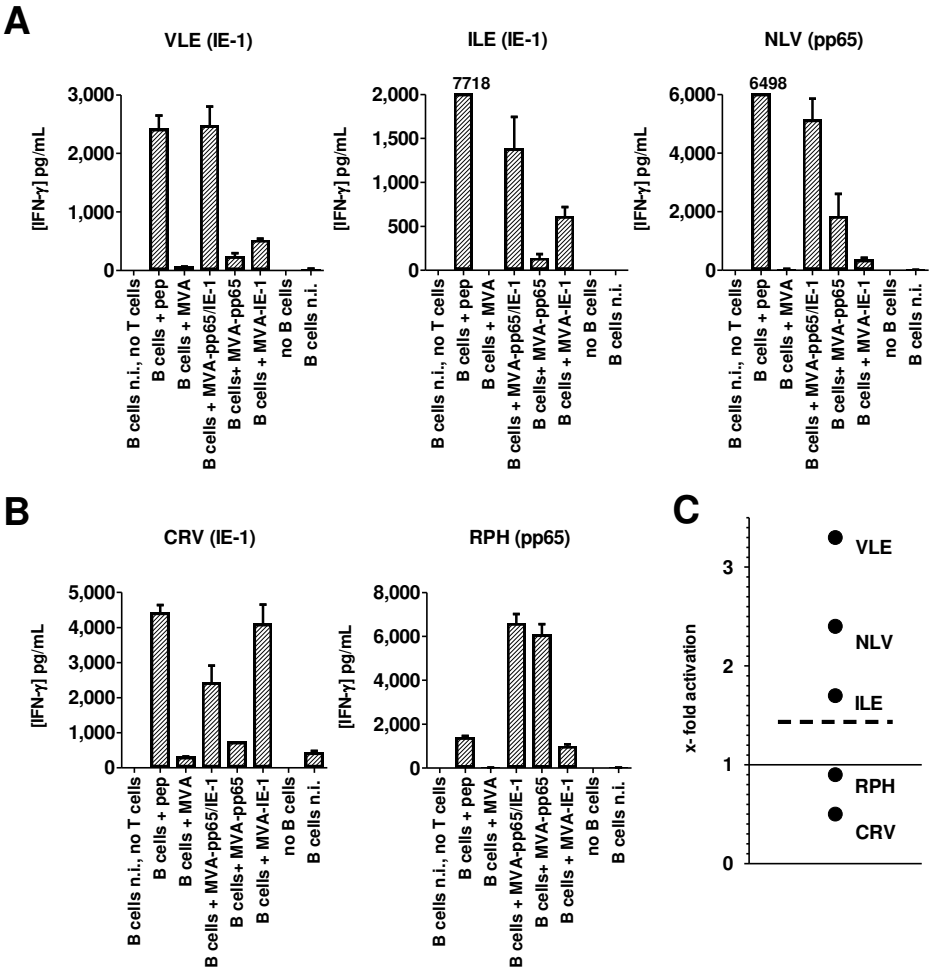


Fig. 11: IFN-γ detection of activated HCMV-specific polyclonal T cells by MVA-infected CD40 B cells. (A,B) Polyclonal T cells were generated by stimulation of PBMC with HCMV peptides as indicated in headlines for 10 days. B cells were infected with MVA, MVA-IE-1, MVA-pp65, or MVA-pp65/IE-1 and incubated with autologous specific T cells for 16-18 h. Concentrations of IFN-γ in supernatants were determined using ELISA. Error bars indicate mean and range of triplicates in one experiment. (C) x-fold activation was calculated by dividing the detected IFN-γ values of B cells + MVA-pp65/IE-1 by the sum of B cells + MVA-IE-1 and B cells + MVA-pp65, each. Dashed line indicates geometric mean of the indicated epitopes.

Both donors showed significantly higher IFN-γ levels when CD40 B cells were infected with recombinant HCMV-MVAs compared to non-recombinant MVA or non-infected CD40 B cells. This indicates that CD40 B cells are well suited to present HCMV-specific antigens delivered by recombinant MVAs.

Moreover, results of donor A showed that MVA-pp65/IE-1 infected CD40 B cells were best able to activate polyclonal T cells resulting in highest numbers of IFN-γ secretion (Fig. 11a). This was true for all three epitope-specific polyclonal T cell lines in this experiment.

MVA-pp65-infected CD40 B cells activated the NLV-specific polyclonal T cell line only. In addition, IFN-γ secretion was 3-fold reduced compared to NLV-specific polyclonal T cells recognizing CD40 B cells that were infected with MVA-pp65/IE-1. When MVA-IE-1-infected CD40 B cells were incubated with either VLE- or ILE-specific polyclonal T cells, IFN-γ secretion was 5-fold and 2-fold reduced compared to CD40 B cells infected with MVA-pp65/IE-1, respectively.

Results for donor B point into the same direction (Fig. 11b). Here, CRV- and RPH-specific polyclonal T cells were incubated with MVA-infected CD40 B cells.

As expected, MVA-IE-1 infected CD40 B cells were only able to induce high amounts of IFN-γ secretion of T cells with CRV epitope specificity but not RPH specificity. Conversely, MVA-pp65 infected CD40 B cells were able to activate RPH-specific polyclonal T cells but not CRV-specific T cells. Again, MVA-pp65/IE-1 represented the only virus activating both pp65- and IE-1 specific polyclonal T cells. The measured amounts of IFN-γ secretion of RPH- and CRV-specific T cells were roughly comparable to those of single HCMV-MVAs (approximately 6,580 pg/ml vs. 6,070 pg/ml and 2,410 pg/ml vs. 4,080 pg/ml, respectively).

Additionally, the x-fold activation capability of MVA-pp65/IE-1 was calculated compared to MVA-IE-1 and MVA-pp65, by dividing the amount of IFN-γ using MVA-pp65/IE-1 by the sum of IFN-γ levels using single-recombinant MVAs (Fig. 11c). Notably, MVA-pp65/IE-1 showed a 1.4-fold activation potential on average compared to MVA-IE-1 and MVA-pp65 which ranged between 0.5-fold (CRV) and 3.3-fold (VLE) (Fig. 11c).

Our data confirmed that HCMV-MVA-infected CD40 B cells efficaciously present HCMV epitopes to specific T cells. Therefore, they are a valuable tool for studying the functional properties of HCMV-encoding MVAs. Moreover, in most cases MVA-pp65/IE-1 showed the best activation potential of all viruses.

2.3. MVA-pp65/IE-1 efficiently propagates HCMV-specific CD8 T cells in PBMC of seropositive donors

After proving the ability of CD40 B cells to present HCMV antigens encoded by MVA, they were used to examine the immunological effect of MVA-pp65/IE-1 in PBMC of CMV seropositive donors.

PBMC from donors A and B were co-cultured with either MVA-pp65/IE-1 or MVA-infected CD40 B cells for 10 days. At days 0 and 10, HCMV-specific CD8 T cells of various epitope specificities were quantified by HLA/peptide multimer staining. At least one suitable epitope per antigen and donor was evaluated. For donor A, HLA-A2- and B8-restricted epitopes were studied, for donor B epitopes that were HLA-B7- and HLA-C7-restricted. Exemplary FACS analysis are shown for donor A in Fig. 12.

2.3.1. Donor A

The proportion of T cells specific for each of the tested epitopes within PBMC was increased after 10 days of *in vitro* stimulation using MVA-pp65/IE-1-infected CD40 B cells. Among the three specificities studied, T cells with ELK specificity showed the lowest increase after stimulation (3.5 fold), whereas T cells specific for NLV were increased 10-fold and VLE-specific T cells 20-fold (Fig. 12 and Tab. 5).

In contrast, absolute numbers of specific T cells in PBMC stimulated using control MVA remained stable or even decreased (Tab. 5). There was an approximately 2-fold increase in the proportion of ELK-specific T cells after control MVA stimulation. Since total T cell numbers decreased 3-fold in this condition, however, ELK-specific T cells decreased in absolute terms (Fig. 13a).

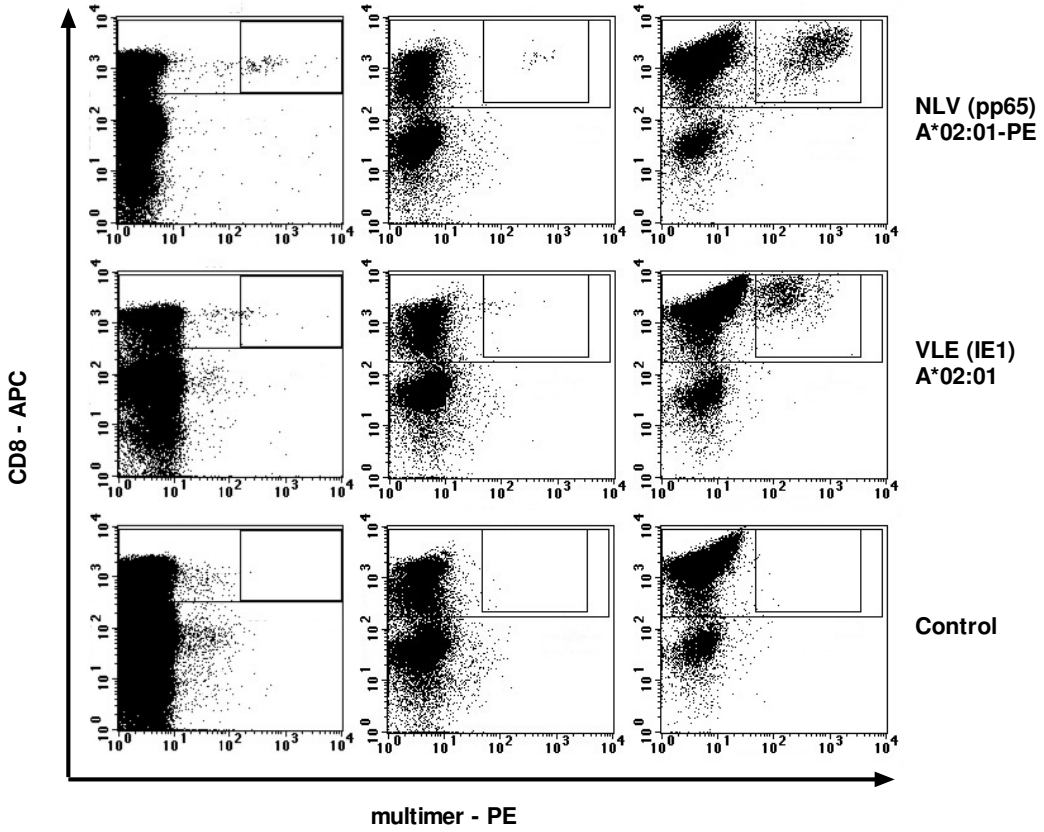


Fig. 12: Analysis of HCMV-specific CD8 T cells after co-culturing of PBMCs with MVA-infected CD40 B cells. HCMV-specific T cells were identified by staining with appropriate HCMV-specific HLA/peptide multimers in PBMCs on day 0 (left column) and after 10-day co-culture with B cells infected with MVA (middle column) or infected with MVA-pp65/IE-1 (right column). Cells were analyzed by flow cytometry. Cells without multimer staining (Control).

Changes in absolute numbers of HCMV-specific T cells (Fig. 13a) and the specific expansion per epitope specificity for both MVA-pp65/IE-1- and MVA-stimulated PBMC were also calculated (Fig. 13c). The specific expansion was defined as the ratio of the absolute numbers of specific T cells on day 10 and day 0.

Furthermore, the total expansion factor of PBMC was determined by dividing the total cell number of day 10 by the initial cell number at day 0 (Tab. 5). Since the total cell number after stimulation using MVA-pp65/IE-1 resembled the number of cells used to initiate the culture, for donor A the absolute increase in specific CD8 T cells was approximately parallel to their relative increase (Fig. 13). In

cultures stimulated with MVA, HCMV-specific absolute cell numbers stagnated or dropped as expected.

donor A	% multimer positive cells in CD8 T cells			% CD8 T cells in total	total expansion factor d0→d10
	NLV	**VLE**	**ELK**		
PBMC d0 w/o virus	0.67	0.26	0.28	23.9	
PBMC d10 + MVA	0.57	0.59	0.64	39.0	0.3
PBMC d10 + MVA-pp65/IE-1	6.50	5.39	0.97	86.6	1.1
donor B	% multimer positive cells in CD8 T cells			% CD8 T cells in total	total expansion factor d0→d10
	TPR	**RPH**	**CRV**		
PBMC d0 w/o virus	1.52	2.65	3.42	23.2	
PBMC d10 + MVA	1.26	2.87	8.89	32.4	0.4
PBMC d10 + MVA-pp65/IE-1	5.89	12.11	22.28	79.1	1.5

Tab. 5: Percentage of HCMV-specific CD8 T cells after co-culturing of PBMC (donor A and donor B) with MVA and MVA-pp65/IE-1 CD40 B cells. HCMV-specific T cells were identified by staining with appropriate HCMV-specific HLA/peptide multimers and analyzed by flow cytometry.

2.3.2. Donor B

The HLA/peptide multimers used to stain HCMV-specific T cells from donor B included the multimer CRV/C*07:02. HLA-C*07:02 is a ligand for the killer Ig-like receptor KIR2DL2/3 (PARHAM, 2005; MOESTA et al., 2008), which can be expressed by some CD8 T cells. Therefore, it is possible that peptide/HLA-C*07:02 multimers may not only stain CD8 T cells with the matched antigen-specific T cell receptor (TCR), but also NK cells and some CD8 T cells that do not express the specific TCR. To exclude such CD8 T cells from our analysis, PBMC stained with HLA-C*07:02/CRV-streptamer were additionally counterstained with an anti-KIR2DL2/3 antibody and KIR2DL2/3-positive cells were excluded from analysis of CRV-specific T cells. As a consequence, potentially existing CD8 T cells that were both specific for CRV/C*07:02 and expressing this KIR were also subtracted from the analysis, which leads to an underestimation of the frequency of CRV-specific T cells. This is not a severe limitation, however, since KIR2DL2/3 is expressed only on a minority of T cells that were stained with the HLA-C*07:02/CRV-streptamer (Fig. 14).

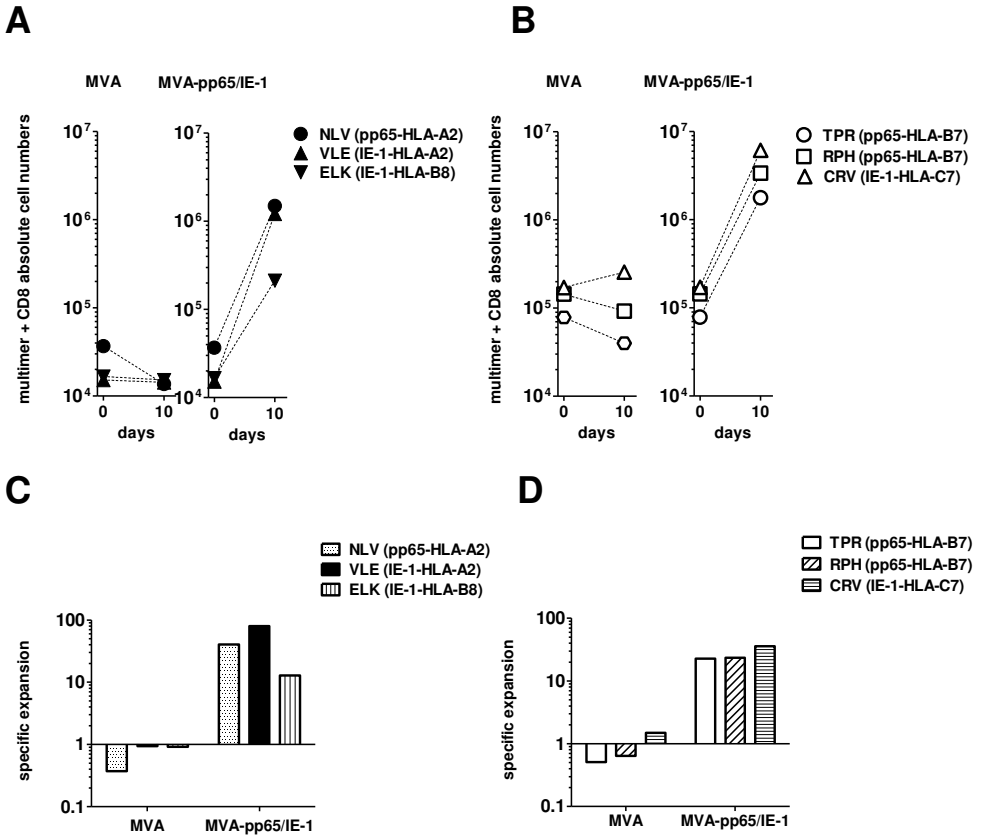


Fig. 13: Propagation of HCMV-specific T cells after MVA-pp65/IE-1 infection. HCMV-specific CD8 T cells were identified by HLA/peptide multimer staining. (A,B) Absolute numbers of HCMV-specific T cells were calculated by multiplying total cell number and the proportion of specific cells identified by multimer staining. Unstimulated PBMC (d0); PBMC stimulated with CD40 B cells infected with MVA or MVA-pp65/IE-1 (d10). (C,D) Specific expansion was calculated by dividing specific cell numbers on day 10 by cell numbers on day 0. Figures show results of donor A (panel A, panel C) and donor B (panel B, panel D).

Interestingly, T cells with CRV specificity were consistently detectable as two distinct populations in donor B (Fig. 14). This phenomenon was most impressive in PBMC after stimulation using MVA-pp65/IE-1 for 10 days. This suggests that CRV-specific T cells from this donor consist of at least two subpopulations that differ either in their level of TCR expression or in TCR avidity to the CRV/C*07:02 complex. The second possibility is more likely, since similar observations have been made for other HCMV epitopes before (PRICE et al., 2005). Similarly to donor A, relative numbers of epitope-specific T cells unambiguously increased under the condition of MVA-pp65/IE-1 stimulation in donor B, whereas in the control condition (MVA) proportions of specific T cells stagnated for two epitopes

(TPR, (WILLS et al., 1996) and RPH), but increased for one epitope (CRV) (Tab. 5). As explained before for donor A, this relative increase in some specific T cells in the control condition did not corresponded to an absolute increase of T cells specific for this epitope (Fig. 13b and d).

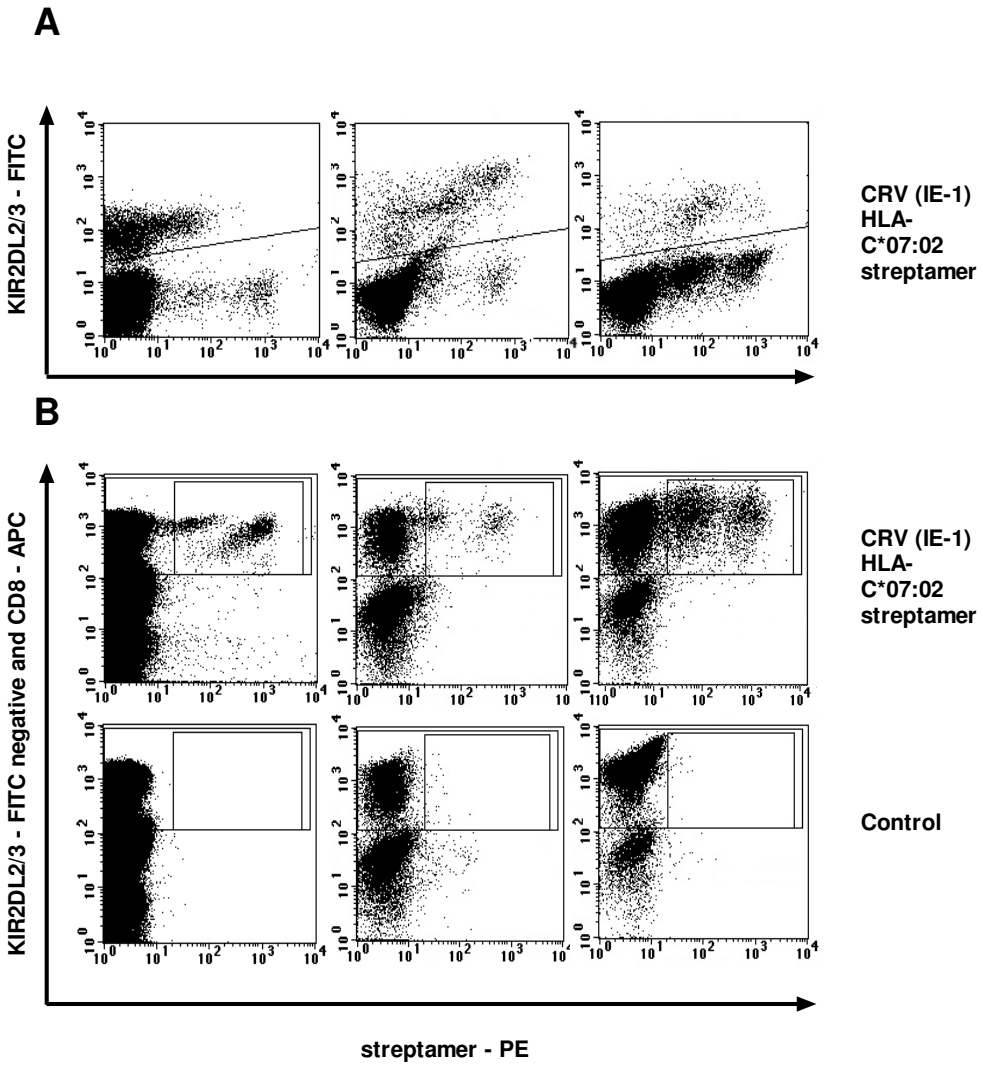


Fig. 14: Analysis of CRV-specific CD8 T cells after co-culturing of PBMC with MVA-infected CD40 B cells. (A) PBMC on day 0 (left column) and after 10-day co-culture with B cells infected with MVA (middle column) or infected with MVA-pp65/IE-1 (right column) were counterstained with anti-KIR2DL2/3 antibody and "KIR-negative" gated to reduce background. (B) CRV-specific T cells were identified by staining with HLA-C*07:02/CRV streptamer-PE. Cells were analyzed by flow cytometry. Cells without HLA-C*07:02/CRV streptamer-PE staining (Control).

Taken together, the results (presented for two donors, Fig. 13) showed that antigen-specific T cells to various epitopes of IE-1 and pp65 could be expanded using recombinant MVA-infected B cells, but not wildtype MVA.

This shows that MVA-pp65/IE-1 drives antigen-specific expansion of virus-specific T cells, an important prerequisite for the development of an HCMV vaccine.

2.4. MVA-pp65/IE-1 has an at least comparable ability to propagate HCMV-specific T cells as MVA-IE-1 and MVA-pp65

The next step was the comparison of MVA-pp65/IE-1's stimulatory capacity to single HCMV-MVAs coding for either pp65 or IE-1.

This time, PBMC from three HLA-A2-positive donors C-E were co-cultured for 10 days with B cells that were infected with MVA-pp65/IE-1, MVA-IE-1, MVA-pp65, or control MVA. Having in mind that the number of cells under the condition of control MVA decreased in the previous experiment, a higher number of PBMC was stimulated with control MVA than with recombinant MVA encoding HCMV antigens for each donor, C-E (40×10^6, 28×10^6, and 16×10^6 PBMC, respectively).

For stimulation with HCMV antigen encoding MVAs, lower numbers of PBMC were used (donor C-E, 16×10^6; 12-16 $\times 10^6$, and 8-12 $\times 10^6$ PBMC, respectively) according to availability. Constant T cell ratios per reaction volume were used throughout.

The study focused on HCMV specific CD8 T cells with VLE, NLV, and IPS (GAVIN et al., 1993) specificities and quantified them by HLA/peptide multimer staining on day 0 and day 10, respectively. At least one suitable epitope per donor was evaluable. For donors C and D, HLA-A2 restricted epitopes (VLE, NLV) were studied. For donor E, HLA-A2 and HLA-B35 epitopes (VLE, NLV, IPS).

donor C	% multimer positive cells in CD8 T cells		% CD8 T cells in total	total expansion factor d0→d10
	NLV	**VLE**		
PBMC d0 w/o virus	0.31	0.16	30.9	
PBMC d10 + MVA	0.49	0.23	22.3	0.4
PBMC d10 + MVA-IE-1	0.17	10.0	41.7	0.7
PBMC d10 + MVA-pp65	51.4	0.06	52.4	0.9
PBMC d10 + MVA-pp65/IE-1	28.0	12.25	57.0	1.5
donor D	**VLE**		% CD8 T cells in total	total expansion factor d0→d10
PBMC d0 w/o virus	1.84		13.9	
PBMC d10 + MVA	1.83		18.5	0.5
PBMC d10 + MVA-IE-1	39.1		47.0	1.1
PBMC d10 + MVA-pp65	0.41		44.5	1.0
PBMC d10 + MVA-pp65/IE-1	19.8		51.8	1.2
donor E	**NLV**	**VLE**	**IPS**	% CD8 T cells in total	total expansion factor d0→d10
PBMC d0 w/o virus	0.34	0.10	0.40	13.3	
PBMC d10 + MVA	0.62	0.17	0.81	17.6	0.8
PBMC d10 + MVA-IE-1	0.56	9.65	0.51	26.2	0.3
PBMC d10 + MVA-pp65	36.6	0.11	11.2	77.1	3.1
PBMC d10 + MVA-pp65/IE-1	34.3	2.03	10.2	66.7	2.9

Tab. 6: Percentage of HCMV-specific CD8 T cells after co-culturing of PBMC (donor C - B) with MVA, MVA-IE-1, MVA-pp65, and MVA-pp65/IE-1 CD40 B cells. HCMV- specific T cells were identified by staining with appropriate HCMV-specific HLA/peptide multimers and analyzed by flow cytometry.

In all of the three donors, the proportion of T cells specific for each of the tested epitopes within PBMC strongly increased during the 10 days of *in vitro* stimulation using MVA-pp65/IE-1 infected CD40 B cells. As expected, within PBMC stimulated using MVA-IE-1 only IE-1 specific T cells and within PBMC stimulated using MVA-pp65 only pp65-specific T cells unambiguously increased during stimulation, respectively. In contrast, relative proportions of all tested HCMV T cell specificities remained stable or decreased in PBMC stimulated using control MVA (Tab. 6).

Corresponding results were obtained when comparing the calculated absolute cell numbers of each epitope's specificity (Fig. 15a). Furthermore, an absolute increase in CD8 T cells was only detectable in PBMC stimulated using any of the recombinant HCMV-MVAs, but not control MVA (Tab. 6).

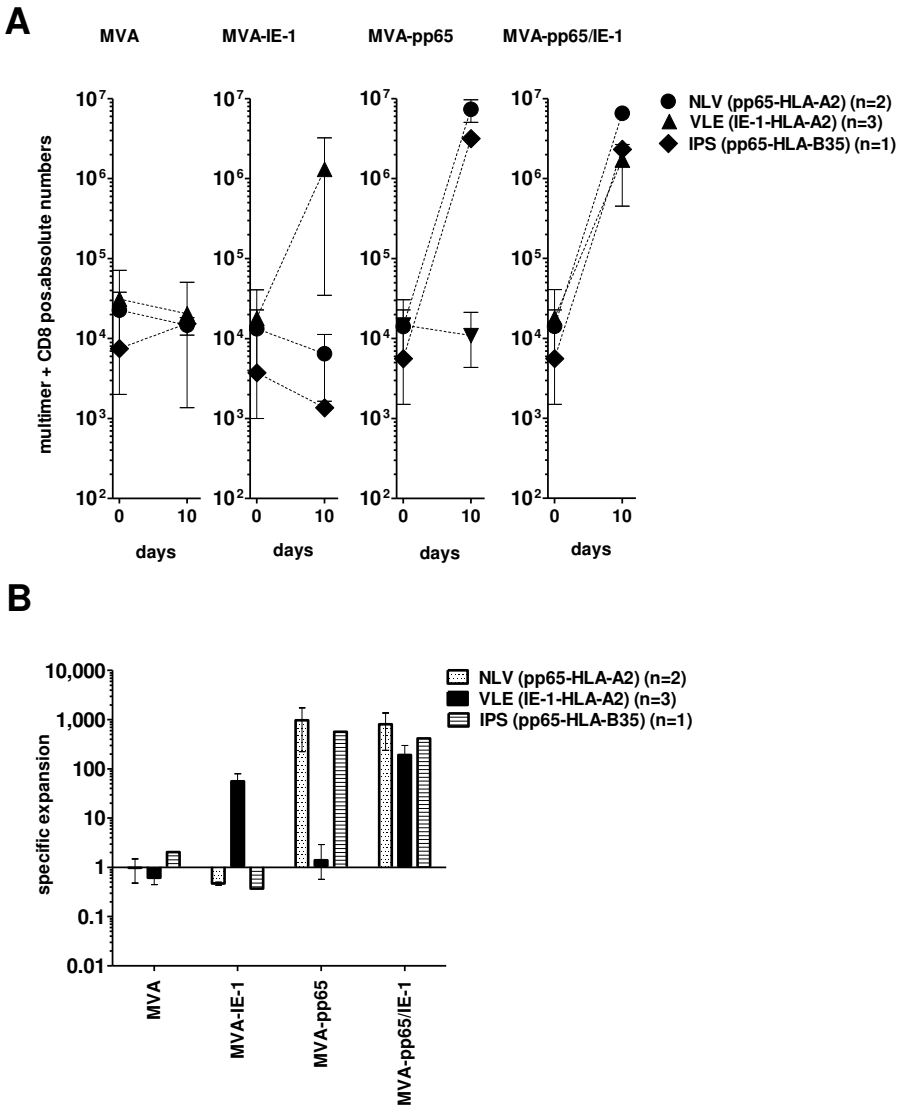


Fig. 15: Infection of PBMC with MVA-IE-1, MVA-pp65 and MVA-pp65/IE-1 to propagate HCMV-specific T cells. HCMV-specific CD8 T cells were identified by HLA/peptide multimer staining. (A) Absolute numbers of HCMV-specific T cells were calculated by multiplying total cell number and proportion of specific cells identified by multimer staining. Unstimulated PBMC (d0); PBMC stimulated with CD40 B cells infected with the indicated MVA viruses (d10). (B) Specific expansion was calculated by dividing specific cell numbers on day 10 by cell numbers on day 0. Error bars indicate mean and range of two (NLV) or three (VLE) different donors.

Overall, the total expansion factor of PBMC stimulated using one of the recombinant HCMV-MVAs was higher compared to those stimulated using control MVA. The only exception was the MVA-IE-1-stimulated culture from donor E, who had a particularly low frequency of IE-1 specific (VLE-specific) T cells in PBMC (Tab. 6).

Interestingly, only stimulation with MVA-pp65-IE-1-infected B cells, but not the three other MVAs, consistently increased total cell numbers in cultures from all three donors (total expansion factor: 1.2–2.9).

Comparing MVA-pp65/IE-1 and MVAs expressing single HCMV antigens, each of the recombinant HCMV-MVAs was similarly able to stimulate the corresponding HCMV-specific T cells within PBMC. This was true for absolute cell numbers of HCMV-specific T cells (Fig. 15a) and for specific expansion (Fig. 15b) of T cells with each of the different epitope specificities.

These results confirmed that the immunological impact of MVA-pp65/IE-1 in stimulating HCMV-specific T cells within PBMC was at the same level as the impact of MVA-IE-1 and MVA-pp65, respectively. Potentially, an MVA coding for a large fusion protein of two antigens might be less suitable for T cell stimulation than MVAs coding for single antigens, since competition of epitopes of MHC molecules or competition of T cells for MVA-infected antigen-presenting cells may take place. However, the present results showed that such deleterious effects were either absent in this situation, or were compensated by the improved construction of MVA-pp65/IE-1.

2.5. IFN-γ secretion by HCMV IE-1-specific T cells generated with recombinant HCMV-MVAs

As we were not only interested in the stimulation capability of the recombinant HCMV-MVAs but also in their functional behavior, reactivity of the previously generated HCMV-specific T cells was studied within PBMC of donors C and D. We examined whether those T cells were able to recognize CD40 B cells loaded with various IE-1 peptides. Responses to four different IE-1-specific epitopes were analyzed per donor, restricted through HLA-A*02:01, A*03:01, and B*40:01, by quantifying IFN-γ secretion of HCMV-specific T cell lines in ELISPOT and ELISA assay. In parallel, ELISPOT assays with unstimulated PBMC were performed to estimate the increase of IE-1-epitope specific T cells during MVA stimulation (Fig. 16a and d). Both donor C and donor D showed lower frequencies of HCMV-specific T cells than detected by multimer staining by a factor of three on average. Despite of this, the IFN-γ ELISPOT remains a valuable assay, since it sensitively detects and quantifies an important antiviral effector function of virus-specific T cells.

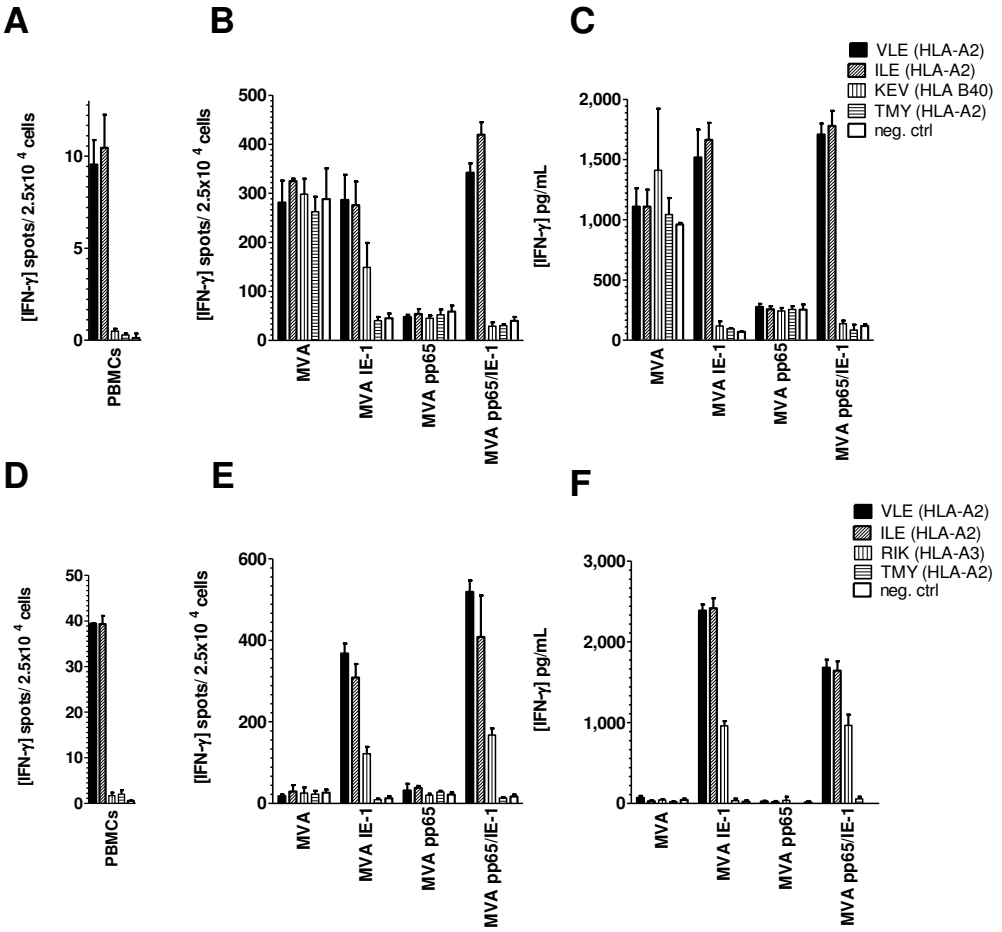


Fig. 16: Propagation of HCMV IE-1-specific T cells from PBMC. Unstimulated PBMC were incubated with HCMV IE-1 derived peptides (A and D) or PBMC stimulated using recombinant MVAs were incubated with HCMV IE-1-peptide-loaded CD40 B cells (B-C and E-F) for 16-18h. IE-1 specific T cells were analyzed with IFN-γ ELISPOT (B and E) or concentrations of IFN-γ in supernatants were determined using ELISA (C and F). Donor C (A-C) and donor D (D-F). Columns and error bars above indicate mean and maximum of triplicates in one experiment.

2.5.1. Donor C

Surprisingly, a quite pronounced IFN-γ secretion in PBMC stimulated under the condition of control MVA was measured in all tested IE-1-epitopes also in the negative control in both assays, ELISPOT (Fig 16b) and ELISA (Fig. 16c). These observations did not disturb the interpretation of the other results, as each set of epitope-loaded B cells was controlled by a separate negative control, B cells not loaded with peptide (Fig. 16, white columns). Potential reasons for this apparently B cell-specific background reactivity are discussed in Section V.

HCMV-specific T cells in non-stimulated PBMC of donor C only recognized VLE- and ILE-, but not KEV- and TMY-peptide-loaded B cells (Fig. 16a). Furthermore, IFN-γ secretion was 30- to 40-fold increased within PBMC

stimulated using MVA-pp65/IE-1, whereas in PBMC stimulated using MVA-IE-1 an only 25-fold increase of cells recognizing VLE- and ILE-loaded B cells was detectable. As expected, HCMV-specific T cell lines stimulated using MVA-pp65 failed to recognize any IE-1-peptide. Besides, T cells in PBMC stimulated using MVA-IE-1 also recognized KEV-loaded B cells by ELISPOT (Fig. 16b) but not in ELISA (Fig. 16c).

Comparable results were obtained when measuring the IFN-γ secretion in ELISA. Highest reactivity of T cells in PBMC stimulated using MVA-pp65/IE-1 and MVA-IE-1 was detected against VLE- and ILE-loaded B cells (Fig. 16c). As expected and already detected via ELISPOT, PBMC stimulated using MVA-pp65 did not recognize IE-1 peptide loaded B cells.

2.5.2. Donor D

As analyzed by ELISPOT, HCMV-specific T cells in non-stimulated PBMC of donor D were again only able to recognize B cells loaded with peptides VLE and ILE, but not RIK and TMY (Fig. 16a).

The ability of T cells in PBMC stimulated for 10 days by MVA-pp65/IE-1- and MVA-IE-1-infected CD40 B cells to recognize VLE- and ILE-loaded CD40 B cells was quite comparable in both assays (Fig. 16e and f). Moreover, RIK-loaded CD40 B cells were recognized from T cells of both stimulated PBMC in spite of their undetectable frequency *ex vivo*. Admittedly, the level of IFN-γ secretion was not comparable to VLE- and ILE-loaded CD40 B cells, but obviously positive compared to TMY-loaded CD40 B cells and CD40 B cells without peptide. This time as expected, HCMV-specific T cell lines within PBMC stimulated using either MVA-pp65 or control MVA failed to recognize any of the IE-1-peptides presented by CD40 B cells. Taken together, functional T cell responses to various IE-1 peptides were amplified by stimulation of PBMC with MVAs encoding IE-1 individually or as a fusion protein with pp65. The absence of responses to the TMY peptide was not surprising, since responses to this peptide can be elicited in HLA-A2-transgenic mice, but are often absent in humans (ELKINGTON et al., 2003).

V. DISCUSSION

For the past 40 years, intensive effort has been placed in developing a vaccine against HCMV which can cause lifelong problems and require expensive and toxic treatment of infected newborns and transplant recipients. In 2000, the U.S. classified the creation of an HCMV vaccine as a national priority (INSTITUTE OF MEDICINE, 2000). Although intensified research activities were catalyzed by this classification, the development of a satisfying vaccine has not been successful until now.

This study evaluated a new HCMV-MVA vaccine candidate (MVA-pp65/IE-1) that had recently been constructed in our laboratory. This recombinant MVA expresses a fusion protein of the immunodominant antigens pp65 and IE-1. The construct has several novel features that were designed to optimize its potency. A detailed genetic and immunological characterization of MVA-pp65/IE-1 was performed. Thereby, the focus was to detail MVA-pp65/IE-1's capacity to activate HCMV-specific CD8 T cells both quantitatively and functionally. Replication capacity of MVA-pp65/IE-1 in primary CEF cells proved to yield high titers (10^7 PFU/ml) that were comparable to non-recombinant MVA. In contrast, MVA-pp65/IE-1 was unable to grow in human cells, as expected. Furthermore, this study confirmed that HCMV-MVA-infected CD40 B cells are well suited to effectively present HCMV-specific antigens to specific T cells. Moreover, antigen-specific T cells to various epitopes from IE-1 and pp65 could be expanded *in vitro* by stimulation with MVA-pp65/IE-1-infected B cells. Finally, IFN-γ secretion of these T cells co-cultured with IE-1-loaded B cells was detectable. The expansion and functionality of virus-specific T cells and the consistent high cell numbers from tested donors achieved only when MVA-pp65/IE-1 was used. This indicated high immunogenicity and constituted an important prerequisite for the development of an HCMV vaccine.

Taken together, these data support the usage of MVA-pp65/IE-1 as a promising vaccine candidate in patients undergoing immunosuppressive therapy.

Reasons for using a pp65/IE-1 fusion gene and the MVA vector platform for the construction of an HCMV vaccine

We chose MVA as a vaccine platform because it is known to deliver foreign antigens with high stability to the host cell thus enabling an efficient antigen-specific immune response. Moreover, MVA constitutes an ideal candidate to immunize patients during immunosuppressive treatment due to its remarkable safety profile. Following well-established protocols for the generation of recombinant MVAs available in our lab, we included HCMV genes into the existing deletion site III of MVA's genome and placed them under the control of the strong early-late promoter PmH5 (MEYER et al., 1991; WYATT et al., 1996; KREMER et al., 2012). Cytotoxic T cell responses are considered to play a crucial role in CMV infection and in CMV prevention (QUINNAN et al., 1982; REDDEHASE et al., 1987; RIDDELL et al., 1992). As HCMV antigens to be vaccinated against, we chose pp65 and IE-1, because they constitute two major targets of T cells and generally induce immunodominant responses (GIBSON et al., 2004; SYLWESTER et al., 2005). Moreover, T cells directed against these antigens are considered to play important roles in preventing primary infection and reactivation of HCMV, and the two antigens are complementary in this respect. Specifically, pp65 is an abundant structural protein of the tegument. Thus it offers the possibility of a fast processing and presentation to T cells during primary infection in recently infected cells (McLAUGHLIN-TAYLOR et al., 1994), and pp65 is also abundant in the late phase of the replication cycle. In contrast, IE-1 is deemed to play a more important role in viral reactivation than pp65 because it is expressed earlier in HCMV replication (STINSKI et al., 1983). In our approach, we decided to combine both immunogenic antigens in the form of a fusion gene, because it is still unclear whether pp65- or IE-1-specific T cells are more important to prevent HCMV disease in transplant recipients. Some studies described IE-1-specific T cells to be most beneficial against HCMV (BUNDE et al., 2005; SACRE et al., 2008), whereas others showed that the presence or the transfer of pp65-specific T cells was associated with reduced or controlled HCMV disease (WALTER et al., 1995; GRATAMA et al., 2008). T cells have been targeted against each of the antigens during adoptive T cell transfer (COBBOLD et al., 2005; SCHMITT et al., 2011), but available data is too limited to clearly identify which antigen has superior protective capacity. The results of

recently completed controlled clinical studies on T cell transfer (BOECKH et al., 2015), expected to be published soon, may provide more information on this open question.

In contrast to other groups who used mixtures of single recombinant HCMV-MVAs encoding for either pp65 or parts of the IE-1 gene (WANG et al., 2004), our approach with a single recombinant MVA encoding both genes offers the possibility to decrease the number of viral vectors required for an effective vaccination. Consequently, safer, more practical, and substantially more economic immunization of humans may be achieved through this approach.

Wang et al. generated a single recombinant MVA vaccine including exon 4 of IE-1 (WANG et al., 2004) to avoid the transactivation properties of IE-1 (CASTILLO & KOWALIK, 2002). However, exon 4 still contains a STAT2 binding region that may potentially decrease immunogenicity of the construct, since it interferes with the cellular interferon response (HUH et al., 2008). Perhaps elimination of elements with transactivation functions and abolition of nuclear targeting may decrease the likelihood of unwanted effects. Accordingly, we excluded the known NLS region encoded by exon 2 (LEE et al., 2007) and the STAT2 binding region from the IE-1 sequence used in the pp65/IE-1 fusion construct. Admittedly, by excluding these two regions, the remaining IE-1 sequence is shorter than the complete exon 4. However, exon 3 encodes relevant T cell epitopes (KHAN et al., 2007), whereas the STAT2 binding region is dispensable as a source of epitopes (KHAN, 2007). In light of these modifications, we hypothesized for an improved immunogenicity of our pp65/IE-1 fusion antigen. Moreover, our inclusion of exon 3 is proposed to increase antigen stability (HUH et al., 2008), which is a desired property for efficacious vaccination with MVA.

Wang et al. constructed another HCMV-MVA with pp65 and exon 4 of IE-1 separately placed into deletion site II of the MVA genome (WANG et al., 2007). In contrast, we investigated the construction of a fusion gene of pp65 and modified IE-1. A fusion gene offers several advantages including the simultaneous expression of its constituents, followed by joint uptake by APCs and antigen presentation. Moreover, a fusion gene is under the control of one promoter, and hence expression of the constituents is uniform. On the contrary, a construction

with 2 distinct genes – as e.g. in Wang et al. – requires a promoter for each gene which is potentially problematic. The use of two different promoters results in differences in the levels of gene expression, whereas a combination of two similar promoters introduces homologous nucleotide sequences and thus potential instability of the viral vector. Wang et al. solved this problem by using two synthetic promoters (Psyn I and Psyn II) for the generation of their HCMV-MVA. These promoters had already been frequently used in the 1990's but are currently considered more rarely due to the synthesis of very high levels of recombinant gene products that may lead to a reduced replication capacity of the recombinant MVA (WYATT et al., 1996). Such replication deficiency is a disadvantage for large scale GMP production of any candidate vector vaccine. Therefore, we constructed our MVA-pp65/IE-1 using the current gold-standard promoter system, the strong early-late promoter PmH5 (WYATT et al., 1996).

In general, the construction of a fusion gene bears a high risk of incorrect folding of the protein leading to instabilities. Furthermore, such synthetic fusion proteins might develop new unwanted properties, such as increased cytotoxic characteristics. In our studies, MVA-pp65/IE-1 did not show such unfavorable properties and remained stable over very long periods of time with no toxicity observed. Despite the fact that we were not the first group who constructed a fusion gene composed of pp65 and IE-1 (BERNSTEIN et al., 2009), our MVA-pp65/IE-1 encodes for a novel synthetic immunogen originated from pp65 and IE-1 that is designed to optimize immunogenicity.

MVA-pp65/IE-1 stably expresses its transgene and retains the specific safety properties of MVA

Since earlier studies using MVA as a viral vector rarely observed genetic instabilities of recombinant MVA (WYATT et al., 1996; WYATT et al., 2009), the stable expression of the HCMV fusion gene by MVA-pp65/IE-1 was tested. The results showed that ~90% single MVA-pp65/IE-1 plaques stably expressed the HCMV protein of interest after five passages in BHK. Moreover, Western blot analysis illustrated that the majority of the fusion protein was intact 48 hours after infection. 96 hours post infection, the majority of the fusion protein was cleaved, giving rise to a dominant 50 kDa band stained by an anti-IE-1 antibody, but the complete fusion protein remained detectable after this extended infection period.

This result showed that the intact pp65/IE-1 fusion protein is expressed for several days. This is an important prerequisite for the *in vivo* immunogenicity of this MVA vaccine candidate, since it was shown by Gasteiger et al. that cross-presentation of MVA-encoded antigen may be the major mechanism leading to priming of antigen-specific T cells after MVA vaccination (GASTEIGER et al., 2007). In contrast, destabilized MVA-expressed antigens prime specific T cells with much lower efficiency (GASTEIGER et al., 2007).

The results of the multi-step growth analysis showed that important requirements for large scale MVA-pp65/IE-1 vaccine manufacturing are fulfilled. The growth kinetics confirmed that MVA-pp65/IE-1 did not productively grow in human cells. In addition, its amplification capacity in CEF was comparable to non-recombinant MVA (Fig. 9). This implies that MVA-pp65/IE-1 can be handled and produced under biosafety level 1 conditions (ZKBS, 1997).

Immunological characterization of MVA-pp65/IE-1

In the first approach to study the immunological effect of MVA-pp65/IE-1, we followed a modified version of the protocol described in La Rosa et al. 2006. We were not able to reproduce the authors' results of an *in vitro* expansion of HCMV-specific T cells or a survival in PBMC. One potential reason for the lack of success is the high infectious dose of MVA recommended by La Rosa et al. (an MOI of 5). Perhaps different protocols were used to determine viral titers. Moreover, the quality of virus titers might be influenced by stock purification methods which could potentially differ along various lines: (1) the duration of virus amplification, (2) the type of cells used for amplification, (3) whether purification was performed using 36% sucrose gradient centrifugation or not, and (4) the method of resuspension of the final virus pellet (PBS supplemented with 5% lactose vs. 10 mM Tris-HCl, pH 9.0). Furthermore, purified MVA loses some of its infectivity when stored at -20°C and not at -80°C. This is another potential explanation for the higher MOIs necessary for sufficient infection rates in the earlier study (LA ROSA et al., 2006). For that purpose, all recombinant MVAs used in this study were stored at -80°C and the virus was strictly handled on ice until usage; thus ensuring the stability of the virus titer. This goes in line with the observation that a much lower MOI of 0.1 to 0.2 led to robust infection, T cell activation, and expansion in all experiments that were performed thereafter, and

led to better survival of infected cells.

Induction of IFN-γ-secretion of HCMV-specific polyclonal T cells after co-incubation with MVA-infected CD40 B cells

After confirming that CD40 B cells were well suited to efficaciously present HCMV-specific antigens delivered by recombinant MVAs to specific T cells, this method was used to study the functional properties of HCMV-encoding MVAs in greater detail. Remarkably, MVA-pp65/IE-1-infected CD40 B cells were best able to activate polyclonal T cells, resulting in the highest numbers of IFN-γ secretion for all epitopes tested of donor A when this virus was used (Fig. 11a). For donor B, the activation potential was more comparable to single HCMV-MVAs (Fig.11b). On average, MVA-pp65/IE-1 showed the best activation potential of all studied viruses when evaluating the functional properties of different recombinant HCMV-MVAs (Fig. 11c). Furthermore, only stimulation with MVA-pp65/IE-1-infected B cells, but not the three other MVAs, consistently increased total cell numbers in cultures from all three donors (total expansion factor: 1.2–2.9, Tab. 6). This data gave the indication of a potential increase in immunogenicity due to the unique modifications of the IE-1 gene sequence.

In addition, the possibility of immunomodulatory effects of pp65 needed to be considered, since an initial report described that pp65 is able to reduce presentation of IE-1 (GILBERT et al., 1996). However, it appears that these early results were never replicated, and recent studies did not confirm them, but found no undesirable effect of pp65 on IE-1 presentation and T cell activation (AMERES et al., 2013). Negative effects were also absent within these studies. This is also consistent with the finding of Wang et al., who used MVAs encoding two separate genes within one virus (WANG et al., 2007), as well as with this study using an MVA encoding for one fusion gene composed of pp65 and modified IE-1.

Expansion of HCMV-specific T cells in PBMC of donors A and B by stimulation with MVA-pp65/IE-1-infected B cells compared to B cells infected with control MVA was closely monitored by HLA/peptide multimer staining. Distinct populations of antigen-specific T cells were detected for all epitopes analyzed (donor A and B). The observation that CRV-specific T cells were detectable as

two distinct populations in PBMC after stimulation using MVA-pp65/IE-1 suggests that CRV-specific T cells from donor B consist of at least two subpopulations that most probably differ in their TCR avidity to the CRV/C*07:02 complex (Fig. 14). This data is analogous to Price et al. who described oligoclonal CD8 T cell responses against the NLV-epitope. They observed a general dominance of one or two prevalent clonotype responses against NLV that differed in multimer staining intensity and correlated with avidity (PRICE et al., 2005). Data on clonotypic composition of CRV/C*07:02-specific T cells have not been published, but unpublished data indicate that these T cells have a highly complex TCR repertoire (Liang and Moosmann, personal communication), which may explain the variability of multimer staining.

Interestingly, staining PBMC and T cell lines with the CRV/HLA-C*07:02 multimer resulted in different subpopulations while this was not the case with the NLV/HLA-A*02:01 multimer. A probable explanation for this might be the high concentration of multimers that were used throughout this experiment. Price et al. who found that the subpopulations with low intensive multimer staining by multimers increased only under lower multimer concentrations. However, at least CRV-specific T cells appeared as two subpopulations in flow cytometry indicating that they might differ in their avidity. The property of MVA-pp65/IE-1-infected B cells to stimulate clonotypic diversity within HCMV-specific T cells should be considered as an additional advantage in counteracting complex pathogens, e.g. HCMV.

In general, an MVA coding for a large fusion protein of two antigens might harbor the danger that competition of epitopes of MHC molecules or competition of T cells for MVA-infected antigen-presenting cells may take place. However, this data showed no decrease in efficacy of antigen presentation through MVA presenting the fusion protein (Fig. 13 and 15). This finding either indicates that MVA-pp65/IE-1 is able to compensate these effects by its improved construction, or competition effects were absent. Therefore, MVA-pp65/IE-1 effectively induces immune responses as the corresponding single recombinant HCMV-MVAs, and can be considered a promising HCMV vaccine candidate.

Considering that cross-presentation is a major pathway for CD8 T cell priming *in vivo* (GASTEIGER et al., 2007), it is very unlikely that following vaccination the

amount of antigen available for cross-presentation will be high enough to give rise to competition for MHC binding in the cross-presenting cell.

As for the other donors, HCMV-specific T cells from donor C could be specifically expanded by stimulation with HCMV antigen-encoding MVAs. Surprisingly, PBMC of donor C stimulated under the condition of non-recombinant (control) MVA showed a pronounced, but unspecific IFN-γ secretion (Fig. 16b and c). The most probable explanation for this result is that peptide-loaded B cells propagated other, but non-HCMV-specific T cells in this setting due to the lack of HCMV-specific antigens. These conditions might enable the propagation of T cells against irrelevant antigens of different origin. Likely, these include auto-reactive T cells that are specific for (presently unknown) autoantigens on activated B cells. In the presence of viral antigens that are recognized by virus-specific T cells from the donor, such autoreactive T cells are suppressed or lost due to competition, but in the absence of stimulation of virus-specific T cells the autoreactive T cells may expand (Moosmann, personal communication). Moreover, T cells with FCS-antigen specificities also might be propagated because the cell culture medium is supplemented with FCS. Lastly and less likely, T cells specific for murine antigens might have an influence since the B cells were cultured on LL8, a murine cell line. These observations are similar to earlier findings that stimulation with LCLs (EBV-transformed B cells) may activate T cells specific for self-antigens expressed by activated B cells (ADHIKARY et al., 2007; LINNERBAUER et al., 2014). Although their physiological origin and function is not clear, such B cell autoreactive T cells do not seem to be problematic in clinical application, since LCL-stimulated T cells have been safety used for EBV-specific T cell therapy in more than 100 patients in the absence of major toxicity (HESLOP et al., 2010).

The amino acid sequences of T cell epitopes from HCMV antigens may differ according to the HCMV strain. This is particularly relevant for IE-1, which is less conserved between HCMV strains than pp65. Thus, there are polymorphisms in several immunodominant CD8 T cell epitopes in IE-1, notably the epitopes VLE/ILE (Tab. 3), CRV, and ELR/ELK. However, the intensity of IFN-γ T cell responses against VLE- or ILE- peptide-loaded B cells did not differ when using either MVA-IE-1 (AD169) or MVA-pp65/IE-1 (TB40E) for PBMC stimulation,

although these strains differ in their IE-1 gene sequence (e.g. regarding the epitope VLE in AD169 and ILE in TB40E) (Fig. 16). The initial values of VLE- and ILE-specific T cells in PBMC at day 0 analyzed by ELISPOT did not appear to influence this observation. Indicating none or only a minor influence of epitope-variations, these findings generally support the view that a sufficient proportion of T cells specific for such polymorphic epitopes are able to recognize both variants of the epitope. Therefore, it is likely that MVA-pp65/IE-1 can be used to prevent disease caused by different HCMV strains. Admittedly, a high peptide concentration (5 µg/ml) was used for recognition of peptide-specific T cells in this assay which might have activated T cells with VLE-specificity by ILE-loaded B cells, although they have a lower affinity to the ILE- than to the VLE-peptide, and vice versa. Thus, the present analysis is not definitive, and further analyses of MVA-expanded HCMV-specific T cells should be performed that address the question more specifically, either by using target T cells loaded with the different polymorphic versions of the peptides at limiting dilution, or by employing titratable gene vectors that code for individual polymorphic HCMV epitopes.

Our data did not report T cell responses to the TMY peptide although it has been reported that TMY-specific responses can be elicited in HLA-A2-transgenic mice (GALLEZ-HAWKINS et al., 2003). Our finding is not surprising as it corresponds to other studies that showed an absence of these responses in humans as well (ELKINGTON et al., 2003). The reasons for this different epitope being used in humans and mice are not well understood. However, it is possible that some HCMV-specific T cells might be missing in humans that are present in mice due to negative selection, as both organisms have different endogenous protein repertoires. Moreover, it might be that the murine and human proteasomes differ in antigen processing. Hence, not the same epitope repertoire is presented to T cells with matching epitope specificity in HLA-A2-transgenic mice and humans.

The literature described KEV and RIK as further epitopes of IE-1 that could only be observed in some but not in a majority of humans (KHAN et al., 2007; AMERES et al., 2013). When examining these epitopes using MVA-pp65/IE-1, RIK-specific responses could only be partially detected while KEV-specific responses were absent. An analysis of a larger number of donors would clarify

whether T cells specific for these epitopes can be expanded from every donor who carries these specificities. So far, we could not obtain evidence that HCMV-specific T cells could not be expanded in such a situation.

This indicates that MVA-pp65/IE-1 is not only able to stimulate HCMV-specific T cells within PBMC, but that they also have functional reactivity properties. These important observations further confirmed that MVA-pp65/IE-1 is a promising vaccine candidate against HCMV. The interesting observation, that only stimulation of PBMC (donor C-E) with MVA-pp65-IE-1-infected B cells, but not the three other MVAs, consistently increased total cell numbers (total expansion factor: 1.2-2.9, Tab. 6) implies a potential advantage in immunogenicity of this promising vaccine candidate.

Further avenues of research/next steps

Within this study, the immunological characterization of MVA-pp65/IE-1 was primarily conducted by evaluating CD8 T cells. In order to include all different cell types of the cellular immune response that are important in the recovery of HCMV disease (BIRON et al., 1989; GABANTI et al., 2014), CD4 T cells as well as NK cells should also be part of the examination in further experiments.

Before MVA-pp65/IE-1 may reach clinical trials, it requires further preclinical characterization. A potential intermediate step towards this would be the usage of HLA-A2-transgenic mice, an *in vivo* model that allows for the evaluation of HCMV-specific T cell responses.

In general, it is difficult to find an appropriate animal model to test the efficacy of HCMV vaccines, as a challenge model of HCMV is not feasible due to the very strict host specificity of CMV. Nonetheless, several different animal models partly resemble the HCMV infection in humans. Firstly, a lot of important research on CMV was conducted by studying the murine cytomegalovirus (MCMV) in mice (REDDEHASE et al., 1985; MUTTER et al., 1988). Moreover, using guinea pigs to study the guinea pig cytomegalovirus (GPCMV) seems to be the most promising model to evaluate the transmission of CMV to the fetus during pregnancy by crossing the placenta because this animal model is very similar to humans (SCHLEISS et al., 2015). Most notably, rhesus cytomegalovirus (RhCMV) disease in non-human primates is consided to be the animal model

most comparable to HCMV disease in humans (YUE et al., 2008), but nevertheless findings in this model are still not fully transferable. Thus, a fully satisfying animal model is lacking for the evaluation of a successful vaccine to prevent HCMV disease.

Encouraged by the *in vitro* data collected concerning MVA-pp65/IE-1 and considering the convincing safety and immunogenicity of other recombinant MVA vaccines in preclinical trials, we are confident that the MVA-pp65/IE-1 vaccine candidate will perform well in *in vivo* experiments.

Possible therapeutic uses of MVA-pp65/IE-1

MVA-pp65/IE-1 offers several possible applications in humans. Firstly, MVA-pp65/IE-1 can be directly used for vaccination of patients before transplantation. Among patients who receive solid organ transplantation, seronegative recipients obtaining a transplant from a seropositive donor would benefit most from a vaccination before transplantation, because they could then build up immunity with HCMV-specific T cells before receiving an HCMV-positive organ during immunosuppressive therapy. In contrast, HCMV-positive recipients of HSC would profit most from a pre-transplantational vaccination of an HCMV-negative HSC donor, because grafts from such a donor are otherwise unable to reconstitute HCMV-specific immunity in the patient. Since HCMV reactivation in HSCT patients is also associated with graft-versus-host disease as well as transplant rejection, such a vaccine may have wide-ranging beneficial effects on transplantation success.

An alternative use of MVA-pp65/IE-1 would be to employ it for *in vitro* amplification of HCMV-specific T cells before adoptive transfer to patients. Other approaches in adoptive immunotherapy obtain the HCMV-specific T cells of interest by staining of PBMC with multimers. This procedure is only able to enrich, not expand, those T cells and thus requires large numbers of PBMC. As confirmed in this study, MVA-pp65/IE-1 in combination with CD40 B cells efficiently stimulates HCMV-specific T cells after one round of stimulation. Admittedly, there are other ways to expand HCMV-specific T cells *in vitro* for adoptive T cell transfer such as complete HCMV viruses or adenoviral vectors encoding for HCMV genes (REUSSER et al., 1991; ZHONG et al., 2008).

However, compared to these approaches MVA vector vaccines do a better job in meeting GMP requirements and should thus be preferred.

In the case of HCMV, another important task for a vaccine is to prevent the transmission during pregnancy. Remarkably, our MVA-pp65/IE-1 is a feasible backbone for including further major antigens to widen the scope of possible applications in pregnant women to prevent infection of the unborn child, as well as to improve its applicability in the case of transplant recipients even further. Thereby, the humoral immune response is especially important. As it is known that MVA is not only able to stimulate the cellular but also the humoral pathway by production of antigen-specific antibodies (SUTTER et al., 1994), this vector offers all requirements to also include antigens that are crucial for that. In this context, the gH/gL/UL128/UL130/UL131A is a pentamer complex known for its importance in entering endothelial as well as epithelial cells (WANG & SHENK, 2005; RYCKMAN et al., 2008). Antibodies against this pentameric glycoprotein appear to be superior in neutralization of infection (CHIUPPESI et al., 2015). Therefore, this complex could potentially be added to the MVA-pp65/IE-1 backbone. This improvement of the MVA-pp65/IE-1 would widen the scope of the vaccine even further, since a combined virus would induce CD8 T cells, CD4 T cells, and neutralizing antibodies. This would be favorable for its application in pregnant women.

To further improve the use of MVA-pp65/IE-1 in the case of transplantation recipients, a selection of major antigens of the various infectious agents that are harmful for transplant patients could be combined into one MVA vector (e.g. HCMV, EBV, adenovirus). Efforts to cover many of those pathogens in a single therapy have already been conducted by developing multispecific-T cells for adoptive transfer (LEEN et al., 2006; PAPADOPOULOU et al., 2014). Since prophylactic application of adoptive T cell therapy is logistically challenging and may not be easy to implement in large patient cohorts, it would be much more beneficial if a vaccine was developed that is able to control multiple harmful and life-threatening infectious agents in patients undergoing transplantations.

VI. SUMMARY

Human cytomegalovirus (HCMV) is a member of the β-Herpesvirinae subfamily and plays an important role in transplant recipients and infected newborns because primary infection and reactivation of HCMV cause lifelong problems and require expensive and harmful treatment. This constitutes an even greater problem as seroprevalence of human cytomegalovirus (HCMV) ranges between 30 and 90% in the human population. Despite huge efforts in the development of a satisfying vaccine against HCMV in the last decades, not a single vaccine candidate has been successfully evaluated until now.

Live attenuated Modified Vaccinia virus Ankara (MVA) is one of the most promising viral vector systems as it is replication deficient in human cells but efficiently delivers recombinant proteins encoded by heterologous genes. Thereby, MVA enables the induction of cellular immune responses and humoral ones. Moreover, large scale production of recombinant MVAs is feasible under GMP conditions. Hence, MVA meets several important requirements for the production of an adequate vaccine.

Within this study, a recently constructed HCMV vaccine candidate (MVA-pp65/IE-1) based on MVA was characterized genetically and immunologically. CD40 B cells infected with recombinant MVAs were established as a valuable tool for immunological characterization of recombinant HCMV-MVAs. By growth kinetics, replication deficiency of MVA-pp65/IE-1 was proven in mammalian cells (HaCaT) and replication competence was shown in primary chicken embryo cells (CEF). A 120 kDa protein that was expressed in MVA-pp65/IE-1-infected cells was recognized by HCMV-specific antibodies. Antigen-specific T cells to various epitopes of IE-1 and pp65 were expandable *in vitro* by stimulation with MVA-pp65/IE-1-infected B cells. Furthermore, functional T cell responses to various IE-1 peptides of IE-1-specific T cells were detected by IFN-γ measurement.

These data indicate that MVA-pp65/IE-1 meets important requirements as vaccine against HCMV. Thus, subsequent studies focusing on the further development of this promising vaccine candidate should be aspired.

VII. ZUSAMMENFASSUNG

Das humane Zytomegalievirus (HCMV) gehört zur Unterfamilie der β-Herpesvirinae und spielt vor allem in Transplantationsempfängern und infizierten Neugeborenen eine bedeutende Rolle, da Primärinfektionen oder Reaktivierungen mit HCMV ein lebenslanges Leiden verursachen, welches lediglich mit teuren und schädlichen Medikamenten behandelt werden kann. Dies stellt ein besonders großes Problem dar, da die Seroprävalenz von HCMV innerhalb der menschlichen Bevölkerung zwischen 30 und 90% beträgt. Obwohl bereits seit Jahrzenten an der Entwicklung eines wirksamen Impfstoffes gegen HCMV geforscht wird, konnte bisher kein einziger Impfstoff erfolgreich getestet werden.

Das Modifizierte Vacciniavirus Ankara (MVA) gilt aufgrund seiner besonderen Eigenschaften als eines der vielversprechendsten viralen Vektorsysteme für Impfstoffe, da es zwar in menschlichen Zellen nicht repliziert, aber auf effiziente Art und Weise rekombinante Proteine exprimieren kann, die durch heterologe Gene kodiert werden. Dies ermöglicht sowohl die Induktion der zellulären als auch der humoralen Immunantwort. Ein weiterer Vorteil des MVA Vektorsystems besteht darin, dass die Viren in großen Mengen und unter GMP Bedingungen hergestellt werden können. Hiermit erfüllt MVA wichtige Voraussetzungen für die Herstellung eines erfolgreichen Impfstoffes.

Diese Arbeit beschreibt die genetische und immunologische Charakterisierung eines neuen auf der Basis von MVA entwickelten Impfstoffkandidaten gegen HCMV (MVA-pp65/IE-1). Dabei wurde die Infektion von CD40 B Zellen mit rekombinanten MVA Viren etabliert und zur immunologischen Charakterisierung dieser Viren verwendet.

Das rekombinante Virus MVA-pp65/IE-1 war in primären Hühner-embryofibroblasten (CEF) vermehrungsfähig, nicht aber in humanen Zellen (HaCaT). Ein 120 kDa großes Protein konnte in MVA-pp65/IE-1 infizierten Zellen exprimiert und durch HCMV-spezifischen Antikörper nachgewiesen werden. Mit MVA-pp65/IE-1 infizierten B Zellen war es möglich, T Zellen spezifisch gegen eine Vielzahl von IE-1 und pp65 Epitopen zu expandieren. Hierbei konnte zudem

eine funktionelle T Zellantwort gegen eine Vielzahl von verschiedenen IE-1 Peptiden durch IFN-γ-Messung gezeigt werden.

Diese Ergebnisse zeigen erstmals, dass MVA-pp65/IE-1 wichtige Voraussetzungen als Impfvirus gegen HCMV erfüllt. Daher ist eine weitergehende Erforschung und Entwicklung dieses vielversprechenden Impfstoffkandidaten anzustreben.

VIII. REFERENCES

Adhikary D, Behrends U, Boerschmann H, Pfunder A, Burdach S, Moosmann A, Witter K, Bornkamm GW, Mautner J. Immunodominance of lytic cycle antigens in Epstein-Barr virus-specific CD4+ T cell preparations for therapy. PLoS One 2007; 2: e583.

Adler SP, Plotkin SA, Gonczol E, Cadoz M, Meric C, Wang JB, Dellamonica P, Best AM, Zahradnik J, Pincus S, Berencsi K, Cox WI, Gyulai Z. A canarypox vector expressing cytomegalovirus (CMV) glycoprotein B primes for antibody responses to a live attenuated CMV vaccine (Towne). J Infect Dis 1999; 180: 843-6.

Adler SP, Finney JW, Manganello AM, Best AM. Prevention of child-to-mother transmission of cytomegalovirus among pregnant women. J Pediatr 2004; 145: 485-91.

Albarran YCA, de la Garza A, Cruz Quiroz BJ, Vazquez Zea E, Diaz Estrada I, Mendez Fuentez E, Lopez Contreras M, Andrade-Manzano A, Padilla S, Varela AR, Rosales R. MVA E2 recombinant vaccine in the treatment of human papillomavirus infection in men presenting intraurethral flat condyloma: a phase I/II study. BioDrugs 2007; 21: 47-59.

Ameres S, Mautner J, Schlott F, Neuenhahn M, Busch DH, Plachter B, Moosmann A. Presentation of an immunodominant immediate-early CD8+ T cell epitope resists human cytomegalovirus immunoevasion. PLoS Pathog 2013; 9: e1003383.

Antoine G, Scheiflinger F, Dorner F, Falkner FG. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology 1998; 244: 365-96.

Antrobus RD, Lillie PJ, Berthoud TK, Spencer AJ, McLaren JE, Ladell K, Lambe T, Milicic A, Price DA, Hill AV, Gilbert SC. A T cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years. PLoS One 2012; 7: e48322.

Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA. EBV persistence in memory B cells in vivo. Immunity 1998; 9: 395-404.

Backes S, Sperling KM, Zwilling J, Gasteiger G, Ludwig H, Kremmer E, Schwantes A, Staib C, Sutter G. Viral host-range factor C7 or K1 is essential for modified vaccinia virus Ankara late gene expression in human and murine cells, irrespective of their capacity to inhibit protein kinase R-mediated phosphorylation of eukaryotic translation initiation factor 2alpha. J Gen Virol 2010; 91: 470-82.

Barron MA, Gao D, Springer KL, Patterson JA, Brunvand MW, McSweeney PA, Zeng C, Baron AE, Weinberg A. Relationship of reconstituted adaptive and innate cytomegalovirus (CMV)-specific immune responses with CMV viremia in hematopoietic stem cell transplant recipients. Clin Infect Dis 2009; 49: 1777-83.

Bennink JR, Yewdell JW, Smith GL, Moller C, Moss B. Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. Nature 1984; 311: 578-9.

Berencsi K, Gyulai Z, Gonczol E, Pincus S, Cox WI, Michelson S, Kari L, Meric C, Cadoz M, Zahradnik J, Starr S, Plotkin S. A canarypox vector-expressing cytomegalovirus (CMV) phosphoprotein 65 induces long-lasting cytotoxic T cell responses in human CMV-seronegative subjects. J Infect Dis 2001; 183: 1171-9.

Bernstein DI, Reap EA, Katen K, Watson A, Smith K, Norberg P, Olmsted RA, Hoeper A, Morris J, Negri S, Maughan MF, Chulay JD. Randomized, double-blind, Phase 1 trial of an alphavirus replicon vaccine for cytomegalovirus in CMV seronegative adult volunteers. Vaccine 2009; 28: 484-93.

Berthoud TK, Hamill M, Lillie PJ, Hwenda L, Collins KA, Ewer KJ, Milicic A, Poyntz HC, Lambe T, Fletcher HA, Hill AV, Gilbert SC. Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. Clin Infect Dis 2011; 52: 1-7.

Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med 1989; 320: 1731-5.

Blanchard TJ, Alcami A, Andrea P, Smith GL. Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. J Gen Virol 1998; 79 (Pt 5): 1159-67.

Boeckh M, Leisenring W, Riddell SR, Bowden RA, Huang ML, Myerson D, Stevens-Ayers T, Flowers ME, Cunningham T, Corey L. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. Blood 2003; 101: 407-14.

Boeckh M, Ljungman P. How we treat cytomegalovirus in hematopoietic cell transplant recipients. Blood 2009; 113: 5711-9.

Boeckh M, Murphy WJ, Peggs KS. Recent advances in cytomegalovirus: an update on pharmacologic and cellular therapies. Biol Blood Marrow Transplant 2015; 21: 24-9.

Born WK, Kemal Aydintug M, O'Brien RL. Diversity of gammadelta T-cell antigens. Cell Mol Immunol 2013; 10: 13-20.

Borysiewicz LK, Graham S, Hickling JK, Mason PD, Sissons JG. Human cytomegalovirus-specific cytotoxic T cells: their precursor frequency and stage specificity. Eur J Immunol 1988; 18: 269-75.

Britt WJ, Vugler L, Butfiloski EJ, Stephens EB. Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response. J Virol 1990; 64: 1079-85.

Brocke P, Garbi N, Momburg F, Hammerling GJ. HLA-DM, HLA-DO and tapasin: functional similarities and differences. Curr Opin Immunol 2002; 14: 22-9.

Broder CC, Earl PL. Recombinant vaccinia viruses. Design, generation, and isolation. Mol Biotechnol 1999; 13: 223-45.

Bryant P, Ploegh H. Class II MHC peptide loading by the professionals. Curr Opin Immunol 2004; 16: 96-102.

Bunde T, Kirchner A, Hoffmeister B, Habedank D, Hetzer R, Cherepnev G, Proesch S, Reinke P, Volk HD, Lehmkuhl H, Kern F. Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. J Exp Med 2005; 201: 1031-6.

Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol 2010; 20: 202-13.

Carroll MW, Moss B. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. Virology 1997; 238: 198-211.

Castillo JP, Kowalik TF. Human cytomegalovirus immediate early proteins and cell growth control. Gene 2002; 290: 19-34.

Chiuppesi F, Wussow F, Johnson E, Bian C, Zhuo M, Rajakumar A, Barry PA, Britt WJ, Chakraborty R, Diamond DJ. Vaccine-derived neutralizing antibodies to the human cytomegalovirus gH/gL pentamer potently block primary cytotrophoblast infection. J Virol 2015; 89: 11884-98.

Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H, Assenmacher M, Billingham L, Steward C, Crawley C, Olavarria E, Goldman J, Chakraverty R, Mahendra P, Craddock C, Moss PA. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. J Exp Med 2005; 202: 379-86.

Conboy TJ, Pass RF, Stagno S, Alford CA, Myers GJ, Britt WJ, McCollister FP, Summers MN, McFarland CE, Boll TJ. Early clinical manifestations and intellectual outcome in children with symptomatic congenital cytomegalovirus infection. J Pediatr 1987; 111: 343-8.

Cresswell P, Bangia N, Dick T, Diedrich G. The nature of the MHC class I peptide loading complex. Immunol Rev 1999; 172: 21-8.

Cresswell P. Intracellular surveillance: controlling the assembly of MHC class I-peptide complexes. Traffic 2000; 1: 301-5.

Crotzer VL, Blum JS. Autophagy and adaptive immunity. Immunology 2010; 131: 9-17.

Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. Clin Microbiol Rev 2009; 22: 76-98.

Cui X, Meza BP, Adler SP, McVoy MA. Cytomegalovirus vaccines fail to induce epithelial entry neutralizing antibodies comparable to natural infection. Vaccine 2008; 26: 5760-6.

Dargan DJ, Douglas E, Cunningham C, Jamieson F, Stanton RJ, Baluchova K, McSharry BP, Tomasec P, Emery VC, Percivalle E, Sarasini A, Gerna G, Wilkinson GW, Davison AJ. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. J Gen Virol 2010; 91: 1535-46.

Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988; 334: 395-402.

Davison AJ, Dolan A, Akter P, Addison C, Dargan DJ, Alcendor DJ, McGeoch DJ, Hayward GS. The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. J Gen Virol 2003; 84: 17-28.

Diamond DJ, York J, Sun JY, Wright CL, Forman SJ. Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. Blood 1997; 90: 1751-67.

Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu Rev Immunol 1997; 15: 617-48.

Drexler I, Heller K, Wahren B, Erfle V, Sutter G. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. J Gen Virol 1998; 79 (Pt 2): 347-52.

Elek SD, Stern H. Development of a vaccine against mental retardation caused by cytomegalovirus infection in utero. Lancet 1974; 1: 1-5.

Elkington R, Walker S, Crough T, Menzies M, Tellam J, Bharadwaj M, Khanna R. Ex vivo profiling of CD8+-T-cell responses to human cytomegalovirus reveals broad and multispecific reactivities in healthy virus carriers. J Virol 2003; 77: 5226-40.

European Medicines Agency. Imvanex: modifiziertes Vacciniavirus Ankara lebend. In: EMA/490157/2013. London, United Kingdom: European Medicines Agency 2013.

Falagas ME, Snydman DR, Ruthazer R, Griffith J, Werner BG, Freeman R, Rohrer R. Cytomegalovirus immune globulin (CMVIG) prophylaxis is associated with increased survival after orthotopic liver transplantation. The Boston Center for Liver Transplantation CMVIG Study Group. Clin Transplant 1997; 11: 432-7.

Fouts AE, Chan P, Stephan JP, Vandlen R, Feierbach B. Antibodies against the gH/gL/UL128/UL130/UL131 complex comprise the majority of the anti-cytomegalovirus (anti-CMV) neutralizing antibody response in CMV hyperimmune globulin. J Virol 2012; 86: 7444-7.

Freed DC, Tang Q, Tang A, Li F, He X, Huang Z, Meng W, Xia L, Finnefrock AC, Durr E, Espeseth AS, Casimiro DR, Zhang N, Shiver JW, Wang D, An Z, Fu TM. Pentameric complex of viral glycoprotein H is the primary target for potent neutralization by a human cytomegalovirus vaccine. Proc Natl Acad Sci U S A 2013; 110: E4997-5005.

Frey SE, Harrison C, Pass RF, Yang E, Boken D, Sekulovich RE, Percell S, Izu AE, Hirabayashi S, Burke RL, Duliege AM. Effects of antigen dose and immunization regimens on antibody responses to a cytomegalovirus glycoprotein B subunit vaccine. J Infect Dis 1999; 180: 1700-3.

Gabanti E, Bruno F, Lilleri D, Fornara C, Zelini P, Cane I, Migotto C, Sarchi E, Furione M, Gerna G. Human cytomegalovirus (HCMV)-specific CD4+ and CD8+ T cells are both required for prevention of HCMV disease in seropositive solid-organ transplant recipients. PLoS One 2014; 9: e106044.

Gallez-Hawkins G, Villacres MC, Li X, Sanborn MC, Lomeli NA, Zaia JA. Use of transgenic HLA A*0201/Kb and HHD II mice to evaluate frequency of cytomegalovirus IE1-derived peptide usage in eliciting human CD8 cytokine response. J Virol 2003; 77: 4457-62.

Gamadia LE, Remmerswaal EB, Weel JF, Bemelman F, van Lier RA, Ten Berge IJ. Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease. Blood 2003; 101: 2686-92.

Gasteiger G, Kastenmuller W, Ljapoci R, Sutter G, Drexler I. Cross-priming of cytotoxic T cells dictates antigen requisites for modified vaccinia virus Ankara vector vaccines. J Virol 2007; 81: 11925-36.

Gavin MA, Gilbert MJ, Riddell SR, Greenberg PD, Bevan MJ. Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. J Immunol 1993; 151: 3971-80.

Geginat J, Paroni M, Maglie S, Alfen JS, Kastirr I, Gruarin P, De Simone M, Pagani M, Abrignani S. Plasticity of human CD4 T cell subsets. Front Immunol 2014; 5: 630.

Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 1994; 76: 287-99.

Gerna G, Sarasini A, Patrone M, Percivalle E, Fiorina L, Campanini G, Gallina A, Baldanti F, Revello MG. Human cytomegalovirus serum neutralizing antibodies block virus infection of endothelial/epithelial cells, but not fibroblasts, early during primary infection. J Gen Virol 2008; 89: 853-65.

Gibson L, Piccinini G, Lilleri D, Revello MG, Wang Z, Markel S, Diamond DJ, Luzuriaga K. Human cytomegalovirus proteins pp65 and immediate early protein 1 are common targets for CD8+ T cell responses in children with congenital or postnatal human cytomegalovirus infection. J Immunol 2004; 172: 2256-64.

Gil A, Shen S, Coley S, Gibson L, Diamond DJ, Wang S, Lu S. DNA vaccine prime followed by boost with live attenuated virus significantly improves antigen-specific T cell responses against human cytomegalovirus. Hum Vaccin Immunother 2013; 9: 2120-32.

Gilbert MJ, Riddell SR, Plachter B, Greenberg PD. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. Nature 1996; 383: 720-2.

Gilbert SC. Clinical development of Modified Vaccinia virus Ankara vaccines. Vaccine 2013; 31: 4241-6.

Goodrich JM, Mori M, Gleaves CA, Du Mond C, Cays M, Ebeling DF, Buhles WC, DeArmond B, Meyers JD. Early treatment with ganciclovir to prevent cytomegalovirus disease after allogeneic bone marrow transplantation. N Engl J Med 1991; 325: 1601-7.

Gratama JW, Brooimans RA, van der Holt B, Sintnicolaas K, van Doornum G, Niesters HG, Lowenberg B, Cornelissen JJ. Monitoring cytomegalovirus IE-1 and pp65-specific CD4+ and CD8+ T-cell responses after allogeneic stem cell transplantation may identify patients at risk for recurrent CMV reactivations. Cytometry B Clin Cytom 2008; 74: 211-20.

Greiner S, Humrich JY, Thuman P, Sauter B, Schuler G, Jenne L. The highly attenuated vaccinia virus strain modified virus Ankara induces apoptosis in melanoma cells and allows bystander dendritic cells to generate a potent anti-tumoral immunity. Clin Exp Immunol 2006; 146: 344-53.

Gromme M, Uytdehaag FG, Janssen H, Calafat J, van Binnendijk RS, Kenter MJ, Tulp A, Verwoerd D, Neefjes J. Recycling MHC class I molecules and endosomal peptide loading. Proc Natl Acad Sci U S A 1999; 96: 10326-31.

Haagmans BL, van den Brand JM, Raj VS, Volz A, Wohlsein P, Smits SL, Schipper D, Bestebroer TM, Okba N, Fux R, Bensaid A, Solanes Foz D, Kuiken T, Baumgartner W, Segales J, Sutter G, Osterhaus AD. An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. Science 2016; 351: 77-81.

Hamilton ST, van Zuylen W, Shand A, Scott GM, Naing Z, Hall B, Craig ME, Rawlinson WD. Prevention of congenital cytomegalovirus complications by maternal and neonatal treatments: a systematic review. Rev Med Virol 2014; 24: 420-33.

Heineman TC, Schleiss M, Bernstein DI, Spaete RR, Yan L, Duke G, Prichard M, Wang Z, Yan Q, Sharp MA, Klein N, Arvin AM, Kemble G. A phase 1 study of 4 live, recombinant human cytomegalovirus Towne/Toledo chimeric vaccines. J Infect Dis 2006; 193: 1350-60.

Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, Bollard CM, Liu H, Wu MF, Rochester RJ, Amrolia PJ, Hurwitz JL, Brenner MK, Rooney CM. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood 2010; 115: 925-35.

Hirsch VM, Fuerst TR, Sutter G, Carroll MW, Yang LC, Goldstein S, Piatak M, Jr., Elkins WR, Alvord WG, Montefiori DC, Moss B, Lifson JD. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. J Virol 1996; 70: 3741-52.

Honey K, Rudensky AY. Lysosomal cysteine proteases regulate antigen presentation. Nat Rev Immunol 2003; 3: 472-82.

Huh YH, Kim YE, Kim ET, Park JJ, Song MJ, Zhu H, Hayward GS, Ahn JH. Binding STAT2 by the acidic domain of human cytomegalovirus IE1 promotes viral growth and is negatively regulated by SUMO. J Virol 2008; 82: 10444-54.

Humar A, Michaels M. American Society of Transplantation recommendations for screening, monitoring and reporting of infectious complications in immunosuppression trials in recipients of organ transplantation. Am J Transplant 2006; 6: 262-74.

Institute of Medicine. In: Vaccines for the 21st Century: A Tool for Decisionmaking. Commitee to Study Prorities for Vaccine Division of Health Promotion and Disease Prevention. Stratton KR, Durch JS, Lawrence RS, eds. Washington (DC): National Academies Press (US) 2000.

Jabs DA, Enger C, Bartlett JG. Cytomegalovirus retinitis and acquired immunodeficiency syndrome. Arch Ophthalmol 1989; 107: 75-80.

Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nat Rev Immunol 2012; 12: 557-69.

Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. Nat Rev Immunol 2012; 12: 749-61.

Kennedy RB, Ovsyannikova I, Poland GA. Smallpox vaccines for biodefense. Vaccine 2009; 27 Suppl 4: D73-9.

Khan N, Cobbold M, Keenan R, Moss PA. Comparative analysis of CD8+ T cell responses against human cytomegalovirus proteins pp65 and immediate early 1 shows similarities in precursor frequency, oligoclonality, and phenotype. J Infect Dis 2002; 185: 1025-34.

Khan N. The immunological burden of human cytomegalovirus infection. Arch Immunol Ther Exp (Warsz) 2007; 55: 299-308.

Khan N, Best D, Bruton R, Nayak L, Rickinson AB, Moss PA. T cell recognition patterns of immunodominant cytomegalovirus antigens in primary and persistent infection. J Immunol 2007; 178: 4455-65.

Krause PR, Bialek SR, Boppana SB, Griffiths PD, Laughlin CA, Ljungman P, Mocarski ES, Pass RF, Read JS, Schleiss MR, Plotkin SA. Priorities for CMV vaccine development. Vaccine 2013; 32: 4-10.

Kreijtz JH, Suezer Y, van Amerongen G, de Mutsert G, Schnierle BS, Wood JM, Kuiken T, Fouchier RA, Lower J, Osterhaus AD, Sutter G, Rimmelzwaan GF. Recombinant modified vaccinia virus Ankara-based vaccine induces protective immunity in mice against infection with influenza virus H5N1. J Infect Dis 2007; 195: 1598-606.

Kreijtz JH, Suezer Y, de Mutsert G, van den Brand JM, van Amerongen G, Schnierle BS, Kuiken T, Fouchier RA, Lower J, Osterhaus AD, Sutter G, Rimmelzwaan GF. Preclinical evaluation of a modified vaccinia virus Ankara (MVA)-based vaccine against influenza A/H5N1 viruses. Vaccine 2009; 27: 6296-9.

Kreijtz JH, Suzer Y, Bodewes R, Schwantes A, van Amerongen G, Verburgh RJ, de Mutsert G, van den Brand J, van Trierum SE, Kuiken T, Fouchier RA, Osterhaus AD, Sutter G, Rimmelzwaan GF. Evaluation of a modified vaccinia virus Ankara (MVA)-based candidate pandemic influenza A/H1N1 vaccine in the ferret model. J Gen Virol 2010; 91: 2745-52.

Kreijtz JH, Gilbert SC, Sutter G. Poxvirus vectors. Vaccine 2013; 31: 4217-9.

Kremer M, Volz A, Kreijtz JH, Fux R, Lehmann MH, Sutter G. Easy and efficient protocols for working with recombinant vaccinia virus MVA. Methods Mol Biol 2012; 890: 59-92.

La Rosa C, Wang Z, Brewer JC, Lacey SF, Villacres MC, Sharan R, Krishnan R, Crooks M, Markel S, Maas R, Diamond DJ. Preclinical development of an adjuvant-free peptide vaccine with activity against CMV pp65 in HLA transgenic mice. Blood 2002; 100: 3681-9.

La Rosa C, Wang Z, Lacey SF, Lalimarmo MM, Krishnan A, Longmate J, Diamond DJ. In vitro expansion of polyclonal T-cell subsets for adoptive immunotherapy by recombinant modified vaccinia Ankara. Exp Hematol 2006; 34: 497-507.

La Rosa C, Longmate J, Lacey SF, Kaltcheva T, Sharan R, Marsano D, Kwon P, Drake J, Williams B, Denison S, Broyer S, Couture L, Nakamura R, Dadwal S, Kelsey MI, Krieg AM, Diamond DJ, Zaia JA. Clinical evaluation of safety and immunogenicity of PADRE-cytomegalovirus (CMV) and tetanus-CMV fusion peptide vaccines with or without PF03512676 adjuvant. J Infect Dis 2012; 205: 1294-304.

Lane JM, Ruben FL, Neff JM, Millar JD. Complications of smallpox vaccination, 1968. N Engl J Med 1969; 281: 1201-8.

Lee HR, Huh YH, Kim YE, Lee K, Kim S, Ahn JH. N-terminal determinants of human cytomegalovirus IE1 protein in nuclear targeting and disrupting PML-associated subnuclear structures. Biochem Biophys Res Commun 2007; 356: 499-504.

Leen AM, Myers GD, Sili U, Huls MH, Weiss H, Leung KS, Carrum G, Krance RA, Chang CC, Molldrem JJ, Gee AP, Brenner MK, Heslop HE, Rooney CM, Bollard CM. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. Nat Med 2006; 12: 1160-6.

Lehmann MH, Kastenmuller W, Kandemir JD, Brandt F, Suezer Y, Sutter G. Modified vaccinia virus Ankara triggers chemotaxis of monocytes and early respiratory immigration of leukocytes by induction of CCL2 expression. J Virol 2009; 83: 2540-52.

Li CR, Greenberg PD, Gilbert MJ, Goodrich JM, Riddell SR. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood 1994; 83: 1971-9.

Lillie PJ, Berthoud TK, Powell TJ, Lambe T, Mullarkey C, Spencer AJ, Hamill M, Peng Y, Blais ME, Duncan CJ, Sheehy SH, Havelock T, Faust SN, Williams RL, Gilbert A, Oxford J, Dong T, Hill AV, Gilbert SC. Preliminary assessment of the efficacy of a T-cell-based influenza vaccine, MVA-NP+M1, in humans. Clin Infect Dis 2012; 55: 19-25.

Linnerbauer S, Behrends U, Adhikary D, Witter K, Bornkamm GW, Mautner J. Virus and autoantigen-specific CD4+ T cells are key effectors in a SCID mouse model of EBV-associated post-transplant lymphoproliferative disorders. PLoS Pathog 2014; 10: e1004068.

Longmate J, York J, La Rosa C, Krishnan R, Zhang M, Senitzer D, Diamond DJ. Population coverage by HLA class-I restricted cytotoxic T-lymphocyte epitopes. Immunogenetics 2001; 52: 165-73.

Loughney JW, Rustandi RR, Wang D, Troutman MC, Dick LW, Jr., Li G, Liu Z, Li F, Freed DC, Price CE, Hoang VM, Culp TD, DePhillips PA, Fu TM, Ha S. Soluble human cytomegalovirus gH/gL/pUL128-131 pentameric complex, but not gH/gL, inhibits viral entry to epithelial cells and presents dominant native neutralizing epitopes. J Biol Chem 2015; 290: 15985-95.

Ludwig A, Hengel H. Epidemiological impact and disease burden of congenital cytomegalovirus infection in Europe. Euro Surveill 2009; 14: 26-32.

Ludwig B, Kraus FB, Kipp M, Preiser W, Schwerdtfeger R, Doerr HW, Buxbaum S. Cytomegalovirus-specific CD4 T-cell and glycoprotein B specific antibody response in recipients of allogenic stem cell transplantation. J Clin Virol 2006; 35: 160-6.

Lurain NS, Chou S. Antiviral drug resistance of human cytomegalovirus. Clin Microbiol Rev 2010; 23: 689-712.

Macagno A, Bernasconi NL, Vanzetta F, Dander E, Sarasini A, Revello MG, Gerna G, Sallusto F, Lanzavecchia A. Isolation of human monoclonal antibodies that potently neutralize human cytomegalovirus infection by targeting different epitopes on the gH/gL/UL128-131A complex. J Virol 2010; 84: 1005-13.

Mackett M, Smith GL, Moss B. Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc Natl Acad Sci U S A 1982; 79: 7415-9.

Marty FM, Ljungman P, Papanicolaou GA, Winston DJ, Chemaly RF, Strasfeld L, Young JA, Rodriguez T, Maertens J, Schmitt M, Einsele H, Ferrant A, Lipton JH, Villano SA, Chen H, Boeckh M. Maribavir prophylaxis for prevention of cytomegalovirus disease in recipients of allogeneic stem-cell transplants: a phase 3, double-blind, placebo-controlled, randomised trial. Lancet Infect Dis 2011; 11: 284-92.

Mayr A, Munz E. [Changes in the vaccinia virus through continuing passages in chick embryo fibroblast cultures]. Zentralbl Bakteriol Orig 1964; 195: 24-35.

Mayr A, Hochstein-Mintzel V, Stickl H. Abstammung, Eigenschaften und Verwendung des attenuierten Vaccinia-Stammes MVA. Infection 1975; 3: 6-14.

Mayr A, Stickl H, Muller HK, Danner K, Singer H. [The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl)]. Zentralbl Bakteriol B 1978; 167: 375-90.

McLaughlin-Taylor E, Pande H, Forman SJ, Tanamachi B, Li CR, Zaia JA, Greenberg PD, Riddell SR. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J Med Virol 1994; 43: 103-10.

Messori A, Rampazzo R, Scroccaro G, Martini N. Efficacy of hyperimmune anti-cytomegalovirus immunoglobulins for the prevention of cytomegalovirus infection in recipients of allogeneic bone marrow transplantation: a meta-analysis. Bone Marrow Transplant 1994; 13: 163-7.

Meyer H, Sutter G, Mayr A. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J Gen Virol 1991; 72 (Pt 5): 1031-8.

Micklethwaite K, Hansen A, Foster A, Snape E, Antonenas V, Sartor M, Shaw P, Bradstock K, Gottlieb D. Ex vivo expansion and prophylactic infusion of CMV-pp65 peptide-specific cytotoxic T-lymphocytes following allogeneic hematopoietic stem cell transplantation. Biol Blood Marrow Transplant 2007; 13: 707-14.

Mocarski ES, Shenk T, Griffith PD, Pass RF. Cytomegaloviruses. In: Field's Virology 6th edition. Knipe DM, Howley PM, eds. United States: Lippincott Williams & Wilki 2013: 1960-2014.

Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimer M, Parham P. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. J Immunol 2008; 180: 3969-79.

Moosmann A, Bigalke I, Tischer J, Schirrmann L, Kasten J, Tippmer S, Leeping M, Prevalsek D, Jaeger G, Ledderose G, Mautner J, Hammerschmidt W, Schendel DJ, Kolb HJ. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. Blood 2010; 115: 2960-70.

Moss B. Vaccinia virus: a tool for research and vaccine development. Science 1991; 252: 1662-7.

Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A 1996; 93: 11341-8.

Munoz I, Gutierrez A, Gimeno C, Farga A, Alberola J, Solano C, Prosper F, Garcia-Conde J, Navarro D. Lack of association between the kinetics of human cytomegalovirus (HCMV) glycoprotein B (gB)-specific and neutralizing serum antibodies and development or recovery from HCMV active infection in patients undergoing allogeneic stem cell transplant. J Med Virol 2001; 65: 77-84.

Murphy K, Travers P, Walport M. An introduction to immunobiology and innate immunity (Part I) - The adaptive immune response (Part IV). In: Janeway's Immunobiology 7th edition. New York, London: Garland Science 2008: 1-458.

Mutter W, Reddehase MJ, Busch FW, Buhring HJ, Koszinowski UH. Failure in generating hemopoietic stem cells is the primary cause of death from cytomegalovirus disease in the immunocompromised host. J Exp Med 1988; 167: 1645-58.

Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol 2011; 11: 823-36.

Panicali D, Paoletti E. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad Sci U S A 1982; 79: 4927-31.

Paoletti E. Applications of pox virus vectors to vaccination: an update. Proc Natl Acad Sci U S A 1996; 93: 11349-53.

Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, Leung K, Carrum G, Gee AP, Vera JF, Krance RA, Brenner MK, Rooney CM, Heslop HE, Leen AM. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. Sci Transl Med 2014; 6: 242ra83.

Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol 2005; 5: 201-14.

Pascutti MF, Rodriguez AM, Falivene J, Giavedoni L, Drexler I, Gherardi MM. Interplay between modified vaccinia virus Ankara and dendritic cells: phenotypic and functional maturation of bystander dendritic cells. J Virol 2011; 85: 5532-45.

Pass RF, Stagno S, Myers GJ, Alford CA. Outcome of symptomatic congenital cytomegalovirus infection: results of long-term longitudinal follow-up. Pediatrics 1980; 66: 758-62.

Pass RF, Duliege AM, Boppana S, Sekulovich R, Percell S, Britt W, Burke RL. A subunit cytomegalovirus vaccine based on recombinant envelope glycoprotein B and a new adjuvant. J Infect Dis 1999; 180: 970-5.

Peggs K, Verfuerth S, Mackinnon S. Induction of cytomegalovirus (CMV)-specific T-cell responses using dendritic cells pulsed with CMV antigen: a novel culture system free of live CMV virions. Blood 2001; 97: 994-1000.

Pepperl S, Munster J, Mach M, Harris JR, Plachter B. Dense bodies of human cytomegalovirus induce both humoral and cellular immune responses in the absence of viral gene expression. J Virol 2000; 74: 6132-46.

Plotkin SA, Farquhar J, Horberger E. Clinical trials of immunization with the Towne 125 strain of human cytomegalovirus. J Infect Dis 1976; 134: 470-5.

Polic B, Hengel H, Krmpotic A, Trgovcich J, Pavic I, Luccaronin P, Jonjic S, Koszinowski UH. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. J Exp Med 1998; 188: 1047-54.

Price DA, Brenchley JM, Ruff LE, Betts MR, Hill BJ, Roederer M, Koup RA, Migueles SA, Gostick E, Wooldridge L, Sewell AK, Connors M, Douek DC. Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses. J Exp Med 2005; 202: 1349-61.

Prichard MN, Penfold ME, Duke GM, Spaete RR, Kemble GW. A review of genetic differences between limited and extensively passaged human cytomegalovirus strains. Rev Med Virol 2001; 11: 191-200.

Quinnan GV, Jr., Kirmani N, Rook AH, Manischewitz JF, Jackson L, Moreschi G, Santos GW, Saral R, Burns WH. Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. N Engl J Med 1982; 307: 7-13.

Radha R, Jordan S, Puliyanda D, Bunnapradist S, Petrosyan A, Amet N, Toyoda M. Cellular immune responses to cytomegalovirus in renal transplant recipients. Am J Transplant 2005; 5: 110-7.

Ramirez JC, Gherardi MM, Rodriguez D, Esteban M. Attenuated modified vaccinia virus Ankara can be used as an immunizing agent under conditions of preexisting immunity to the vector. J Virol 2000; 74: 7651-5.

Reap EA, Morris J, Dryga SA, Maughan M, Talarico T, Esch RE, Negri S, Burnett B, Graham A, Olmsted RA, Chulay JD. Development and preclinical evaluation of an alphavirus replicon particle vaccine for cytomegalovirus. Vaccine 2007; 25: 7441-9.

Reddehase MJ, Weiland F, Munch K, Jonjic S, Luske A, Koszinowski UH. Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. J Virol 1985; 55: 264-73.

Reddehase MJ, Mutter W, Munch K, Buhring HJ, Koszinowski UH. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J Virol 1987; 61: 3102-8.

Reddehase MJ, Jonjic S, Weiland F, Mutter W, Koszinowski UH. Adoptive immunotherapy of murine cytomegalovirus adrenalitis in the immunocompromised host: CD4-helper-independent antiviral function of CD8-positive memory T lymphocytes derived from latently infected donors. J Virol 1988; 62: 1061-5.

Reits E, Griekspoor A, Neijssen J, Groothuis T, Jalink K, van Veelen P, Janssen H, Calafat J, Drijfhout JW, Neefjes J. Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. Immunity 2003; 18: 97-108.

Reusken CB, Farag EA, Haagmans BL, Mohran KA, Godeke GJt, Raj S, Alhajri F, Al-Marri SA, Al-Romaihi HE, Al-Thani M, Bosch BJ, van der Eijk AA, El-Sayed AM, Ibrahim AK, Al-Molawi N, Muller MA, Pasha SK, Drosten C, AlHajri MM, Koopmans MP. Occupational Exposure to Dromedaries and Risk for MERS-CoV Infection, Qatar, 2013-2014. Emerg Infect Dis 2015; 21: 1422-5.

Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood 1991; 78: 1373-80.

Revello MG, Lazzarotto T, Guerra B, Spinillo A, Ferrazzi E, Kustermann A, Guaschino S, Vergani P, Todros T, Frusca T, Arossa A, Furione M, Rognoni V, Rizzo N, Gabrielli L, Klersy C, Gerna G. A randomized trial of hyperimmune globulin to prevent congenital cytomegalovirus. N Engl J Med 2014; 370: 1316-26.

Rickinson AB, Rowe M, Hart IJ, Yao QY, Henderson LE, Rabin H, Epstein MA. T-cell-mediated regression of "spontaneous" and of Epstein-Barr virus-induced B-cell transformation in vitro: studies with cyclosporin A. Cell Immunol 1984; 87: 646-58.

Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science 1992; 257: 238-41.

Rist M, Cooper L, Elkington R, Walker S, Fazou C, Tellam J, Crough T, Khanna R. Ex vivo expansion of human cytomegalovirus-specific cytotoxic T cells by recombinant polyepitope: implications for HCMV immunotherapy. Eur J Immunol 2005; 35: 996-1007.

Robain M, Carre N, Dussaix E, Salmon-Ceron D, Meyer L. Incidence and sexual risk factors of cytomegalovirus seroconversion in HIV-infected subjects. The SEROCO Study Group. Sex Transm Dis 1998; 25: 476-80.

Rochlitz C, Figlin R, Squiban P, Salzberg M, Pless M, Herrmann R, Tartour E, Zhao Y, Bizouarne N, Baudin M, Acres B. Phase I immunotherapy with a modified vaccinia virus (MVA) expressing human MUC1 as antigen-specific immunotherapy in patients with MUC1-positive advanced cancer. J Gene Med 2003; 5: 690-9.

Rosales R, Lopez-Contreras M, Rosales C, Magallanes-Molina JR, Gonzalez-Vergara R, Arroyo-Cazarez JM, Ricardez-Arenas A, Del Follo-Valencia A, Padilla-Arriaga S, Guerrero MV, Pirez MA, Arellano-Fiore C, Villarreal F. Regression of human papillomavirus intraepithelial lesions is induced by MVA E2 therapeutic vaccine. Hum Gene Ther 2014; 25: 1035-49.

Ryckman BJ, Rainish BL, Chase MC, Borton JA, Nelson JA, Jarvis MA, Johnson DC. Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates entry into epithelial and endothelial cells. J Virol 2008; 82: 60-70.

Sacre K, Nguyen S, Deback C, Carcelain G, Vernant JP, Leblond V, Autran B, Dhedin N. Expansion of human cytomegalovirus (HCMV) immediate-early 1-specific CD8+ T cells and control of HCMV replication after allogeneic stem cell transplantation. J Virol 2008; 82: 10143-52.

Saveanu L, Carroll O, Lindo V, Del Val M, Lopez D, Lepelletier Y, Greer F, Schomburg L, Fruci D, Niedermann G, van Endert PM. Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. Nat Immunol 2005; 6: 689-97.

Schleiss MR, Bierle CJ, Swanson EC, McVoy MA, Wang JB, Al-Mahdi Z, Geballe AP. Vaccination with a live attenuated cytomegalovirus devoid of a protein kinase R inhibitory gene results in reduced maternal viremia and improved pregnancy outcome in a guinea pig congenital infection model. J Virol 2015; 89: 9727-38.

Schmitt A, Tonn T, Busch DH, Grigoleit GU, Einsele H, Odendahl M, Germeroth L, Ringhoffer M, Ringhoffer S, Wiesneth M, Greiner J, Michel D, Mertens T, Rojewski M, Marx M, von Harsdorf S, Dohner H, Seifried E, Bunjes D, Schmitt M. Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. Transfusion 2011; 51: 591-9.

Schoppel K, Schmidt C, Einsele H, Hebart H, Mach M. Kinetics of the antibody response against human cytomegalovirus-specific proteins in allogeneic bone marrow transplant recipients. J Infect Dis 1998; 178: 1233-43.

Sia IG, Patel R. New strategies for prevention and therapy of cytomegalovirus infection and disease in solid-organ transplant recipients. Clin Microbiol Rev 2000; 13: 83-121.

Smith GL, Benfield CT, Maluquer de Motes C, Mazzon M, Ember SW, Ferguson BJ, Sumner RP. Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. J Gen Virol 2013; 94: 2367-92.

Snydman DR, Werner BG, Heinze-Lacey B, Berardi VP, Tilney NL, Kirkman RL, Milford EL, Cho SI, Bush HL, Jr., Levey AS, et al. Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients. N Engl J Med 1987; 317: 1049-54.

Song F, Fux R, Provacia LB, Volz A, Eickmann M, Becker S, Osterhaus AD, Haagmans BL, Sutter G. Middle East respiratory syndrome coronavirus spike protein delivered by modified vaccinia virus Ankara efficiently induces virus-neutralizing antibodies. J Virol 2013; 87: 11950-4.

Staib C, Lowel M, Erfle V, Sutter G. Improved host range selection for recombinant modified vaccinia virus Ankara. Biotechniques 2003; 34: 694-6, -8, 700.

Stickl H, Hochstein-Mintzel V, Mayr A, Huber HC, Schafer H, Holzner A. [MVA vaccination against smallpox: clinical tests with an attenuated live vaccinia virus strain (MVA) (author's transl)]. Dtsch Med Wochenschr 1974; 99: 2386-92.

Stinski MF. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J Virol 1978; 26: 686-701.

Stinski MF, Thomsen DR, Stenberg RM, Goldstein LC. Organization and expression of the immediate early genes of human cytomegalovirus. J Virol 1983; 46: 1-14.

Stittelaar KJ, Kuiken T, de Swart RL, van Amerongen G, Vos HW, Niesters HG, van Schalkwijk P, van der Kwast T, Wyatt LS, Moss B, Osterhaus AD. Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques. Vaccine 2001; 19: 3700-9.

Sutter G, Moss B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc Natl Acad Sci U S A 1992; 89: 10847-51.

Sutter G, Wyatt LS, Foley PL, Bennink JR, Moss B. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine 1994; 12: 1032-40.

Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, Sleath PR, Grabstein KH, Hosken NA, Kern F, Nelson JA, Picker LJ. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med 2005; 202: 673-85.

Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI, Davis SW, van der Hoeven J, Meignier B, Riviere M, et al. NYVAC: a highly attenuated strain of vaccinia virus. Virology 1992; 188: 217-32.

Tay CH, Yu LY, Kumar V, Mason L, Ortaldo JR, Welsh RM. The role of LY49 NK cell subsets in the regulation of murine cytomegalovirus infections. J Immunol 1999; 162: 718-26.

Thiele F, Tao S, Zhang Y, Muschaweckh A, Zollmann T, Protzer U, Abele R, Drexler I. Modified vaccinia virus Ankara-infected dendritic cells present CD4+ T-cell epitopes by endogenous major histocompatibility complex class II presentation pathways. J Virol 2015; 89: 2698-709.

Valdez Graham V, Sutter G, Jose MV, Garcia-Carranca A, Erfle V, Moreno Mendoza N, Merchant H, Rosales R. Human tumor growth is inhibited by a vaccinia virus carrying the E2 gene of bovine papillomavirus. Cancer 2000; 88: 1650-62.

van den Doel P, Volz A, Roose JM, Sewbalaksing VD, Pijlman GP, van Middelkoop I, Duiverman V, van de Wetering E, Sutter G, Osterhaus AD, Martina BE. Recombinant modified vaccinia virus Ankara expressing glycoprotein E2 of Chikungunya virus protects AG129 mice against lethal challenge. PLoS Negl Trop Dis 2014; 8: e3101.

Volz A, Sutter G. Protective efficacy of modified vaccinia virus Ankara in preclinical studies. Vaccine 2013; 31: 4235-40.

Volz A, Kupke A, Song F, Jany S, Fux R, Shams-Eldin H, Schmidt J, Becker C, Eickmann M, Becker S, Sutter G. Protective efficacy of recombinant modified vaccinia virus Ankara delivering middle east respiratory syndrome coronavirus spike glycoprotein. J Virol 2015; 89: 8651-6.

Waibler Z, Anzaghe M, Ludwig H, Akira S, Weiss S, Sutter G, Kalinke U. Modified vaccinia virus Ankara induces Toll-like receptor-independent type I interferon responses. J Virol 2007; 81: 12102-10.

Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, Riddell SR. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med 1995; 333: 1038-44.

Wang D, Shenk T. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. Proc Natl Acad Sci U S A 2005; 102: 18153-8.

Wang Z, La Rosa C, Mekhoubad S, Lacey SF, Villacres MC, Markel S, Longmate J, Ellenhorn JD, Siliciano RF, Buck C, Britt WJ, Diamond DJ. Attenuated poxviruses generate clinically relevant frequencies of CMV-specific T cells. Blood 2004; 104: 847-56.

Wang Z, La Rosa C, Li Z, Ly H, Krishnan A, Martinez J, Britt WJ, Diamond DJ. Vaccine properties of a novel marker gene-free recombinant modified vaccinia Ankara expressing immunodominant CMV antigens pp65 and IE1. Vaccine 2007; 25: 1132-41.

Wiesner M, Zentz C, Mayr C, Wimmer R, Hammerschmidt W, Zeidler R, Moosmann A. Conditional immortalization of human B cells by CD40 ligation. PLoS One 2008; 3: e1464.

Wills MR, Carmichael AJ, Mynard K, Jin X, Weekes MP, Plachter B, Sissons JG. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J Virol 1996; 70: 7569-79.

Wilson SR, Wilson JH, Buonocore L, Palin A, Rose JK, Reuter JD. Intranasal immunization with recombinant vesicular stomatitis virus expressing murine cytomegalovirus glycoprotein B induces humoral and cellular immunity. Comp Med 2008; 58: 129-39.

Winston DJ, Saliba F, Blumberg E, Abouljoud M, Garcia-Diaz JB, Goss JA, Clough L, Avery R, Limaye AP, Ericzon BG, Navasa M, Troisi RI, Chen H, Villano SA, Uknis ME. Efficacy and safety of maribavir dosed at 100 mg orally twice daily for the prevention of cytomegalovirus disease in liver transplant recipients: a randomized, double-blind, multicenter controlled trial. Am J Transplant 2012; 12: 3021-30.

Wloch MK, Smith LR, Boutsaboualoy S, Reyes L, Han C, Kehler J, Smith HD, Selk L, Nakamura R, Brown JM, Marbury T, Wald A, Rolland A, Kaslow D, Evans T, Boeckh M. Safety and immunogenicity of a bivalent cytomegalovirus DNA vaccine in healthy adult subjects. J Infect Dis 2008; 197: 1634-42.

Wussow F, Chiuppesi F, Martinez J, Campo J, Johnson E, Flechsig C, Newell M, Tran E, Ortiz J, La Rosa C, Herrmann A, Longmate J, Chakraborty R, Barry PA, Diamond DJ. Human cytomegalovirus vaccine based on the envelope gH/gL pentamer complex. PLoS Pathog 2014; 10: e1004524.

Wyatt LS, Shors ST, Murphy BR, Moss B. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. Vaccine 1996; 14: 1451-8.

Wyatt LS, Earl PL, Xiao W, Americo JL, Cotter CA, Vogt J, Moss B. Elucidating and minimizing the loss by recombinant vaccinia virus of human immunodeficiency virus gene expression resulting from spontaneous mutations and positive selection. J Virol 2009; 83: 7176-84.

Yue Y, Wang Z, Abel K, Li J, Strelow L, Mandarino A, Eberhardt MK, Schmidt KA, Diamond DJ, Barry PA. Evaluation of recombinant modified vaccinia Ankara virus-based rhesus cytomegalovirus vaccines in rhesus macaques. Med Microbiol Immunol 2008; 197: 117-23.

Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med 2012; 367: 1814-20.

Zegri Reiriz I, Gomez-Bueno M, Segovia Cubero J. Successful use of maribavir for drug-resistant cytomegalovirus colitis in a heart transplant recipient. Rev Esp Cardiol (Engl Ed) 2015; 68: 908-9.

Zentz C, Wiesner M, Man S, Frankenberger B, Wollenberg B, Hillemanns P, Zeidler R, Hammerschmidt W, Moosmann A. Activated B cells mediate efficient expansion of rare antigen-specific T cells. Hum Immunol 2007; 68: 75-85.

Zhong J, Rist M, Cooper L, Smith C, Khanna R. Induction of pluripotent protective immunity following immunisation with a chimeric vaccine against human cytomegalovirus. PLoS One 2008; 3: e3256.

ZKBS. Position statement on handling recombinant vaccinia viruses. In: Ref. No. 6790-10-14. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit. Berlin, Germany: Zentrale Kommission für die Biologische Sicherheit 1997.

IX. APPENDICES

1. Buffers, solutions, and SDS-gel

Buffers and solutions	Conditions
Loading buffer (6×)	10 mM Tris-HCl pH 7,6 0.03% bromophenol blue 60% glycerol 60 mM EDTA add. distilled water
Lysis buffer (1×)	62.5 mM Tris-HCl pH 6,8 2% SDS 10% glycerol 50 mM DTT 0.01% bromophenol blue add. distilled water
PBS (1×, pH 7.4)	140 mM NaCl KCl $Na_2HPO_4 + 7H_2O$ KH_2PO_4 add. distilled water
TAE (50×. pH 7.8)	2 M Tris-acetate 0.5 M NaCl 50 mM EDTA add. distilled water
TBS (10×, pH 7,6)	200 mM Tris base 1.4 M NaCl add. distilled water
Transfer buffer (1×)	25 mM Tris base 200 mM glycine 20% ethanol add. distilled water

SDS-gel	Conditions
Resolving gel (10%)	9.9 ml (30% acrylamide) 7.5 ml (1.5 M Tris-HCl pH 8.8) 0.15 ml (20% SDS) 0.9 ml (10% ammoniumpersulfate) 24 µl TEMED add. 30 ml distilled water
Stacking gel (5%)	1.5 ml (1.0 M Tris-HCl pH 6.8) 45 µl (20% SDS) 0.27 ml (10% ammoniumpersulfate) 18 µl TEMED add. 9 ml distilled water

2. Commercial kits

Commercial kits	Supplier
AP Conjugate Substrate Kit	Bio-Rad, München, Germany
Clarity™ ECL Western Blotting Substrate	Bio-Rad, München, Germany
EnzygnostR Anti-CMV/IgG	Siemens, Erlangen, Germany
Human IFN-γ ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
Human IFN-γ ELISPOT Kit (ALP)	Mabtech, Nacka Strand, Sweden
Trans-Blot Turbo Mini Nitrocellulose Transfer pack	Bio-Rad, München, Germany

3. Media and additives

Media and additives	Supplier
HEPES Buffer (1 M)	Sigma-Aldrich, St.Louis, USA
Fetal calf serum (FCS)	Gibco Invitrogen, Karlsruhe, Germany
MEM Non-Essential Amino Acid Solution (100×)	Sigma-Aldrich, St.Louis, USA

Minimum Essential Medium Eagle (MEM)	Sigma-Aldrich, St.Louis, USA
PBS Dulbecco (w/o Mg^{2+})	PAN Biotech, Aidenbach, Germany
Penicillin (10,000U/ml)/ Streptomycin (10mg/ml)	Sigma-Aldrich, St.Louis, USA
RPMI-1640	Gibco Invitrogen, Karlsruhe, Germany
Sodium selenite	ICN Biochemicals, Aurora, USA
Trypsin-EDTA solution	Sigma-Aldrich, St.Louis, USA Gibco Invitrogen, Karlsruhe, Germany
VLE Dulbecco's MEM (DMEM)	Merck Millipore, Biochrom GmbH

4. Laboratory equipment and software

Laboratory equipment	Supplier
Avanti® J-26 XP Centrifuge	Beckman Coulter, Krefeld, Germany
ChemiDoc™MP, Imaging System	Bio-Rad, München, Germany
EL800, Universal Microplate Reader	BIO TEK Instruments, Winooski, USA
FACS Calibur cytofluorometer	Becton Dickinson, Heidelberg, Germany
ImmunoSpot 5.0 Analyzer ProCD	C.T.L., Shaker Heights, USA
Irradiation device Gammacell 40 (Cs-137)	Atomic Energy of Canada Limited, Ottawa, Kanada
Mupid®-One electrophoresis system	Mupid CO. LTD, Tokyo, Japan
Neubauer counting chamber	Paul Marienfeld, Lauda-Königshofen, Germany
PeqSTAR 2X Thermocycler	PEQLAB, Erlangen, Germany
Power Ease 500 protein electrophoresis	Invitrogen life technologies, Carlsbad, USA
Optima™LE-80K Ultracentrifuge	Beckman Coulter, Krefeld, Germany
Sonoplus	Bandelin electronic, Berlin, Germany

Software	Supplier
NCBI BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
CellQuest™ Pro Software	BD Biosciences, Heidelberg, Germany
CTL ImmunoSpot Software	C.T.L., Shaker Heights, USA
KC4	BIO TEK Instruments, Winooski, USA
Image Lab 5.0 Software	Bio-Rad, München, Germany
Prism 5	GraphPad Software, La Jolla, USA

5. Reagents, materials, and chemicals

Reagents/materials/chemicals	Supplier
Acetic acid ($C_2H_4O_2$)	Carl-Roth GmbH, Karlsruhe, Germany
Acrylamide 30% (C_3H_5NO)	AppliChem, Darmstadt, Germany
Ammoniumpersulfate ($H_8N_2O_8S_2$)	AppliChem, Darmstadt, Germany
Biozym LE Agarose	Biozym, Hessisch Oldendorf, Germany
Bovine serum albumine (BSA)	Sigma-Aldrich, St.Louis, USA
Bromophenol blue	Merck, Darmstadt, Germany
CpG-A (ODN 2216)	Miltenyi, Bergisch-Gladbach, Germany
CpG-B (ODN 2006)	Metabion, Planegg, Germany
Cyclosporin A	Novartis, Nürnberg, Germany
Distilled water	In-house production, München, Germany
DMSO (C_2H_6OS)	Carl-Roth GmbH, Karlsruhe, Germany
DTT ($C_4H_{10}O_2S_2$)	Sigma-Aldrich, St.Louis, USA
EDTA ($C_{10}H_{16}N_2O_8$)	ICN Biomedicals, Ohio, USA
FACS Flow/Clean/Rinse	BD Bioscienes, Heidelberg, Germany
Geld Red™	Biotrend, Köln, Germany
Glycerol ($C_3H_8O_3$)	Carl-Roth GmbH, Karlsruhe, Germany
Glycine ($C_2H_5NO_2$)	Carl-Roth GmbH, Karlsruhe, Germany
Hydrochloric acid (6 N,HCl)	Carl-Roth GmbH, Karlsruhe, Germany
Immunoplates MaxiSorp® 96-well flat bottom	Nunc A/S, Roskilde, Denmark

Monopotassium phosphate (KH_2PO_4)	Carl-Roth GmbH, Karlsruhe, Germany
HTS-HA (MSHAN4510) 96-well	Millipore, Billerica, USA
Nonfat dried milk powder	AppliChem, Darmstadt, Germany
Pathogen-free chicken eggs	Charles River Laboratories, Massachusetts, USA ; VALO Biomedia GmbH, Osterholz-Scharmbeck, Germany
Para-nitrophenyl phosphate (p-NPP)	Carl-Roth GmbH, Karlsruhe, Germany
Potassium chloride (KCl)	Merck, Darmstadt, Germany
Precision Dual Color protein ladder	Bio-Rad, München, Germany
Protran™ Amersham™ Nitrocellulose membrane, 0.2 µm	GE Healthcare, München, Germany
Pro5 Fluorotag-PE	Proimmune, Oxford, UK
Recombinant human interleukin-2(rhIL-2)	Novartis, Nürnberg, Germany
Ready to use DNA standard	Thermo Fisher Scientific, Massachusetts, USA
SDS ($C_{12}H_{25}NaO_4S$)	Carl-Roth GmbH, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl-Roth GmbH, Karlsruhe, Germany
Sodium Monohydrogen Phosphate Heptahydrate ($Na_2HPO_4 + 7H_2O$)	Carl-Roth GmbH, Karlsruhe, Germany
TEMED ($C_6H_{16}N_2$)	Sigma-Aldrich, St.Louis, USA
Tris-glycine buffer (10×)	Bio-Rad, München, Germany
Tris-ultrapure ($C_4H_{11}NO_3$)	AppliChem, Darmstadt, Germany
Trypan blue	Sigma-Aldrich, St.Louis, USA-Aldrich Merck, Darmstadt, Germany
Tween20	Sigma-Aldrich, St.Louis, USA-Aldrich
4-20% Criterion TGX stain free gel	Bio-Rad, München, Germany

Other plasticware (plates/flasks) used for	Supplier
CD40 B cells, polyclonal T cell lines, and Immunological assays	
6-/12-/24-/48-/96-well flat bottom plates	BD Bioscienes, Heidelberg, Germany
Cell culture flasks (25/80/175 cm^2)	Nunc A/S, Roskilde, Denmark
Viruses, PCR, and Western blot	
6-/12-/24-/96-well flat bottom plates	Sarstedt, Nümbrecht, Germany
Cell culture flasks (75/175 cm^2)	Sarstedt, Nümbrecht, Germany

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