

Human Cytomegalovirus (HCMV)-based Therapeutic Cancer Vaccines

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Mohammed Omar Abdelaziz Yassen

From Cairo, Egypt

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1st Reviewer: Prof. Dr. Günther Schönrich, Institute of Virology, Charité Universitätsmedizin
Berlin, Berlin.

2nd Reviewer: Prof. Dr. Rupert Mutzel, Institute for Biology – Microbiology, Freie Universität
Berlin, Berlin.

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"The important thing is not to stop questioning. Curiosity has its own reason for existing."

Albert Einstein

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List of abbreviations

AIDS	Acquired immune deficiency syndrome
amp	Ampicillin
AP	Antarctic phosphatase
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
cam	chloramphenicol
CBTRUS	Central Brain Tumor Registry of the United States
cDNA	Complementary DNA
CMV	Cytomegalovirus
CNS	Central nervous system
CPE	Cytopathic effect
CTL	Cytotoxic CD8 T lymphocyte
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ds	Double strand
<i>E. coli</i>	<i>Escherichia coli</i>
EF-1	Human elongation factor-1 alpha
E-genes	Early genes
EGFP	Enhanced GFP
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS or FCS	Fetal Bovine Serum (FBS) or fetal calf serum
GALV	<i>Gibbon ape leukemia virus</i>
GBM	Glioblastoma multiforme
GFP	Green-fluorescent protein
gH	Glycoprotein H
gL	Glycoprotein L
gO	Glycoprotein O
HC2	Hybrid capture 2
HCMV	Human cytomegalovirus
HHV	Human herpes virus
hIL	Human interleukins

HLA	Human leukocyte antigen
HPV	Human papillomavirus
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kan	kanamycin
kbp	Kilo base pair
LB	Luria-Bertani
LCR	Long control region
L-genes	late genes
MCMV	Murine cytomegalovirus
MDSC	Myeloid-derived suppressor cells
MGMT	Methylguanine-DNA methyltransferase
MHC	Major histocompatibility complex
MLV	<i>Murine leukemia virus</i>
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
MVP	Microvascular proliferation
NFAT	Nuclear factor of activated T-cells
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
ng	Nanogram
NK	Natural killer cells
ORF	Open reading frame
p.i.	Post infection
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pCMV	Mammalian expression vector with CMV promoter
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDL-1	Programmed death-ligand 1
pp	Phosphoprotein
pRb	Retinoblastoma protein
PSA	Prostate-Specific Antigen

psm	positive selection marker
RFLP	Restriction fragment length polymorphism
RhCMV	Rhesus macaque CMV
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR
SIV	Simian Immunodeficiency Virus
STING	stimulator of interferon genes
TAE	Tris acetic acid EDTA
TAP	Transporter associated with antigen processing
TB	<i>Mycobacterium tuberculosis</i>
TCR	T cell receptor
TCT	ThinPrep cytological test
TE	Tris-EDTA
TGF	Tumor growth factor
TNF-α	Tumour Necrosis Factor alpha
TRP	Tyrosinase-related-protein
UL	Unique long
US	Unique short
VZV	Varicella zoster virus
WB	Western blot
WHO	World Health Organization
WT	Wild type
ZEBOV	<i>Zaire Ebola virus</i>

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1. Introduction

Herpesviruses are widespread and can infect both human and animals. The family *Herpesviridae* is subdivided into one unassigned genus and three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* based on virus structure, host range and the capability to establish latency in the infected cells [1]. Although viruses of the family *Herpesviridae* can cause primary lytic infection in the infected host, lifelong latency is common in the infection caused by these viruses [2].

1.1 *Betaherpesvirinae*

Members of this subfamily share common characteristics: a narrow cell spectrum of infection, relatively slow replication and they establish latency at the hematopoietic progenitor cells and monocytes [1]. Human cytomegalovirus (HCMV) and roseolovirus subtypes (HHV-6A, HHV-6B, and HHV-7) are the main members of the human *Betaherpesvirinae* subfamily. Although infection by roseoloviruses is always asymptomatic in healthy adults, they constitute an important health concern in neonates and infants. Moreover, roseoloviruses cause severe diseases such as encephalitis in immunosuppressed patients. [3-6]. HHV-6 can be a risk factor for multiple sclerosis and other neuroinflammatory conditions [7]. Importantly, HCMV is a very common pathogen worldwide with infection prevalence depends on socio-economic parameters. In developed countries, HCMV has a seroprevalence of 50-70% [8, 9].

1.2 Human Cytomegalovirus (HCMV)

Cytomegaloviruses (CMVs) are highly species-specific [1]. HCMV can be transmitted through saliva, blood transfusions, sexual contact, transplants, breast milk and across the placenta [10]. HCMV has the ability to infect a variety of different host cells including fibroblasts, epithelial and endothelial cells, monocytes, hepatocytes, smooth muscle cells, macrophages and neuronal cells [11, 12]. Although HCMV infection is usually asymptomatic in healthy individuals [13], it has serious life threatening complications such as retinitis, encephalitis, pneumonia, nephritis or gastroenteritis in immunosuppressed people, especially acquired immune deficiency syndrome (AIDS) or transplant patients [12, 14]. Moreover, HCMV is one of the major causes for graft rejection in kidney transplant recipients [15].

1.2.1 General features of HCMV

1.2.1.1 HCMV structure

HCMV is one of largest viruses with a diameter of 150 to 200 nm and shares the highly characteristic herpesviruses structure [1, 16]. HCMV virion is composed of DNA, nucleocapsid, a proteinaceous matrix and a lipid bilayer [1, 17] (Figure 1).

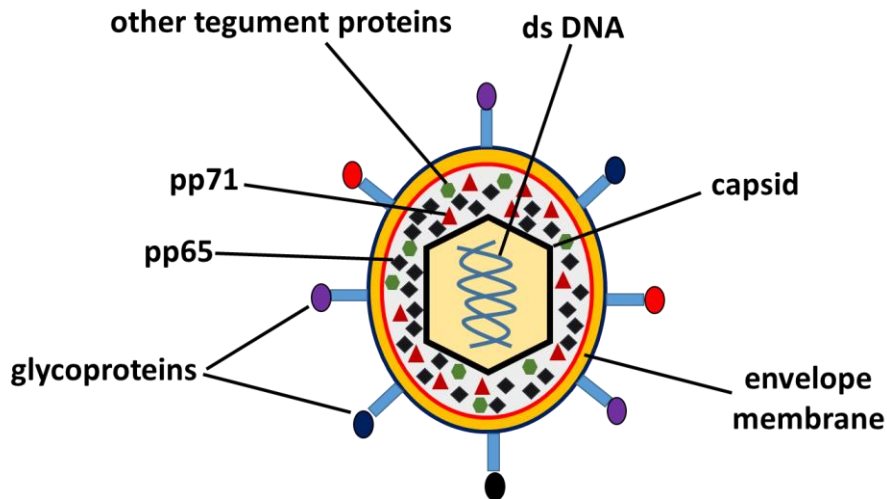


Figure 1. Schematic illustration of HCMV. HCMV has a relatively large genome of approximately 230 kbp located in the nucleocapsid which is embedded in the protein-rich matrix. The outer layer is a lipid envelope which contains the viral entry glycoproteins. Modified from John Paul Tomtishen III, 2012 [18].

The mature infectious HCMV virion contains a double-stranded linear DNA core, which is located in an icosahedral nucleocapsid (90 to 130 nm in diameter), enveloped by a proteinaceous matrix (the tegument) [1, 16]. The tegument is an electron-dense matrix, which consists of a protein mixture of up to twenty components. The viral protein pp65 represents the main tegument component and it has immunodominant properties which drive the major immune-specific responses [18, 19]. The pp65 protein has several roles, mainly it promotes the development of virions and assembly of virus particles into the tegument [18]. Moreover, it facilitates the viral evasion of the host innate and adaptive immune compartments [20, 21]. Another important tegument protein is pp71, which regulates the expression of immediate early proteins [18].

The envelope of HCMV consists of a double lipid membrane, in which several highly conserved glycoproteins are incorporated such as gB, gH and gL [22]. These glycoproteins allow receptor binding, adsorption and penetration [23]. Furthermore, they are major antigens for the induction

of virus neutralizing antibodies [24, 25]. The glycoprotein gB has a role as virus fusogen, which is triggered by gH/gL complexes [26-28]. The glycoproteins gH and gL form a trimer complex with gO (gH/gL/gO) which facilitates virus entry into all cell types while a pentamer complex of gH/gL with other viral proteins (gH/gL/UL128/UL130/UL131) is required for entry into epithelial and endothelial cells but is dispensable for infection of fibroblasts [29-35].

1.2.1.2 HCMV genome

HCMV has one of the largest genomes of all herpesviruses. Nevertheless, HCMV has a typical herpesvirus genome structure with unique long (U_L), unique short (U_S), and repeat regions. Since each long and short region can be oriented in either direction, four genome isomers are produced during replication (Class E genome) (Figure 2). The U_L and U_S regions are both flanked by a pair of inverted repeats; terminal repeat long/internal repeat long (TR_L/IR_L) and internal repeat short/terminal repeat short (IR_S/TR_S) (Figure 2) [1, 36].

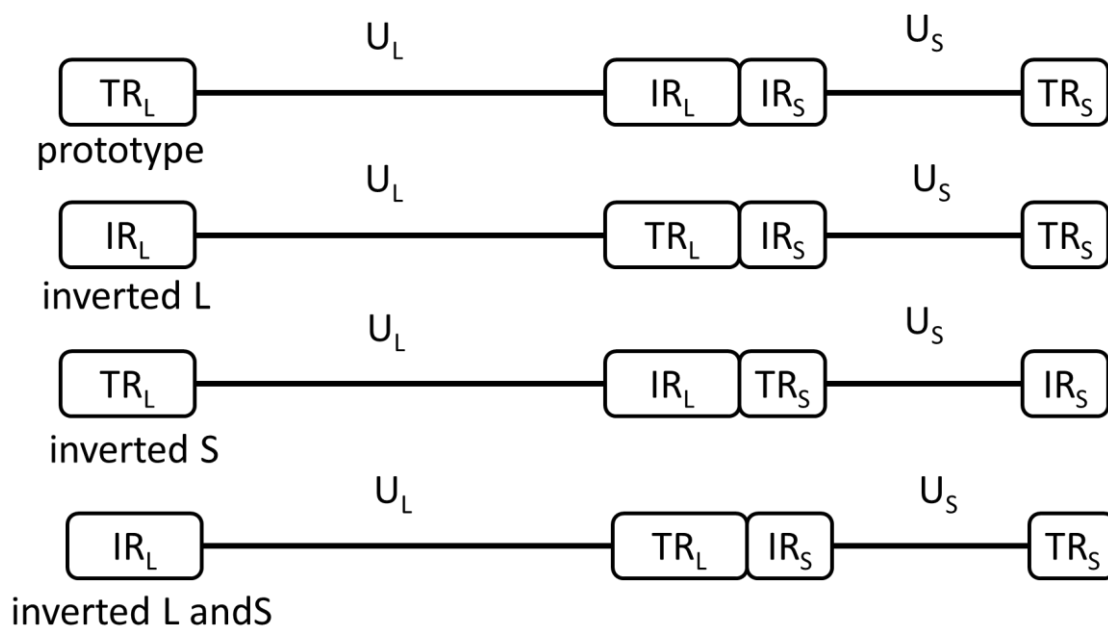


Figure 2. Schematic diagram of HCMV genome. The U_S and U_L regions can be arranged in different ways resulting in four different isomers. Modified from Fields Virology, 2013 [1].

The laboratory HCMV strain AD169 genome is composed of 229354 bp which encodes 208 open reading frames (ORFs) of 100 or more amino acids [37]. These ORFs are numbered sequentially according to their location within the unique (U_S and U_L) and the repeated regions [37, 38]. In contrast to the laboratory strain AD169, additional ORFs have been identified and characterized in strain TB40/E and other clinical strains of HCMV [39-41]. HCMV strain

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AD169 has been extensively passaged in cell culture, and has lost a large number of genes in the UL/b' region of the genome, as well as elsewhere in the genome [39, 40].

Genomic and proteomic analysis has shown that HCMV shares more than 40 ORFs with high homology to proteins encoded by alpha- and gamma- herpesviruses which provides an evidence of a common origin of the three subfamilies [1]. About 25% of HCMV ORFs encode proteins involved in viral DNA metabolism and replication, while the other 75% are involved in the maturation, structural organization of virions and other viral-mediated functions and pathogenesis such as immune evasion [1, 38]. The ORFs-encoded proteins in U_L regions are mainly involved in DNA replication and repair or in virion structure. They are grouped in seven conserved gene blocks (A–G) which are also found in other herpesviruses genomes, however, they exist in a different order in the alpha- and gamma- herpesviruses. On the other hand, the U_S ORFs or other ORFs encoded within the repeated regions of the HCMV genome are less well conserved compared to other herpesviruses [37, 42]. Analysis of wild type strains and genetically modified viruses encoding mutations of different ORFs showed that more than 50 ORFs-encoding proteins are not essential for viral replication *in vitro*. However, these ORFs-encode proteins involved in viral growth *in vivo*, pathogenesis, and evasion of the host immune system [38, 43].

Like other herpesviruses, HCMV can establish lytic infection and latency with intermittent reactivation and shedding of infectious virions. HCMV has three overlapping phases in the productive lytic infection cycles. In these phases, HCMV replicates and infectious virions are eventually released from the infected cells. These phases are immediate-early (IE) (the first two hours) in which CD8+ T lymphocytes (CTLs) are directed against IE-1 transcription factor, however that can be selectively blocked by kinase activity of tegument protein pp65 [21, 44], delayed early (E) phase (the first 24 hours) and the late (L) phase (after 24 hours) [45]. HCMV IE gene products are required to initiate the expression of E genes. Consequently, The proteins of the E phase are then needed for the expression of L genes [46].

During HCMV latency, the virus stays dormant within the host cell without replication. HCMV reactivation from latency can cause severe multi-organ damage in congenitally infected newborns and individuals with deficient immune system such as organ transplant patients [47-51]. The factors responsible for the persistence of viral genome in infected cells during latency and switching between latency and lytic infection are still elusive [52].

1.2.2 Host defense and HCMV-encoded immune evasion

An intact adaptive immune response is required to control HCMV replication during primary infection and reactivation. However, the innate immunity contributes to host defense against HCMV and adaptive immunity priming [14].

In order to establish latency, HCMV has evolved numerous intriguing strategies to subvert the host immune defense and prevent its elimination from the human cells [53, 54]. More than 40 HCMV gene products are recognized to have a role in modulating the host immune response following the viral infection [54]. As virus clearance is mainly dependant on cell-mediated immunity, which is mediated by CTLs and natural killer (NK) cells [55], HCMV encodes a set of proteins to efficiently escape from both cell types [54].

1.2.2.1 Adaptive immune response

1.2.2.1.1 Cell-mediated immunity

The viral antigens are normally processed and presented by major histocompatibility complex (MHC) class I (MHC-I) molecules on the surface of infected cells [56]. The classical MHC-I molecules are also expressed as human leukocyte antigen (HLA -A, -B and -C alleles) [57]. CMV-specific CTLs recognizing peptides loaded on MHC-I molecules play a crucial role in antiviral-immune responses and recovery from primary CMV infection [53, 54]. In animals, suppression of CTLs causes reactivation and dissemination of latent CMV infection [58]. Specific CTLs against structural and non-structural viral proteins were identified in HCMV infections [59]. HCMV-specific CTLs for pp65 or IE1 showed higher proliferation than those specific for gB or other viral envelope proteins. These findings suggest pp65 and IE1 are the main targets for the CTL-mediated immune response [60-62]. However, It has been demonstrated that HCMV can interfere with antigen presentation by MHC-I molecules which stimulates specific CTLs [53]. Till now, HCMV is known to encode four immune evasion protein (US2, US3, US6 and US11) which are implicated in evading MHC-I antigen presentation [54]. The assembly of MHC-I molecules with peptides requires the interaction of a multi-protein complex in the endoplasmic reticulum (ER). Tapasin protein forms a protein complex through interaction with both the transporter associated with antigen processing (TAP) and MHC- I molecules, this interaction is required for peptide loading [63]. US3 retains MHC-I molecules within the ER by inhibiting the interaction of tapasin with TAP [64, 65]. Additionally, US6 inhibits binding of ATP by TAP1 which is required for peptide transport.

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US6 associates with TAP which results in stabilization of a confirmation in TAP1 and therefore inhibits ATP binding and peptide translocation [66]. Moreover, HCMV encodes two other proteins that facilitate MHC-I heavy chain proteasomal degradation in HCMV-infected cells. US2 and US11 cause newly synthesized MHC-I molecules to be ubiquitinated which results in their degradation by the proteasome [67, 68]. Other cellular proteins such as Derlin-1 and SEL1L are essentially required for US11-mediated MHC-I degradation, while signal peptide peptidase is critical for US2 function [69-71].

On the other hand, CD4⁺ T cells play a key role in initiating and sustaining the viral-specific immunity through priming CTL-specific immunity and guiding the naïve B cells to become plasma cells that are able to secrete viral specific antibodies [72-74]. The investigation of lymphocyte proliferation in HCMV-seropositive but immunocompetent adults showed a high frequency of CD4⁺ T cells that are specific for MHC class II (MHC-II)-presented viral peptides derived from HCMV proteins such as gB, gH, pp65, and IE proteins [75]. Moreover, CD4⁺ T cells are important for the suppression of CMV lytic infection [76, 77]. In MCMV infection, CD4⁺ T cells play an important role in controlling primary infection in long term CTL-depleted mice [78]. In healthy HCMV-infected children, prolonged HCMV salivary shedding was associated with persistent deficiency of HCMV-specific CD4⁺ T cells immunity [79]. In order to subvert CD4⁺ T cells-mediated immune response, HCMV US2-encoded protein plays another role in immune evasion by downregulating MHC-II molecules. US2 targets the HLA-DR- α and HLA-DM- α chains for cellular degradation and therefore blocks antigen presentation to CD4⁺ T cells [80, 81].

1.2.2.1.2 Humoral adaptive immunity

Neutralizing antibodies play the main role for vaccine-induced protection against viral infections [82]. HCMV-infected immunocompetent individuals show high level of HCMV-specific immunoglobulin (Ig)M antibodies upon primary infection which lasts for 3–4 months. After a few weeks, HCMV-specific IgG antibodies develop and persist for lifelong and the main protective antibody was found to be against viral gB [83]. In animals, the gB-specific antibodies could protect mice and guinea pig from lethal CMV challenges [84, 85].

Several studies have been performed to evaluate the ability of gB administration to drive a strong and protective antibody response in humans [84, 86]. Nevertheless, the administration of gB-expressing ALVAC vaccines showed limited protective response as they failed to

produce high protective antibodies titre that react with the surface of the virion and infected cells. Thus, there is yet no approved protective HCMV vaccine. [87]. Anti-gH antibodies also have a potent and complement-independent neutralizing activity [88, 89]. Other viral proteins such as pp65 (UL83), and pp150 (UL32) can also drive strong and long lasting antibody responses [90]. Other HCMV vaccine trials demonstrated that several viral proteins are recognized by humoral immunity and a protective response could be derived by the administration of attenuated HCMV strain Towne or passive immunisation with high-titer anti-HCMV immunoglobulins [87].

1.2.2.2 Innate immune response

The NK cells play an important role together with CTLs for host defense with memory-like immune responses [91]. In mouse models, NK cells play a crucial role in MCMV clearance [92, 93]. Moreover, adoptive transfer of NK cells can protect mice against MCMV infection [94]. In humans, the importance of NK cells in the control of HCMV infection has been observed in NK cells-deficient patients [95-97]. However, HCMV encodes several proteins that block NK-mediated immune defense [95].

HLA-E is a non classical MHC-I molecule which is expressed in variety of cells including monocytes and macrophages and it plays an important role in controlling NK cells function. HLA-E binds to NK receptor (CD94/NKG2A) resulting in inhibition of NK-mediated immunity [98-100]. The signal sequence of HCMV UL40 gene product is a canonical ligand for HLA-E and negatively regulates NK cells [101]. Moreover, HCMV-encoded UL141 protein downregulates the NK-activating ligand CD155, which results in further suppression of NK-mediated immunity [102]. Furthermore, the HCMV UL18 gene encodes a protein homolog to MHC-I which impairs NK-mediated cell lysis [103].

Taken together, there are numerous HCMV-encoded proteins that interfere with antiviral innate and immune and adaptive immune responses in addition to those mentioned in the previous paragraphs. The gene product of HCMV UL83 (pp65) inhibits the proteasomal processing of the immunogenic HCMV IE1 protein by phosphorylation [21, 54]. Moreover, the tegument UL82 protein (pp71) and US9 protein impair the antiviral immune response through inhibition of stimulator of interferon genes (STING) signaling [104, 105]. Additionally, HCMV encodes a homolog of human Interleukin-10 (hIL-10), which has a potent immunoregulatory function [106-108]. HCMV UL111A generates two isoforms of viral IL-10 by alternative splicing. One

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of these proteins (cmvIL-10) is 175 amino acids long and expressed during the late phase of lytic infection. The second isoform, which is called LAcmvIL-10, is shorter (139 amino acids) and expressed not only during lytic infection but also during latency [109, 110]. Similar to HCMV, the rhesus macaque CMV (RhCMV) UL111A also encodes viral IL-10 whereas MCMV lacks an IL-10 homolog [111]. cmvIL-10 mediates immune suppression of several effector immune cells and downregulates MHC-I and MHC-II in infected individuals [112]. Importantly, it interferes with dendritic cell (DC) function [108, 110, 113]. The main HCMV gene products involved in immune evasion are summarized in Table 1.

HCMV gene product	Mechanism of immune evasion	References
US2, US3, US6 and US11	MHC-I downregulation and evasion of CTL-mediated immunity.	[53, 54, 114]
US2 and IE1	MHC-II downregulation and evasion of CD4+ T cells-mediated immunity.	[80]
US18 and US20	Evasion of NK-mediated immunity.	[115]
UL18	MHC-I homolog and evasion of NK cells.	[53]
UL16	NK cells function impairment.	[80]
UL40	Evasion of NK cells.	[95]
UL83 (pp65)	Inhibition of IE1 proteasomal processing and inhibition of antiviral gene expression.	[21, 54]
IE2	Overexpression of anti-apoptotic FLIP protein.	[21, 80]
UL82 (pp71)	Inhibition of interferon-mediated immunity.	[104]
UL111A (cmvIL-10)	IL-10 homolog, modulation of MHC-I and MHC-II molecules and impairment of DC function.	[108, 110, 112, 113, 116, 117]
UL141	Downregulation of CD155.	[53, 102]
miR-UL112	Escape from NK-mediated immunity.	[118]
UL141- UL144	Inhibition NK-activating ligands.	[53, 119]
UL142	Suppression of NK- and CTL-mediated immunity.	[80]

Table 1. HCMV-encoded genes involved in immune evasion.

1.2.3 HCMV and memory inflation

Upon viral infection, specific T cells expand and proliferate in order to eliminate the infection. After viral clearance, only small population of specific T cells remain in blood and other compartments such as the bone marrow as memory T cells, the rest are eliminated by apoptosis [120]. However, during latent CMV infection in animals such as MCMV-infected mice, specific T cells recognizing CMV epitopes expand and proliferate and remain at high frequency with effector-memory phenotype [121-123]. It is hypothesized that during establishing persistent infection in healthy individuals, HCMV-specific CTLs are primed and boosted overtime by repetitive reactivation of HCMV which had been observed in cross-sectional studies [124-126]. However, memory inflation has not been proven yet in humans due to limited longitudinal studies [125]. The percentage of CMV-specific CTLs expand with time until becoming extraordinary large, with a size about 20% of total CTLs [123, 126-128]. Interestingly, these CTLs remain functional, non-exhausted and can even occupy up to 50% of the total memory compartment. They are maintained during the lifetime of the infected individual. Moreover, they show restricted contraction following priming with sustained “effector-memory” phenotype. This unique phenomenon is termed as memory inflation (Figure 3) [123, 129-131]. In murine models, there was slight inflationary CD4+ T cells response post MCMV infection [132]. In humans, primary HCMV infection could also drive inflationary CD4+ T cells response in infected individuals [133].

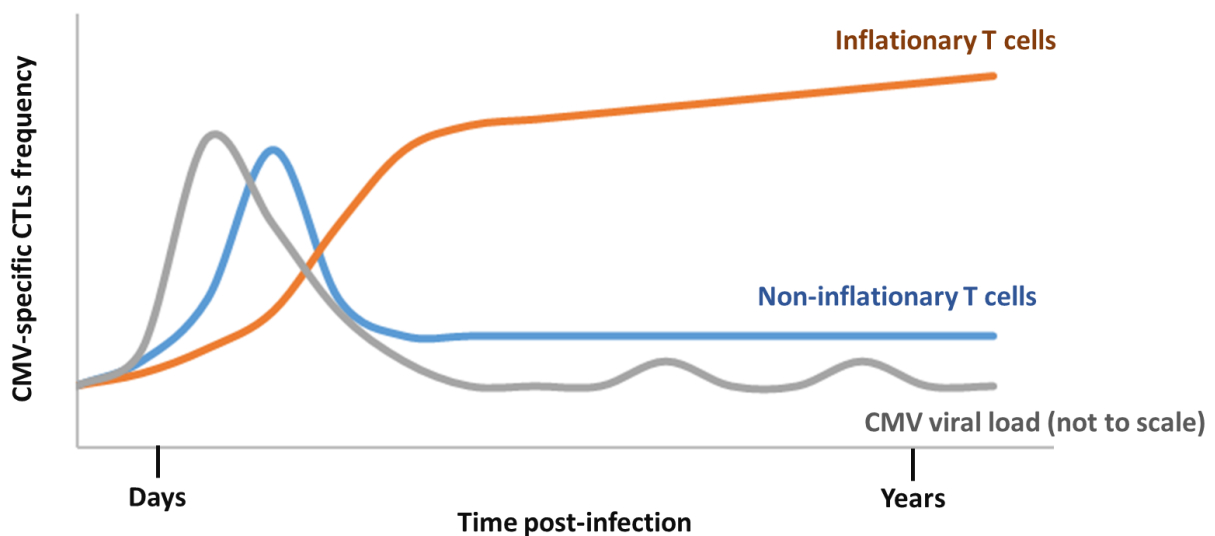


Figure 3. Schematic diagram of Inflationary CTLs. During CMV infection inflationary CTLs expand and proliferate to form a pool of memory T cells. Modified from O’Hara et.al , 2012 [130].

1.2.3.1 Features of inflationary memory T cells

Inflationary CTLs are characterized by an effector memory phenotype, in contrast to non-inflationary T cells which typically have a central memory phenotype [130, 134]. Lymph node homing markers such as CD62L and CCR7 are expressed at a low level on inflationary memory CTLs [128]. Moreover, they express low levels of co-stimulatory receptors such as CD27 and CD28, but high level of inhibitory receptors such as CD85j which is a negative regulatory receptor that associated with aging of CTLs [122, 134-136]. Interestingly, they do not express inhibitory receptor PD-1, this feature may prevent the exhaustion of inflationary memory T cells [128, 137].

Inflationary memory CTLs remain proliferative and able to secrete effector cytokines such as IFN- γ and TNF- α and maintain cytotoxicity [138]. Moreover, they can respond to CMV reactivation or *ex vivo* stimulation [123]. This unique functional feature is characteristic for CMV-specific inflationary memory CTLs, while in cancer patients T cells are exhausted and are not capable of mediating cytotoxicity [137, 139]. Inflationary CMV-specific CTLs show restriction in T cell receptor (TCR) diversity which suggests that T cell populations have higher affinity to preferentially selected CMV epitopes which maintain their clonal selection and dominance [140, 141].

1.2.3.2 Factors involved in memory inflation

Although the main mechanisms explaining this phenomenon remain mysterious, some factors involved in memory inflation of CMV-specific CTLs will be here discussed [128].

1.2.3.2.1 Antigen presentation

It was suggested that viral antigen presentation by infected cells, which are accessible to the blood supply and CTLs, is one of the primary factors to drive memory inflation for MCMV [142]. Although HCMV harbors several genes involved in host immune evasion including those that downregulate MHC-I molecules (see Section 1.2.2), antigen processing and presentation by MHC-I is crucial for driving memory inflation that may be primed due to cross-presentation by DCs [143-146]. Studies on MCMV showed that this inflationary response was facilitated through antigen presentation by non-hematopoietic cells in the lymph nodes during the latent MCMV infection. These expanded CTLs subsequently move to peripheral tissues, where they terminally differentiate and eventually accumulate [147].

1.2.3.2.2 Repetitive antigen exposure

Repetitive antigen exposure is essential to establish memory inflation response. Murine models showed that the MCMV-specific CTLs failed to divide when transferred from MCMV-infected mice to uninfected ones [128]. Moreover, a low infective dose of MCMV curbed the proliferation and accumulation of inflationary CTLs [148].

1.2.3.2.3 Cytokines and co-stimulatory molecules

It has been shown that secreted cytokines and co-stimulatory molecules play an important role in developing inflationary memory responses for CMV [149]. For example, the co-stimulatory 4-1BB and OX40 molecules are expressed on CMV-specific CTLs after activation and they play a role in MCMV-driven memory inflation [150, 151]. Murine models showed also that the MCMV-driven memory inflation is dependent on autocrine secretion of interleukins such as IL-2 [152, 153]. In addition, it was suggested that other cytokines such IL-15 play a role in potentiation of CTL proliferation and driving inflationary memory responses for CMV [154].

1.2.4 CMV-based vaccines

Interestingly, HCMV is able to superinfect individuals who were previously infected with another HCMV strain [155]. This fact is a major advantage of HCMV-based vaccines which makes them capable of infecting both HCMV-seronegative and seropositive individuals [156, 157]. Moreover, the inflationary memory CTLs are not exhausted and can respond efficiently to antigen-presenting cells as previously described (see Section 1.2.3).

It was demonstrated that recombinant CMV-based vaccines can drive robust T cell stimulation and proliferation against heterogenous antigens which were encoded in CMV vectors with mediating therapeutic and prophylactic responses [158-162].

1.2.4.1 Recombinant CMV vectors against infectious diseases

Several studies have investigated prophylactic and therapeutic CMV-based vaccines against different infectious diseases such as simian immunodeficiency virus (SIV) [163, 164], *Mycobacterium tuberculosis* (TB) [161, 165] and *Zaire ebolavirus* (ZEBOV) [166].

It is still unclear how CMV-based vaccines mediate their prophylactic and therapeutic effects. In some studies these effects were mediated by T cells, which were reported in CMV-based

vaccines against SIV in macaques and ZEBOV in mice [163, 166]. However, it could be also mediated by strong antibody-driven response which was previously shown in CMV-based vaccines against Ebola infection in macaques [162].

1.2.4.2 CMV-based vaccines for cancer therapy

Noncommunicable diseases are responsible for the majority of natural deaths, accounting for about 86% of deaths in Europe [167]. Cancers are becoming increasingly prevalent and are expected to be the leading cause of death in the 21st century [168]. In 2015, there were more than 17 million incident cancer cases worldwide and about 8.7 million deaths due to cancer [169].

The successful use of CMV-based vaccines against infectious diseases was expanded to develop CMV-based vaccines against cancer such as prostate cancer, melanoma and HPV-induced cancer which could generate a pool of tumor specific CTLs that mediate anti-tumor prophylactic and therapeutic responses [170].

1.2.4.2.1 CMV-based vaccines for prostate cancer therapy

MCMV has been successfully used to generate a CMV-based vaccine encoding human prostate-specific antigen (PSA) to treat prostate cancer in mouse models. The recombinant MCMV expressing MHC-I-restricted human PSA epitope was able to drive inflationary CTLs responses against PSA in a mouse model. More than 85% of the immunized mice showed total clearance of PSA-expressing tumors [171].

1.2.4.2.2 CMV-based vaccines for melanoma treatment

A single dose of recombinant MCMV expressing unmodified melanoma antigen tyrosinase-related-protein (TRP)-2 showed prophylactic response and significant reduction of tumors in a melanoma mouse model [172]. Interestingly, this immune response was independent of CTLs, instead tumor-specific antibodies were involved in this anti-melanoma response [173]

In another model using a MCMV-based vaccine expressing the melanoma antigen gp100, a tumor-associated self antigen, the vaccine failed to drive CTL tumor-specific responses. However, recombinant MCMV vaccine expressing a modified gp100 antigen that was mutated for higher MHC-I affinity could induce tumor-specific CTL cytotoxicity when used as a prophylactic and therapeutic vaccine in a mouse model [174, 175].

1.3 Human papillomavirus (HPV)-induced cancer

HPV is one of most common sexually transmitted diseases worldwide [176]. HPV causes transient infection that can develop into cancer [177]. A prophylactic vaccine has been generated with a high efficiency against high risk HPV strains such HPV16 and 18 [178]. HPV infection is usually cleared by the immune system in the immunocompetent, although the infected individuals remain at risk of developing lifelong infection which can progress to cancer [179, 180].

1.3.1 HPV epidemiology

It has been demonstrated that more than 7-8% of all human cancers are associated with HPV [181]. Approximately 530,000 women develop cervical cancer worldwide each year with HPV being associated with more than 99% of these cases [182, 183]. In Europe, more than 58,000 women are annually diagnosed with cervical cancer and more than 24,000 cases die because of this disease every year [184]. In the United States, more than 26,000 individuals were diagnosed with HPV-associated cancer per year between the years 2004 and 2008 [185]. Moreover, about 4100 women die annually in the United States due to cervical cancer caused by HPV with the genotype HPV16 being the most implicated [186]. In addition to cervical cancer, HPV is implicated in anal, vulvar, vaginal and oropharyngeal carcinomas (Figure 4) [187].

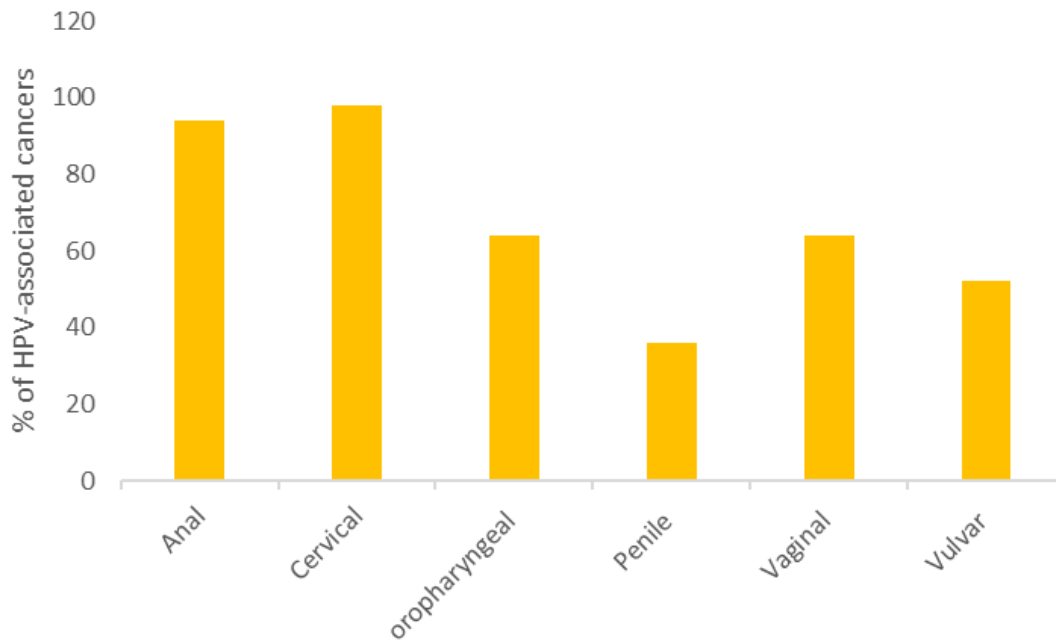


Figure 4. Representation of the percentage of HPV-associated cancers. Modified from Brianti et al., 2017 [177].

1.3.2 General features of HPV

HPV can infect epithelial cells resulting in transformation and tumor development due to the HPV-encoded oncoproteins [177]. HPV has an icosahedral capsid and harbours a double-stranded circular DNA with about 8 kb genome size [188]. HPV encodes early genes (E-genes) involved in replication, transcription and transformation while late genes (L-genes) play a key role in capsid formation. HPV also harbors regulatory long control region (LCR) and a noncoding region [189]. Late HPV genes include the L1 and L2 genes that encode the proteins building the HPV capsid. The HPV capsid contains 72 pentameric capsomeres, each of them composed of five L1 and L2 proteins. HPV assembles in the nucleus of the infected cells with incorporation of L1 and L2 into virus-like particles [179, 190, 191].

The HPV-encoded E6 and E7 are the main conserved oncogenes and can integrate into the genome of infected cells [192]. This step is crucial towards alteration of cell cycle regulation, telomerase maintenance and blocking of tumor suppressor pathways which results in malignant transformation and cancer initiation [193]. The transformed epithelial cells process and present epitopes of both E6 and E7 proteins [194]. High-risk HPVs are of different genus (*Alpha*, *Beta* and *Mu*) (Figure 5). However, the members of the α -genus have been extensively studied due to their high prevalence including HPV16 and 18 genotypes, which account for over than 70% of cervical cancers [177, 195, 196].

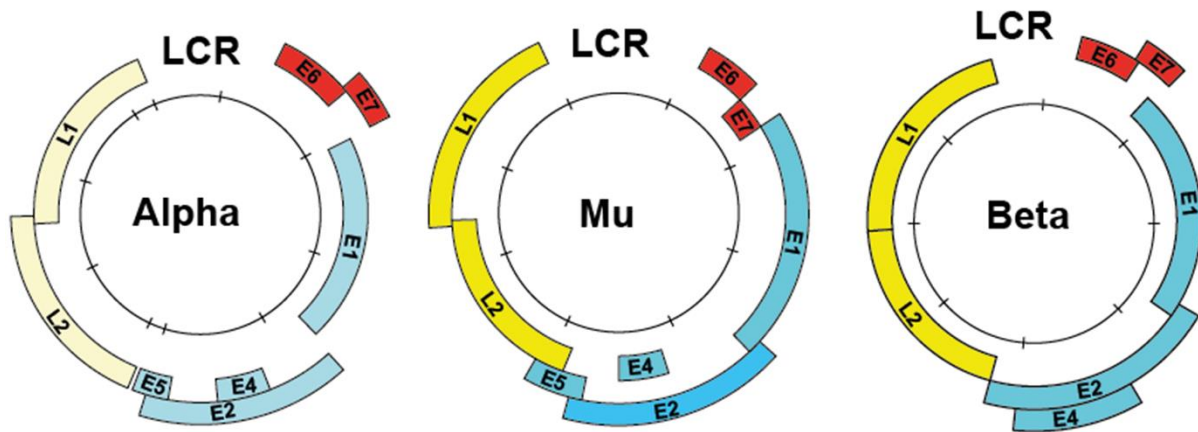


Figure 5. Schematic illustration of high-risk HPV genome. HPV encodes late structural genes (orange), early genes (green) and oncogenes (red). Modified from Doorbar et al., 2015 [195].

High-risk HPV-encoded E6 consists of 158 amino acids. E6 promotes cancer initiation and proliferation by interacting with the tumor suppressor p53 protein which is mediated by a trimeric complex formation of E6, p53 and ubiquitination enzyme E6-AP [192]. This interaction blocks the action of p53, which leads cell growth and tumor formation [197, 198]. Additionally, HPV16-encoded E7 binds with high affinity to cellular retinoblastoma protein (pRb). The Rb protein has a negative cell-cycle regulatory function in the G1/S as well as G2/M transitions [192]. The Rb protein mediates its function by binding to E2F-family transcription factors, which in turn represses the expression of enzyme genes involved in cell replication. E7 protein binds to specific region of Rb protein called pocket domains. These pocket domains of Rb protein are the functional domains for the protein-mediated tumor suppressor function. The binding of E7 with Rb protein alters the Rb-E2F interaction and consequently resulting in E2F release in the active form. The transcriptionally active form of E2F stimulates cell division and replication which promotes cancer formation in the HPV-infected organs (Figure 6) [199-201].

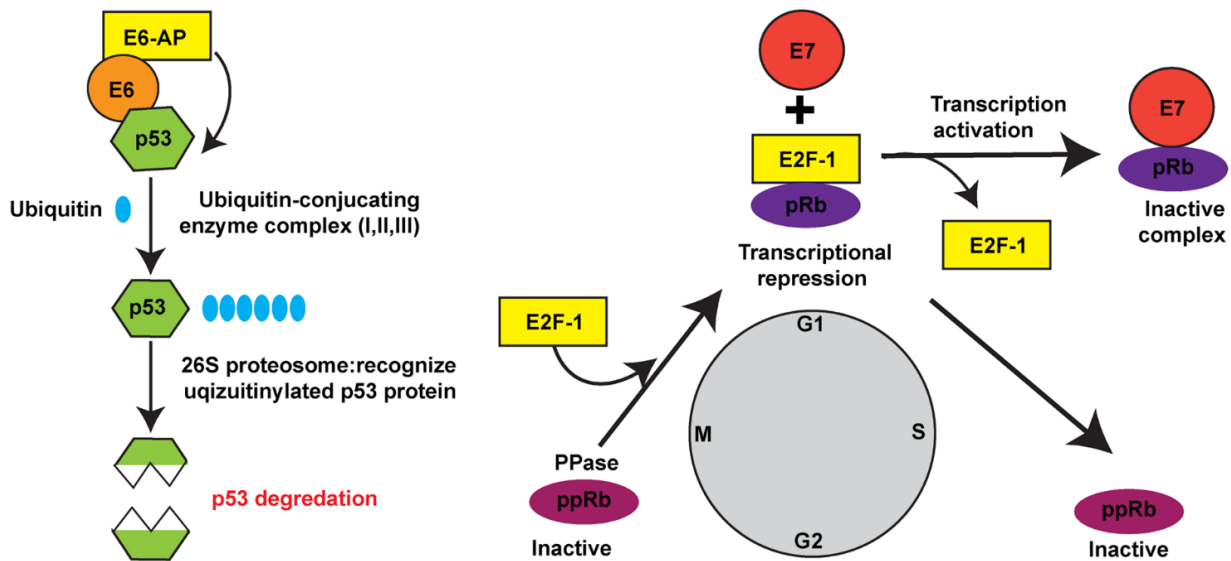


Figure 6. Mechanism of action of HPV16-encoded oncoproteins. HPV16-encoded E6 and E7 interact with p53 and Rb proteins, respectively, which leads to initiation and progression of cancer in HPV-infected cells. Modified from Yim and Park, 2005 [192].

Despite highly effective prophylactic vaccines, there is no approved therapeutic vaccine that triggers cell-mediated immunity against HPV-induced cancer [202]. Although individuals are protected if they are vaccinated prior to the virus exposure, the unvaccinated population will remain at risk lifelong of developing cervical and other related cancers [203]. MCMV was utilized to generate recombinant viruses that express epitopes derived from E7 protein. These MCMV-based vaccines induced robust tumor-specific CTLs that were able to protect the mice from tumor growth. It was also demonstrated that fusing a single HPV E7-derived epitope to the C-terminal of viral proteins is crucial to generate MHC-I-restricted CTLs recognizing this epitope [204, 205].

1.4 Glioblastoma multiforme (GBM)

The glial cells play the main role in supporting and isolating neurons. Moreover, they have an important role in the functional signal processing of the CNS and in the maintenance of homeostasis and brain metabolism [206]. Gliomas are the most common primary tumors of the central nervous system (CNS) [207]. The name refers to its neuroepithelial tissue of origin, the glial cells. The recent World Health Organization (WHO) classification updated in 2016, classifies gliomas into four grades according to histopathological malignancy criteria [208].

1. Introduction

The low-grade gliomas (WHO I and II) show a comparatively good cell differentiation, and thus a high morphological similarity with glial cells [207]. Low-grade malignant gliomas often have a long clinical course due to their rather slow progression [209]. A distinction is made between higher-grade neoplasia, so-called the high-grade gliomas (WHO III and IV). WHO III tumors are characterized by increased cytologic anaplastic signs with increased proliferation rate, diffuse infiltrative growth pattern and developing perifocal edema. Glioblastoma multiforme (GBM), the most common malignant tumor of glial cells, is classified as the most aggressive glioma (WHO IV) which shows additional malignant characteristics such as neoangiogenesis and tumor necrosis [210].

GBM is one of the most aggressive cancers, with median survival of only 14-months after diagnosis [211-213]. The most commonly used treatment options are ineffective for GBM therapy, although different drug combinations have been investigated [214, 215]. Relapse usually occurs, which can no longer be treated and leads to death after a short time period [216, 217]. The fact that GBM is incurable, underlines the need to develop new approaches for the therapy of GBM. To date, the most promising therapeutic strategies for GBM treatment are immunotherapy-based approaches [218].

1.4.1 Incidence of GBM

GBM accounts for about 16% of all CNS tumors and 54% of malignant tumors in the CNS [219]. Every year, there are more than 12,000 new cases diagnosed with GBM in the United States [220]. According to Central Brain Tumor Registry of the United States (CBTRUS) report, the incidence of GBM in the United States is 3.19 per 100,000 individuals. The median age of people with GBM is 64 years. The incidence increases with age and reaches its maximum at 15.27 per 100,000 in the age group 75-84 years [207, 212].

Despite modern therapy concepts, the survival prognosis of GBM patients is severely limited [207, 221]. In the total population, the average 1-year survival is about 40.8%, while the 5-year survival is about 6.8% ,and the 10-year survival is only 0.71% [207, 222].

1.4.2 Pathology of GBM

Recently, it has been established that GBM originates from neural stem cells within subventricular brain zone [223]. The diagnosis of GBM is mainly dependent on the observation of high cellularity, atypical glial cells and microvascular proliferation (MVP) and/or necrosis

[223, 224]. Although GBM is one of the most aggressive cancer types (Figure 7), it rarely metastasizes outside the CNS [225]. Recent studies showed synaptic and electrical integration among glioma and neural cells which promotes glioma progression [226].

1.4.3 Standard GBM treatment strategies

The current gold standard “maximum” therapy consists of tumor resection, followed by combined chemo-radiotherapy. Patients treated with this regimen have a median survival time of about 14 months [221, 227]. GBM almost always relapses near the surgical margins and becomes resistant to therapy [214].

1.4.4 GBM-induced immunosuppression

GBM is characterized by immunosuppression in the “cold” local tumor microenvironment (TME). There is high predominance of CD11b⁺ myeloid cells including brain-resident microglia and myeloid-derived suppressor cells (MDSC), which have immunosuppressive characteristics such as producing IL-10 and tumor growth factor beta (TGF- β) [228-230]. Moreover, the T-cell checkpoint molecule PD-L1 is highly expressed in patients’ GBM tissue [231]. Immunotherapy is one of the promising approaches for GBM therapy by converting the TME from “cold” to “hot” with improved immune response [232].

Moreover, GBM tumors are also able to induce systemic immunosuppression. GBM patients have higher level of MDSC cells in the blood than normal individuals and have elevated serum levels of IL-10. Additionally, circulating monocytes show higher level of PD-L1 expression. In addition, circulating T cells are limited in number and impaired in function as well [233, 234]. The clinical use of PD-1/PD-L1 checkpoint blockades did not offer any survival advantage in GBM patients because GBM shows poor responses to immunotherapies that have been successful in more immunogenic cancers due to immunosuppressive TME [235].

1.4.5 HCMV infection of GBM cells

The relationship between HCMV and GBM tumors has been extensively studied. The detection of HCMV antigens and nucleic acid in GBM cells suggests that HCMV may be implicated in the tumor initiation and malignancy [236, 237], however, HCMV was not detectable in the surrounding tissues [236]. On the other side, HCMV has the ability to reduce the viability of GBM cell lines in the presence or absence of chemotherapeutic agents. Moreover, infection of

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GBM cells with HCMV did not show any increase in resistance to chemotherapeutic drugs [238].

Interestingly, HCMV antigens of HCMV-positive GBM clinical samples were recognized and killed by autologous HCMV-specific CTLs [239]. This is why infecting GBM tumors with HCMV-based vaccines expressing several antigens, which are entirely absent in the tumors, may drive polyclonal CTLs and could be considered as a novel approach for GBM therapy.

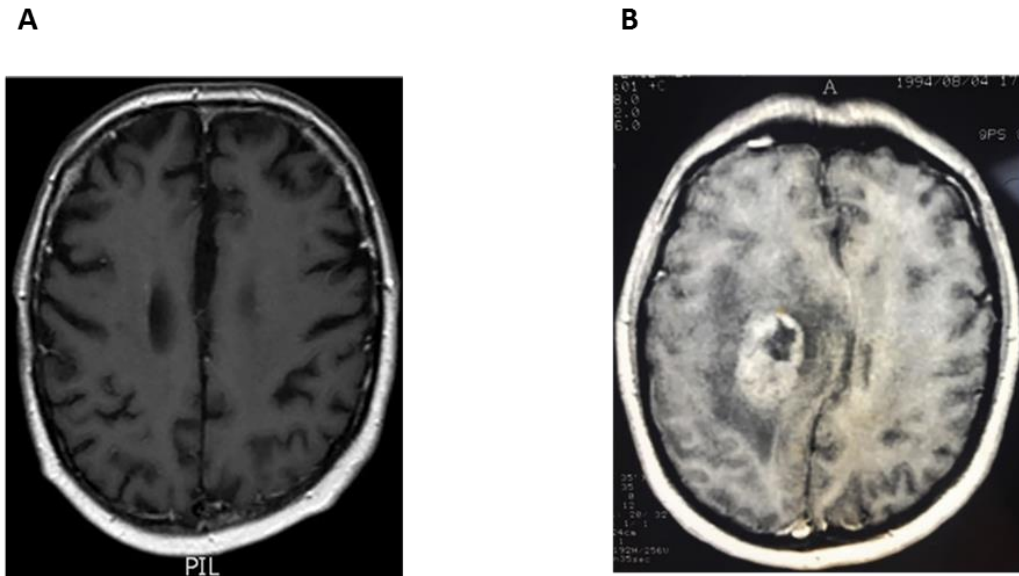


Figure 7. Magnetic resonance imaging (MRI) of brain. (A) The image of MRI does not show any brain pathology. (B) The MRI scan shows a brain tumor in the posterior part of the right frontal lobe with an area of necrosis and surrounded by cerebral edema. Obtained from Caruso et al.,2017 [240].

1.5 Project outline

After HCMV infection, T cells with high avidity for dominant viral antigens are generated and maintained at high frequencies. These cells show a characteristic effector-memory phenotype and lack features of T cell exhaustion. This phenomenon has been called memory inflation and was originally discovered in animal models of CMV infection. Based on this unique feature, HCMV represents a promising vector for the construction of recombinant vaccines that express heterologous (non-HCMV) proteins that are not normally expressed by HCMV wild type (WT). These neoantigens can be subsequently processed and presented (neoepitope). The heterologous proteins should elicit an equally powerful T cell response that could then be exploited for vaccination against chronic infectious disease such as HIV or cancer immunotherapy. Although several studies were carried out in the last few years aiming to develop therapeutic and prophylactic vaccines based on animal CMVs, there is no published study yet for HCMV-based vaccines.

1.3.1 Objectives

HPV16-encoded E6 and E7 are the principle conserved oncogenes responsible for cell transformation and carcinogenesis. Despite the presence of prophylactic vaccines, there is no approved therapeutic vaccine for HPV-induced cancer although different strategies have been attempted. Moreover, we investigated the use of HCMV-based therapeutic viral vaccines to drive the autologous immune system for elimination of tumor cells. GBM cells are permissive to HCMV, thus they can be antigenic and act as a target for CTLs upon HCMV infection through expression and presentation of a neoepitope.

In this study, we aimed to:

- A. Develop therapeutic HCMV-based vaccines against HPV-induced cancer and GBM using two different HCMV strains (Merlin and TB40/E)
- B. Investigate the functionality of HCMV-based vaccine to stimulate specific T cells *in vitro*.

The novelty of this work is the generation of the first optimized HCMV-based cancer vaccine backbone.

1.3.2 Technical approach

A. Construction of HCMV-based therapeutic cancer vaccines

1- HCMV vector modification and optimization

We aimed to knockout MHC-I downregulating genes (US2-US11) using bacterial artificial chromosome (BAC) technology and recombineering to generate HCMV mutants with impaired immune evasion.

2- HPV antigens expression and presentation

HPV16 E6 and E7 proteins were our target antigens. An immunogenic E6/E7 fusion protein without transforming activity (E6/E7) was used in this study to generate HCMV-based vaccines expressing E6/E7 protein as a source of T cell epitopes. Moreover, another set of vaccines was constructed with an E6-derived single epitope fused to HCMV proteins.

B. *In vitro* functional assays of HCMV-based therapeutic vaccines

Primary T cells as well as T cell lines specific for HCMV-encoded epitope or neoepitope were generated by transduction with specific TCRs. HCMV-infected fibroblasts and GBM cells were investigated for their ability to stimulate these specific T cells.

2. Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Primary cells, established cell lines and bacterial strains

2.1.1.1 Primary cells are listed in Table 2

Cells	Feature	Reference
Fi301	Human embryonic fetal fibroblasts with HLA-A2 haplotype.	[241]

Table 2. List of the primary cells.

2.1.1.2 Cell lines are listed in Table 3

Cells	Feature	Reference
CaSki	Cervix cells; derived from metastatic site (small intestine) with HLA-A2 haplotype.	ATCC® CRL-1550™
HEK	Human embryonic kidney cell line.	ATCC® CRL-1573™
HEK293T	Human embryonic kidney derived from HEK cells. Transfected plasmid with SV40 origin of replication can replicate efficiently in HEK293T cells and maintained in a high copy number; which greatly increase the amount of recombinant protein or retrovirus that can be produced from the cells.	ATCC® CRL-3216™
Jurkat E6.1 and Jurkat 76 (J76)	Human T cell lines derived from acute T cell leukemia.	ATCC® TIB-152™
LN18	GBM cell line with HLA-A2 haplotype.	ATCC® CRL-2610™
U251	GBM cell line with HLA-A2 haplotype.	CVCL_4773

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U343	GBM cell line with HLA-A2 haplotype.	ATCC® HTB-17™
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Table 3. List of the cell lines.

2.1.1.3 Bacteria

Laboratory strains of *Escherichia coli* (*E. coli*) were grown in Luria-Bertani (LB) medium (see Section 2.1.6.1) or plated on LB agar plates. The LB media and agar were sterilized by autoclaving. LB agar was supplemented with the appropriate antibiotics and poured into 10 cm diameter Petri dishes. Different antibiotics were used in this project, for example: ampicillin (amp) (100 µg/ml), kanamycin (kan) (50 µg/ml), and chloramphenicol (cam) (20 µg/ml). Bacteria were plated on LB agar by streaking with a wire loop and incubated overnight inverted at 32 °C or 37 °C. Single colonies were inoculated in sterile liquid LB medium supplemented with the appropriate antibiotics. Liquid cultures were incubated at 32 °C or 37 °C overnight in the bacterial incubator with shaking at 220 rpm. Bacterial strains are listed in Table 4

Name of bacteria	Feature	Reference
GS1783	DH10B λcI857Δ(cro-bioA)⟨>araC-P BAD, ISceI	[242]
NEB 10-beta <i>E. coli</i>	A competent <i>E. coli</i> is a derivative of the popular DH10B. It is T1 phage resistant and endonuclease I (<i>endA1</i>) -deficient for high-quality plasmid preparations.	New England Biolabs, Frankfurt, Germany Cat No. C3019H and C3019I

Table 4. List of bacterial strains.

2.1.2 Viruses and BACs

2.1.2.1 Viruses

HCMV viruses used in this project are listed in Table 5

2. Materials and Methods

Virus name	Feature	Reference
TB40/E	HCMV strain TB40/E was derived from throat wash of a bone marrow transplant recipient by propagation for 5 passages in fibroblasts and 22 passages in endothelial cells.	[243]
RV-TB40-BAC _{KL7} -SE-EGFP	An enhanced green fluorescent protein (EGFP)-expressing virus derived from TB40/E. The EGFP gene under the control of the viral major IE promoter/enhancer with a poly(A) site was introduced at an ectopic position downstream of US34A.	[244]

Table 5. List of HCMV viruses.

2.1.2.1 BACs

All modified HCMV and BACs used in this study are listed in Table 6

BAC	Derived virus	Features	Reference
RCMV1161-BAC		HCMV BAC of Merlin strain with repaired RL13 and UL128 regions. It encodes an IRES-EGFP expression cassette which was inserted immediately downstream from UL122 (IE2).	[245]
TB40-BAC4		HCMV BAC of TB40/E strain. The mini-F sequence is replacing the region US2-US6.	[243]
Merlin1161 Δ US2-BAC	Merlin1161 Δ US2	RCMV1161 BAC with US2 deletion.	This work
Merlin1161 Δ US2 Δ 3-BAC	Merlin1161 Δ US2 Δ 3	RCMV1161 BAC with US2-3 deletion.	This work
Merlin1161 Δ US2 Δ 3 Δ 6-BAC	Merlin1161 Δ US2 Δ 3 Δ 6	RCMV1161 BAC with US2-3-6 deletion.	This work

2. Materials and Methods

Merlin1161 Δ US2 Δ 3 Δ 6 Δ 11-BAC	Merlin1161 Δ US2 Δ 3 Δ 6 Δ 11	RCMV1161 BAC with US2-3-6- 11 deletion.	This work
RVTB40 Δ US11- BAC	RVTB40 Δ US11	TB40-BAC4 with US11 deletion.	This work
RVTB40E6/E7intoU S11-BAC	RVTB40_E6/E7int oUS11	RVTB40 Δ US11 with E6/E7 protein expressed under the control of US11 promoter.	This work
RVTB40 Δ US11_EF -1E6/E7-BAC	RVTB40 Δ US11_E F-1E6/E7	RVTB40 Δ US11 with E6/E7 protein expressed und the control of exogenous EF-1 promoter.	This work
RVTB40 Δ US11_E6/ E7intoUL111A- BAC	RVTB40 Δ US11_E 6/E7intoUL111A	RVTB40 Δ US11 with E6/E7 protein expressed und the control of UL111A(vIL-10) promoter.	This work
RVTB40 Δ US11_E6/ E7intoUL83-BAC	RVTB40 Δ US11_E 6/E7intoUL83	RVTB40 Δ US11 with E6/E7 sequence replaced UL83 (pp65).	This work
RVTB40 Δ US11_E6 peptideIE1-BAC	RVTB40 Δ US11_E 6peptideIE1	RVTB40 Δ US11 with HPV16 E6- derived peptide fused to C- terminus of UL123 (IE1).	This work
RVTB40 Δ US11_E6 peptideUL83-BAC	RVTB40 Δ US11_E 6peptideUL83	RVTB40 Δ US11 with HPV16 E6- derived fused to C-terminus of UL83 (pp65).	This work
RVTB40 Δ US11_E6/ E7intoUL83_E6pept ideIE1-BAC	RVTB40 Δ US11_E 6/E7intoUL83_E6p eptideIE1	RVTB40 Δ US11_E6/E7intoUL83 with HPV16 E6-derived fused to C-terminus of UL123 (IE1).	This work

Table 6. List of HCMV BACs.

2.1.3 Chemicals and Reagents

Chemicals and Reagents are listed in Table 7

2. Materials and Methods

Product	Supplier
100bp DNA ladder	Thermo Fisher Scientific, Braunschweig, Germany
1kb DNA ladder	Thermo Fisher Scientific, Braunschweig, Germany
1kb DNA ladder GeneSTA	Geneall, Seoul, South Korea
Acetic acid (CH ₃ COOH)	VWR International, Darmstadt, Germany
Agar-Agar (pure)	Carl Roth, Karlsruhe, Germany
Agarose-Standard Roti® grade	Carl Roth, Karlsruhe, Germany
Ammonium chloride	Sigma-Aldrich, Steinheim, Germany
Ampicillin sodium salt	Carl Roth, Karlsruhe, Germany
Bovine serum albumin (BSA)	HyClone, South Logan, USA
Bromophenol blue (C ₁₉ H ₁₀ Br ₄ O ₅ S)	Sigma-Aldrich, Steinheim, Germany
Chloramphenicol	Carl Roth, Karlsruhe, Germany
Citric acid	Sigma-Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
EDTA (ethylenediaminetetraacetic)	Applichem, Darmstadt, Germany
Ethanol absolute	Applichem, Darmstadt, Germany
Ethidium bromide 1%	Carl Roth, Karlsruhe, Germany
FACS clean	BD, San Jose, USA
FACS rinse	BD, San Jose, USA
G418 (Geneticin)	Thermo Fisher Scientific, Braunschweig, Germany
Glycerol	Applichem, Darmstadt, Germany
Human interleukin 2 (hIL-2)	Peptotech, Rocky Hill, NJ, USA
Hydrochloric acid (37% HCl)	Carl Roth, Karlsruhe, Germany
Ionomycin	Sigma-Aldrich, Steinheim, Germany
Isopropyl alcohol (2-propanol)	AppliChem, Darmstadt, Germany
Kanamycin sulphate	Carl Roth, Karlsruhe, Germany
L-(+)- arabinose	Sigma-Aldrich, Steinheim, Germany
Lipofectamine 2000	Invitrogen, Karlsruhe, Germany
Methanol	VWR International, Darmstadt, Germany
Opti-MEM I	Invitrogen, Karlsruhe, Germany

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Phorbol myristate acetate	Sigma-Aldrich, Steinheim, Germany
Phosphate buffered saline (PBS)	Santa Cruz Biotechnology, Heidelberg, Germany
Polyethylenimine, Linear, MW 25,000 (PEI)	Polysciences, Eppelheim, Germany
Potassium acetate (KCH ₃ CO ₂)	Applichem, Darmstadt, Germany
RNase A	Sigma-Aldrich, Steinheim, Germany
Roti®-Sep 1077 human	Carl Roth, Karlsruhe, Germany
Sodium acetate	Applichem, Darmstadt, Germany
Sodium chloride (NaCl)	Applichem, Darmstadt, Germany
Sodium hydroxide pellets, extra pure	Applichem, Darmstadt, Germany
Sodium pyruvate	Biochrom AG, Berlin, Germany
Tris	Applichem, Darmstadt, Germany
Tris-EDTA (TE)	Sigma-Aldrich, Steinheim, Germany
Trypan blue, 0.4% solution	Lonza, Cologne, Germany
Trypsin/EDTA 0.05%	Gibco, Thermo Fisher Scientific, Braunschweig, Germany
Tryptone	Carl Roth, Karlsruhe, Germany
Water molecular biology grade	Applichem, Darmstadt, Germany
Yeast extract	Carl Roth, Karlsruhe, Germany

Table 7. List of chemicals and reagents.

2.1.4 Consumables and equipment

2.1.4.1. Consumables are listed in Table 8

Consumables	Supplier
0.2 ml thin-walled PCR tube	Applied Biosystems, Darmstadt, Germany
BD Falcon 12 x 75 mm Tubes	BD, San Jose, USA
Cell culture dish 50, 100 and 150 mm	Sarstedt, Nümbrecht, Germany
Cell culture plates	Sarstedt, Nümbrecht, Germany
Cell scraper	Sarstedt, Nümbrecht, Germany
Cell-culture flasks (T-25, T-75, and T-175)	Sarstedt, Nümbrecht, Germany
Cryotubes 1.8 ml	Sarstedt, Nümbrecht, Germany

2. Materials and Methods

Electroporation sterile cuvettes, 1 mm and 2mm	Biodeal, Markkleeberg, Germany
Eppendorf tubes (1.5, 2ml)	Sarstedt, Nümbrecht, Germany
Falcon bacterial tubes (13ml)	Sarstedt, Nümbrecht, Germany
Falcon tubes (15 ml, 50 ml)	BD, San Jose, USA
Parafilm® M	Bems, Neenah, Germany
Pasteur pipette	Sarstedt, Nümbrecht, Germany
Petri dishes	Sarstedt, Nümbrecht, Germany
Pipette tips (1000, 200, 100 and 10)	VWR International, Darmstadt, Germany
Retronectin-coated plates	Takara Bio, Otsu, Japan
Sterile Pipettes (5, 10, 25 ml)	Sarstedt, Nümbrecht, Germany

Table 8. List of consumables.

2.1.4.2. Equipment are listed in Table 9

Equipment	Supplier
Analytical balance (Cubis MSA)	Sartorius AG, Göttingen, Germany
Bacterial incubators Classic Line	Binder, Tuttlingen, Germany
Centrifuges 5424/ 5424 R	Eppendorf AG, Hamburg, Germany
Cloning rings	Hilgenberg GmbH, Malsfeld, Germany
CO ₂ tissue culture incubator (New Brunswick™ Galaxy® 170 R)	Eppendorf AG, Hamburg, Germany
Electrophoresis Power Supply (Power Source™300V)	VWR International, Darmstadt, Germany
FACScalibur Flow Cytometer - 4 Colors	BD, San Jose, USA
Flexcycler Thermocycler	Analytik Jena, Jena, Germany
Fluorescence inverted microscope	Carl Zeiss MicroImaging, Jena, Germany
Freezer -20 °C	Kirsch, Willstätt, Germany
Freezer –80 °C (HERA Freeze™ HFU B Series)	Thermo Fisher Scientific, Braunschweig, Germany
Fume cupboards (DELTAguard)	Wesemann, Wangen im Allgäu, Germany.

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Laminar flow safety cabinet class II	Bleymehl Reinraumtechnik, Inden, Germany
Liquid nitrogen tank	Air liquide, Düsseldorf, Germany
Multiskan™ FC Microplate Photometer	Thermo Fisher Scientific, Braunschweig, Germany
Microscope (AE20)	Motic, Wetzlar, Germany
Microwave oven (R-201A)	Sharp, Osaka, Japan
Mr. Frosty™ Freezing Container	Thermo Fisher Scientific, Braunschweig, Germany
Multiporator	Eppendorf AG, Hamburg, Germany
Nanodrop ND1000	PeqLab, Erlangen, Germany
Neubauer cell counting chamber	VWR International, Darmstadt, Germany
Shaker bacterial incubator	New Brunswick™ Innova, Edison, USA
Orbital shaker (OS-10)	PeqLab, Erlangen, Germany
pH–meter (inoLab pH Level 1)	Wissenschaftlich-Technische Werkstätten, Weilheim, Germany
Pipetboy	INTEGRA Biosciences AG, Biebertal, Germany
Pipettes (single–channel, multichannel)	VWR International, Darmstadt, Germany
PTR-20 360 Degree Vertical Multi-Function Rotator	Grant Bio, Shepreth, UK
Spectrophotometer (Smart spec Plus)	Bio-Rad, Feldkirchen, Germany
Thermomixer	Eppendorf AG, Hamburg, Germany
UV Transilluminator	PeqLab, Erlangen, Germany
Vortex Genie 2™	Bender&Hobein AG, Zurich, Switzerland
Water bath	Eppendorf AG, Hamburg, Germany

Table 9. List of Equipment.

2. Materials and Methods

2.1.5 Buffers and Gels

2.1.5.1 DNA electrophoresis and electroporation buffers are listed in Table 10

Buffer	Composition
1x PBS	9.55 gm from PBS powder 1 L millipore water pH 7.3
1x TAE	40 mM Tris 1 mM Na ₂ EDTA.2H ₂ O 20 mM acetic acid 99%, pH 8.0
1 % agarose gel	1 gm agarose 100 ml of 1x TAE buffer 1 µl ethidium bromide 10 mg/ml
hypo-osmolar buffer for eukaryotic cell electroporation	Eppendorf AG, Hamburg, Germany
Iso-osmolar buffer for eukaryotic cell electroporation	Eppendorf AG, Hamburg, Germany
6x DNA loading buffer	0.2% bromophenol blue 60% glycerol 60 mM EDTA

Table 10. List of DNA electrophoresis and electroporation buffers.

2. Materials and Methods

2.1.5.2 Plasmid and BAC DNA preparation buffers are listed in Table 11

Buffer	Composition
TE buffer	10 mM Tris HCl pH 7 1 mM NaEDTA

Table 11. List of plasmid and BAC DNA preparation buffers.

2.1.6 Media preparation and composition

2.1.6.1 Media and supplements for propagation of bacteria (*E. coli*) are listed in Table 12

Media	Composition
LB medium (1X)	10 g tryptone 5 g yeast extract 10 g NaCl millipore water to 1L
LB agar	10 g tryptone 5 g yeast extract 10 g NaCl 15 gm agar millipore water to 1L

Table 12. List of media and supplements for propagation of bacteria (*E. coli*).

2. Materials and Methods

2.1.6.2 Media and supplements for cultivation of mammalian cells

2.1.6.2.1 Supplements are listed in Table 13

Name	Catalog No	Company
Fetal bovine serum (FBS)	11573397	Gibco, Thermo Fisher Scientific, Braunschweig, Germany
L-alanyl-L-Glutamine	K 0302	Biochrom AG, Berlin, Germany
Minimum Essential Medium Eagle (MEM)	F 0315	Biochrom AG, Berlin, Germany
RPMI 1640	F 1215	Gibco, Thermo Fisher Scientific, Braunschweig, Germany
Gentamycin-sulfate	A 2712	Biochrom AG, Berlin, Germany

Table 13. List of media supplements.

2.1.6.2.2 Cell culture media and buffers are listed in Table 14

Cells	Media
U343	Minimum essential Medium Eagle (MEM)
Fi301	10% heat inactivated FBS (hiFBS)
HEK293T	2mM L-alanyl-L-glutamine
HEK	50 µg/ml gentamicin
LN18	
U251	

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PBMCs and Jurkat cell lines	RPMI 1640 2mM L-alanyl-L-glutamine 10% hiFBS 50 µg/ml gentamicin
RBC lysis buffer	NH ₄ Cl 8.02gm NaHCO ₃ 0.84gm Na ₂ EDTA.2H ₂ O 0.37gm millipore water to 1L

Table 14. List of cell culture media and buffers.

2.1.7 Kits

Kits used in this study are listed in Table 15

Kit	Supplier
Basic Nucleofector™ kit for Primary Mammalian Fibroblasts	Lonza, Cologne, Germany
DNase I kit	Promega, Walldorf, Germany
GeneArt	Life Technologies, Darmstadt, Germany
GeneMATRIX Basic DNA Purification kit	EURx, Gdańsk, Poland
IFN-γ ELISA kit	PeptoTech, Hamburg, Germany
NucleoBond® Xtra BAC	MACHEREY-NAGEL, Düren, Germany
NucleoBond® Xtra Midi	MACHEREY-NAGEL, Düren, Germany
OncoE6 Cervical Test™ kit	Arbor Vita, CA, USA
RecomWell HPV16/18/45 ELISA kit	Mikrogen GmbH, Neuried, Germany
RNeasy Plus Mini kit	Qiagen, Hilden, Germany
SuperScript III Reverse Transcriptase	Invitrogen, Karlsruhe, Germany

Table 15. List of kits.

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2.1.8 Enzymes

All enzymes used in this project were purchased from New England Biolabs, Frankfurt, Germany and are listed in Table 16

Antarctic phosphatase (AP)	<i>KpnI</i>
<i>BamHI</i>	<i>NotI</i>
<i>DpnI</i>	<i>Phusion</i> DNA polymerase
<i>EcoRI</i>	<i>T4</i> DNA Ligase
<i>EcoRV</i>	<i>Taq</i> DNA polymerase

Table 16. List of enzymes.

2.1.9 Antibodies and peptides

2.1.9.1 Antibodies

All antibodies used in this project are listed in Table 17

Antibody	Supplier
anti-CD28 (CD28.2)	BioLegend, London, UK
anti-CD3 (OKT3)	BioLegend, London, UK
anti- HLA-A*0201 antibody clone BB7.2	BioLegend, London, UK

Table 17. List of antibodies.

2.1.9.2 Peptides

All peptides used in this project are purchased from Peptides&elephants GmbH, Hennigsdorf, Germany and listed in Table 18

Peptide	Sequence
CMV pp65 (495- 503)	NLVPMVATV
HPV E6 (29-38)	TIHDIILECV

Table 18. List of peptides.

2. Materials and Methods

2.1.10 Plasmids and vectors

All plasmids and vectors used in this project are listed in Table 19

Name	Feature	Reference
GALV-env	<i>Gibbon ape leukemia virus</i> (GALV) envelope-encoding retroviral vector.	[246]
MLV-gag/pol	<i>Murine leukemia virus</i> (MLV) <i>gag/pol</i> -encoding retroviral vector.	
MP71TCR α	Retroviral vectors harboring TCR (α and β) for HCMV-pp65.	[247]
MP71TCR β		
pcDNA3.1(+)	Mammalian expression vector; Catalog No.V790-20.	Invitrogen
pcDNAE6/E7Kpn-I	pcDNA3.1(+) mammalian expression vector harboring fused consensus sequence of E6/E7 at <i>Kpn-I</i> restriction site.	This work
pEF6/V5-His A	Mammalian expression vector; Catalog #V961-20.	Invitrogen
pEF6E6/E7EcoRI	pEF6/V5-His A mammalian expression vector harboring fused consensus sequence of E6/E7 at <i>EcoRI</i> restriction site.	This work
pEPkan-S	Mammalian expression vector that encodes the kanamycin cassette for mutagenesis.	[242]
pJET1.2/blunt	Plasmid cloning vector for prokaryotic expression.	Thermo Fisher Scientific
pJetConE6E7	pJET1.2/blunt vector harboring fused consensus sequence of E6/E7.	This work
pMP71-PRE-E6	Retroviral vector harboring TCR (α and β) for HPV16 E6 with P2A linker.	This work.

Table 19. List of plasmids and vectors.

2. Materials and Methods

2.1.11 Primers

All primers used in this project were designed using Vector NTI 9.1 software (Invitrogen), ordered and purchased from Integrated DNA Technologies (IDT, Leuven, Belgium). Sequencing reactions were performed by LGC Genomics GmbH, Berlin, Germany (Table 20).

Primers	Sequence (5'-3')	
Deletion of MHC-I down regulating genes		
ΔUS2	F	TTATTGAAACAAACCGCGATCCCGGGCGTCGGTGAAACAGCGTGT TAGGGATAACAGGGT
	R	AAGAGCGTACAGTCCACACGCTGTTTCACCGACGCCCGGGATCGC GCCAGTGTTACAACC
ΔUS3	F	AGGTTTCAGGTACCAGCTGGTTCGTACCTGGGCTCCGAAGGTCCGT AGGGATAACAGGGT
	R	AGCGGAGCCGAGCAGCGGACCTTCGGAGCCCAGGTACGAACCAGC GCCAGTGTTACAACC
ΔUS6	F	ACGCGCTTTTATTGAGACGATAAAACAGCAAGTGAAGAAGGAGCG TAGGGATAACAGGGT
	R	TAAGTACGTCGATATCGCTCCTTCTTCACTTGCTGTTTTATCGTCGC CAGTGTTACAACC
ΔUS11	F	CTCGAGATGCACTCCGCTTCAGTCTATATATCTGTCTTACAAGGGT AGGGATAACAGGGT
	R	CAGGGGAACAGCCTTCCCTTGTAAGACAGATATATAGACTGAAGC GCCAGTGTTACAACC
Sequencing of MHC-I down regulating genes		
SeqUS2-3	F	CTGCCAGTGCTCTCGCTCGAGCAC
	R	CAAAAACACCGTGCAGTCCACACGC
SeqUS6	F	CCGGAGTTGTCTATCGCGACAT
	R	ACAAATATATGGAGTTTGTGTAATGC
SeqUS11	F	CAGGCAGATCGCCAGAGGG
	R	TTCTATCACCTAATCTGTCGTACTIONGTC
Shuttle plasmids design		
FusedclonEc ori	F	ATCCGAATTCGCCACCATGGACTGGACCTG
	R	TCCCGAATTCGCGGCCGCTCATCAGGGTTT

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conE6E7	F	ATCCGAATTCGCCACCATGGACTGGACCTG
Kpn-I	R	TCCCGAATTCGCGGCCGCTCATCAGGGTTT
conE6E7Kan	F	ACCTGGATCCTGTTCTTGGTGGCCGCTAGGGATAACAGGGTAATC
BamH	R	GATTTAT AACAGGATCCAGGTCCAGTCCATGGGCCAGTGTTACAACCAATT AACC
conE6E7Eco	F	GAAGATATCGAATTCGCCACCATGGACTGGACCT
RV	R	CCCAGAAACCCTGATGAGCGGCCGCGATATCCGC
Insertion of E6/E7 sequence in US11		
US11ConE6	F	TTTCCGAGCGACTCGAGATGCACTCCGCTTCAGTCTATATAGCGG
E7	R	CCGCTCATCAGGGTT TGAGTCTAGACAGGGGAACAGCCTTCCCTTGTAAGACAGAGAAT TCGCCACCATGGACTG
E6/E7 expression under the control of exogenous promoter (EF-1)		
EF-1E6/E7	F	GATCCAACACTGAACGCTTTCGTCGTGTTTTTCACGTGAGGCTCC
	R	GGTGCCCGTCAGTGG ACGCTCTCGTCAGGCTTGTCATGGTCTGTAAAAGCTGCAGCGGCC GCTCATCAGGGTTTC
seqEF-	F	TCTGATCCAACACTGAACGCT
1E6/E7	R	CACCTATCATAAAGAATGCAACGCT
Insertion of E6/E7 sequence in UL111a exon		
IL-	F	ACGTAGGTACGGTTTATTGCGACGGTCTTTCTTGAATTCGCCACC
10FusedE6/E	R	ATGGACTGGACCTGG GCTACAAGAGGAAAACACTACGTCACCCGACACGCGGAAGCGGCCG CTCATCAGGGTTTCTG
7		
SeqUL111	F	ATGCTGTCGGTGATGGTCTC
	R	TCGAGTGCAGATACTCTTCGAG
Insertion of E6/E7 sequence in UL83		
UL83FusedE	F	TTATAGAGTCGTCTTAAGCGCGTGCGCGGCCGGGTGGCGCGGCCG
6/E7	R	CTCATCAGGGTTTCT GCCGCTCAGTCGCCTACACCCGTACGCGCAGGCAGCGAATTCGC CACCATGGACTGGAC
SeqUL83	F	AGTGGACGTGGGTTTTTATAGAGT

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	R	ATAACGTTTTTATTTTCGGGTTCCG
UL83RT	F	GCAGAACCAGTGGAAAGAGC
	R	GTGTCGGTACTCAAGCTTGC
RT PCT primer for HPV16 E6 and E7		
E6RT	F	GATCCTGTTCCTGGTGGCC
	R	CAGGTGTCTCTGCAGGGG
E7RT	F	GACCTGCAGCCTGAGACC
	R	GGGTTTCTGGGAGCAGATGG
E6 peptide fusion to C-terminus of UL123 (IE1)		
E6PepIE1	F	TAACAGTAACTGATATATACACACAATAGTTTACACACACTCCAG GATGATGTCGTGGATGGTGGCAGCTAGGGATAACAGGGTAATCG A
	R	ACCCTATGGTGACTAGAAGCAAGGCTGACCAGGCTGCCACCATC CACGACATCATCCTGGAGTGTGTGGCCAGTGTTACAACCAATTA C
SeqIE1	F	GAGGAGTGTTAGTAACCGCGA
	R	AGCAGAGTGATGAGGAACAGG
E6 peptide fusion to C-terminus of UL83 (pp65)		
E6PepUL83	F	GTCGTCTTAAGCGCGTGCGCGGGTGGCTCACACACTCCA GGATGATGTCGTGGATGGTGGCAGCTAGGGATAACAGGGTAATC GA
	R	TGCATCGCCTCGACGCCCAAAAAGCATCGAGGTGCTGCCACCAT CCACGACATCATCCTGGAGTGTGTGGCCAGTGTTACAACCAATTA A
SeqUL83C- ter	F	GGGGAGGGTCAGGGGATG
	R	GCATCCTGGCCCGCAA

Table 20. List of primers.

2.1.12 Software is listed in Table 21

Name	Manufacture
BLAST	NCBI, Bethesda
EndNote X8	THOMSON REUTERS

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FACStation Software version 3.4	BD Biosciences
FinchTV 1.4.0	Geospiza, Inc.
GraphPad Prism 8.1.0	GraphPad Software Inc.
ND-1000 V.3.0.7	PeqLab
Vector NTI Advance™ 9.1	Invitrogen

Table 21. List of software.

2.2 Methods

2.2.1 Standard molecular biology methods

2.2.1.1 Polymerase chain reaction (PCR)

PCR was used to amplify gene sequences using complementary oligonucleotides, so-called primers. For routine PCR, HotstarTaq polymerase (polymerase of the thermophilic eubacterium *Thermus aquaticus*) was used. HotstarTaq polymerases also have the advantage that they only activated after an initiation at 95 °C. The PCR was carried out for 25-30 cycles. The denaturation of DNA takes place at temperatures between 94-96 °C for 30 sec. The temperature of the attachment for the specific primer (annealing) is determined according to their melting temperature, which in turn depends on the GC content. The elongation step was at 72 °C allowing for 1000 bp/1 min extension. The 50 µl reaction contains 200 ng DNA, 1 U polymerase, 1x Taq buffer, 200 µM dNTPs (New England Biolabs, Frankfurt, Germany) and 0.5 µM each forward and reverse primers.

The Phusion Hot Start II High Fidelity DNA polymerase (New England Biolabs, Frankfurt, Germany) was used for cloning, mutagenesis and sequencing. A reaction batch of 50 µl contains 200 ng DNA, 1 U polymerase, 1x Phusion HF buffer, 3% DMSO, 200 µM dNTPs, and 0.5 µM each forward and reverse primer used. The DNA was initially in an initial step denatured at 98 °C for 30 sec, followed by 25 cycles in which a 10 sec in denaturation step at 98 °C, annealing for 30 sec and the elongation of the DNA at 72 °C with 1000 bp/30 sec .

2.2.1.2 Gel electrophoresis

DNA molecules can be sized by agarose gel electrophoresis and be separated from each other. The products of the PCR are used in this procedure were applied to a 1% agarose gel, in an electric field (electrical chamber with TAE buffer as running buffer) and according to their size

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separated. Because DNA molecules are negatively charged, they migrate toward the positive electrode, the anode. Their running speed depends on their size, which means smaller molecules will reach the anode faster than larger ones. The separation of the DNA fragments was carried out at a constant voltage of 100 V for about 30 min in room temperature (RT). To interpret the results, a size standard (e.g. 1kb ladder) was included in each gel electrophoresis. To prepare the agarose gel, appropriate amounts of powdered agarose in TAE buffer was boiled, after a short cooling phase, ethidium bromide was added (to a final concentration of approximately 0.2-0.5 µg/ml) and poured into the mold. Ethidium bromide intercalates into the DNA fragments and makes them visible under UV light. The PCR products were mixed with loading buffer before application. This stabilizes the pH of the sample, facilitates their handling by a color additive and allows the sinking of the sample into the loading pocket of the gels.

2.2.1.3 DNA isolation and purification

Contamination of PCR reactions with primer, nucleotides, polymerase or salts were purified using the GeneMATRIX Basic DNA Purification kit according to manufacturer's instructions.

2.2.1.4 DNA digestion

Restriction enzyme digestion was carried out for screening of plasmids and recombinant BACs as well as for DNA cloning using multiple restriction endonucleases. DNAs were digested according to the manufacturer's recommendations.

2.2.1.5 DNA de-phosphorylation

Antarctic phosphatase (AP) was used to prevent self-ligation by removing of 5' phosphate groups. Briefly, purified digested plasmid DNA with a single restriction enzyme was incubated with AP in the recommended buffer for 1 hour at 37 °C. The AP was inactivated by heating at 65 °C for 15 min.

2.2.1.6 DNA gel extraction

Purification of DNA fragments from agarose gels was carried out by identifying the band of interest from the gel using long wavelength UV light. The band of interest was then carefully cut from the agarose gel with a surgical scalpel and DNA was purified using the GeneMATRIX Basic DNA Purification kit according to manufacturer's instructions.

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2.2.1.7 DNA ligation

DNA ligation was carried out by T4 DNA ligase according to manufacturer's instruction. Ligation reaction contains a molar ratio of 3:1 (insert to vector) that was calculated by NEBio calculator (<http://nebiocalculator.neb.com>). A 1-5 μ l of the ligation reaction was used to transform *E. coli* competent cells (see Section 2.1.1.3).

2.2.1.8 Determination of DNA concentration by spectrophotometry

Concentration and purity of PCR products, plasmids and BACs DNA samples was determined using Nanodrop ND1000 low volume spectrophotometer measuring 220 nm to 750 nm wavelength with software version 3.7.1 (PeqLab, Erlangen). The 260/280 absorbance ratio was used to indicate the contaminants of the DNA samples.

2.2.1.9 Colony PCR

A single bacterial colony was used directly as template for PCR to detect correct clones after ligation reaction. Single colony was transferred to a new agar plate with appropriate antibiotic by a pipette tip and the remains of the cells in the tip were mixed into the PCR reaction using *Taq* DNA polymerase as described previously (see Section 2.2.1.1).

2.2.1.10 RNA isolation and reverse transcriptase PCR (RT-PCR)

RNA was extracted from cells using RNeasy Plus Mini kit according to the manufacturer's instruction. Samples were treated with DNase I kit and cDNA was generated using SuperScript III Reverse Transcriptase kit following manufacturer's instruction. RT-PCR was performed on cDNA with specific primers using *Taq* polymerase as previously described (see Section 2.2.1.1).

2.2.2 Microbiological Methods

2.2.2.1 Bacterial culture

Laboratory strains of *E. coli* were grown on LB medium or plated on LB agar plates (see Section 2.1.6.1). The liquid LB medium and LB agar were sterilized by autoclaving. For positive selection, LB agar was heated by microwave and cooled down to 60 °C and then the

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appropriate antibiotics were added and mixed with LB agar and finally plated in 10 cm petri dishes.

2.2.2.2 Preparation of bacterial stocks for long term storage

Selected bacterial clones with correctly sequenced BACs or plasmids were stored in glycerol at -80 °C for further usage.

To prepare glycerol stock, a single bacterial colony were inoculated in 3-4 ml LB medium with the positively selecting antibiotics for overnight culture at 32 °C or 37 °C with shaking at 220 rpm in bacterial incubator shaker. The next day, 600 µl of the culture was mixed with 200 µl of sterile 60% glycerol in a 1.5 ml eppendorf and stored in -80 °C.

2.2.2.3 Transformation of chemically competent *E. coli*

Chemically competent NEB 10-beta *E. coli* was used for transformation of plasmids and ligation products. NEB 10-beta competent *E. coli* is a derivative of the popular DH10B strain.

Transformation steps were carried out according to manufacturer's protocol. Briefly, tubes of NEB 10-beta Competent *E. coli* cells were left on ice until the last ice crystals disappear. A 1-5 µl containing 1 pg-100 ng of plasmid or ligation product DNA was added to the cell mixture with gently flicking. The cell mixture was then kept for 30 min on ice. The mixture was then given a heat shock at 42 °C for 30 sec and then kept on ice for 5 min. A 950 µl of pre-warmed supplied growth medium was added to the mixture and then kept for 60 min at 37 °C with shaking at 220 rpm in the bacterial incubator shaker. The mixed bacterial cells were then seeded down by centrifugation for 5 min at 5000 rpm and resuspended in 100 µl of media and then plated on the selective LB agar plated for 18-24 hours.

2.2.2.4 DNA isolation from bacteria (Miniprep, Midiprep, and BAC Maxi-prep)

For small-scale plasmid DNA isolation from bacteria (Miniprep), GeneMATRIX Basic DNA Purification kit was used according to manufacturer's instructions.

Plasmid Midiprep and BAC Maxiprep were carried out using NucleoBond® Xtra Midi and NucleoBond® Xtra BAC kits, respectively, following the manufacturer's instructions.

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2.2.2.5 Synthesis of consensus fused HPV16 E6/E7

The fused form of the modified HPV16 consensus E6/E7 DNA sequence (ConE6E7, GenBank accession number: FJ229356) was synthesized and sequenced by Integrated DNA Technologies (IDT, Leuven, Belgium). The synthesized ConE6E7 was digested with *Kpn-I*, cloned into the expression vector pcDNA 3.1(+) under the control of the IE promoter of cytomegalovirus and the sequence of the insert was confirmed by PCR and sequencing, this construct was named as pcDNAE6/E7Kpn-I.

ConE6E7 was amplified and cloned into pJET 1.2 vector at *EcoRV* restriction site using primers conE6E7EcoRVF and conE6E7EcoRVR, this plasmid was named as pJetConE6E7. Then, the kanamycin cassette was amplified from pEPkan-S plasmid using primers conE6E7BamHF and conE6E7BamHR and the amplified fragment was inserted into *BamHI* restriction site, this plasmid was named as pJetConE6E7kan. A shuttle vector was constructed using pEF6/V5-His A plasmid, in which ConE6E7 with kanamycin cassette were inserted at *EcoRI* restriction site using primers pEF_E6/E7F and pEF_E6/E7R, the plasmid was named as pEF6E6/E7EcoRI.

2.2.2.6 Two-step Red-mediated recombination (En passant mutagenesis) [242, 248]

All HCMV recombinant viruses generated in this study were genetically modified using the most efficient mutagenesis that allows introduction of the desired mutations including insertions, deletions, point mutations or tags into herpesvirus DNA called Two-step Red-mediated mutagenesis technique system (En passant mutagenesis). This system was originated from phage λ (lambda phage) and composed of three major protein which are Exo, Beta and Gam proteins components [242, 248]. These proteins can be activated at 42 °C. Linear double stranded DNA inserted to *E. coli* can be degraded by the action of *E. coli* RecB/C/D helicase–nuclease complex; Gam protein of the Red-recombination system inhibits this complex and maintains the inserted linear DNA. On the other hand, Beta protein protects the 5`-3` exonuclease action of homotrimer Exo protein, it also anneals the 3` single strand end produced from the linear DNA with complementary homologous sequences in the desired target. Generally, complementary homologous sequence of 30-50 bp is sufficient to allow integration into replicating DNA in Red recombination system.

All mutagenesis steps were carried out in GS1783 *E. coli* strain harbors HCMV BACs with temperature-inducible Red-recombination system, as well as arabinose induced the *I-SceI* homing endonuclease, and chloramphenicol resistance marker. Shuttle plasmids were initially

2. Materials and Methods

designed which encode the sequence to be inserted into the BAC sequence. These shuttle plasmids were used as PCR template, from which the fragments to be inserted into HCMV BAC are amplified with primers with 40 bp of homology to the desired target sequence in the BAC. The PCR products were digested with *DpnI* enzyme to get rid of the original plasmids and then purified by PCR cleanup or agarose extraction (see Section 2.2.1.3 and 2.2.1.6). A 150 ng of the clean linear PCR were electroporated into electrocompetent GS1783 *E. coli* harboring the HCMV BAC.

To generate electrocompetent GS1783 *E. coli*, GS1783 was grown overnight at 32 °C in 3 ml LB-cam medium. In the next day, 50 ml were inoculated with the 500 µl of overnight culture and incubated at 32 °C under vigorous shaking (220 rpm). The logarithmic growth phase (at OD₆₀₀) was measured after 2-4 hours till it reaches OD₆₀₀ 0.5 - 0.7, then the culture was transferred to water bath shaker at 42 °C for 15 min at 200 rpm to activate the Red-recombination system. The culture then was cooled down on water-ice bath for 15 min and kept on a shaker at RT. The culture was pelleted by centrifugation (5000 rpm, 5 min at 4 °C), followed by three times washed with 25 ml of an ice-cold sterile millipore water and finally resuspended in 100 µl sterile millipore water. A 40-50µl of the bacterial suspension was used for the electroporation step. Electroporation was carried out in 1 mm pre-chilled cuvette at 1.6 kV, for time constant (τ) 5 msec using the Multiporator device.

Samples were resuspended in 1000 µl of pre-warmed LB medium (without antibiotics) and incubated for 1-2 hours at 32 °C with shaking at 220 rpm. The bacteria were afterwards pelleted by centrifugation (5000 rpm, 5 min, RT) and resuspended in 100 µl of the LB medium and plated on selective LB-cam-kan agar plates for 24-48 hours.

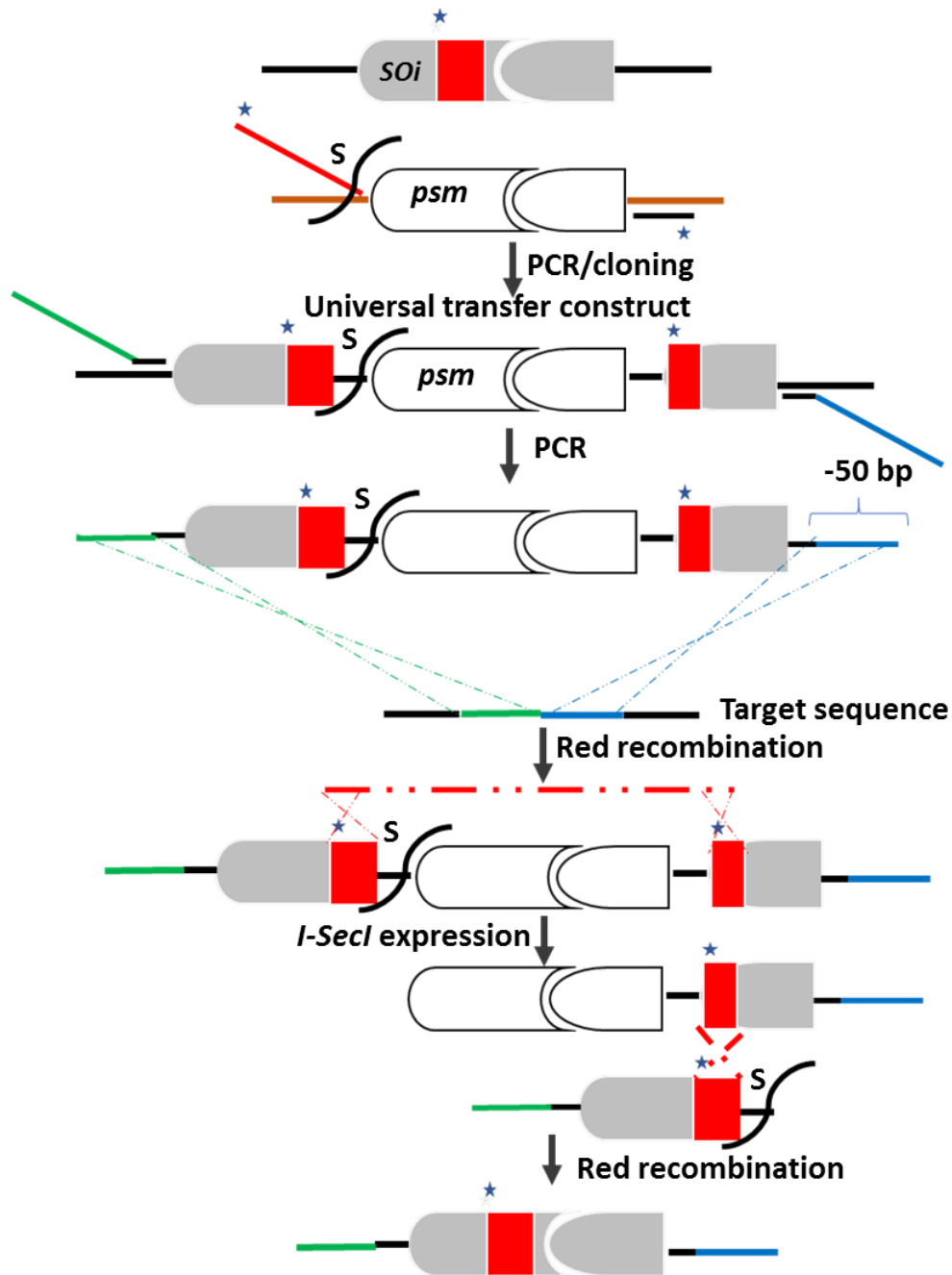


Figure 8. Schematic representation of the two step Red-mediated recombination procedure. Two-step Red-recombination was used for insertion of fragments into HCMV-BAC. First, the kanamycin cassette as positive selection marker (*psm*) obtained by a PCR from pEPkan-S contains *I-SecI* site (S) with homologous sites (red box) is cloned into the single restriction site (*) of the shuttle plasmids. After PCR amplification with primers that have homologous flanks to desired location in BAC (green and blue lines), the first recombination step is performed in which the PCR product is integrated into the genome. The following process, resolution, is the excision of the positive selection marker (kan). Modified from Tischer et al., 2006 [242].

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For scarless excision of the positive selection marker (kan) in the second Red-recombination step (resolution), the correctly modified BACs in GS1783 from overnight culture were grown for 2 hours in 2 ml of LB-cam media at 32 °C with shaking at 220 rpm. To induce the expression of *I-SceI*, 2 ml of fresh LB-cam containing 1% L-(+)- arabinose was added to the culture and incubated for 2 hours at 32 °C with shaking at 220 rpm, then cultures were then transferred to a 42°C water bath shaker (150 rpm) for 30 min to activate the Red-recombination system for the second recombination step between duplicated sequences (Figure 8, shown in red). The bacterial cultures afterwards were grown for another one hour at 32 °C in bacterial incubator shaker with shaking at 220 rpm. Finally, 100 µl of 1x10⁻³ dilution was spread on LB-cam-1% L-(+)- arabinose agar plates, incubated for 48 hours at 32 °C. The final clones were tested for the absence of kanamycin resistance on replica plates with LB-cam-kan and LB-cam. Selected clones were investigated by restriction fragment length polymorphism (RFLP) screening analysis using multiple restriction enzymes and were compared to a predicted banding pattern. Moreover, these mutations were confirmed by PCR and sequencing as well.

2.2.3 Cell culture methods

2.2.3.1 Cell thawing and culture

The cells were thawed in their cryopreservation tubes and placed under sterile transfer conditions to a 15 ml falcon tube containing 10 ml of cell-specific medium. Afterwards, the cells were centrifuged at 300 x g for 5min, RT. The supernatant is discarded and the cell pellet in a sufficient amount of fresh medium resuspended. The cells are transferred to a multi-well plate or cell culture flasks and filled with medium according to their quantity. The cultivation takes place in the cell culture incubator at 37 °C and 5% CO₂.

2.2.3.2 Cell feeding and medium change

Depending on their requirements, the cultured cells were regularly supplied with fresh medium. Sometimes, adding a good amount of fresh medium to it is enough. With a strong consumption of the presented nutrients, it is recommended to partial replace the old medium by 50% with a fresh one. The complete decline of the conditioned medium may cause shock for cells depending on the cell type and this is not recommended.

2. Materials and Methods

2.2.3.3 Cell passaging

Suspension cells can be divided as desired or lower concentrated by a removing certain amount of cells from a culture vessel, transferred to a new one and filled with fresh medium.

For adherent cells, the old medium is removed, and the cell rinsed with 1x PBS with the recommended amount without damaging them. Cells were then detached from the bottom of the cell culture vessels with a small amount of trypsin according to the size of cell culture plate or flask.

After a short reaction time (2-5 min) in the cell culture incubator, cells were treated by the addition of fresh medium and the cells were mixed with medium using sterile cell culture pipettes. The enzymatic reaction of trypsin is stopped, and the complete degradation of the cells prevented by the addition of the fresh medium. The cells can then be counted (see Section 2.2.3.6), distributed to new culture vessels, or in portions to be frozen.

2.2.3.4 Cryopreservation of cells

The suspension cells to be frozen had to be suspended in PBS first. For adherent cells, cells had to be trypsinized first and washed once with the recommended medium and afterwards resuspended in PBS in 15 ml sterile falcon tube.

The cells were then counted (see Section 2.2.3.6), and afterwards centrifuged for 5 min at 300 x g, RT. The PBS was removed, and cells were resuspended in a desired amount of FCS.

Cells were then aliquoted in the 2ml cryopreservation tubes. An equal volume of FCS containing 20% sterile DMSO was added to each tube and the tubes were mixed gently by inverting for 5 times. The final cell number in each tube should not exceed 1×10^7 cells/ml. The cryopreservation vials were then transferred into a Mr. Frosty container (at room temperature) and put into a -80°C freezer. The Mr. Frosty container ensures that the temperature decreases steadily by 1 °C/min. After approximately 24 hours, the cryopreservation vials were transferred from the Mr. Frosty container into liquid nitrogen for long term storage.

2. Materials and Methods

2.2.3.5 Isolation of peripheral blood mononuclear cells (PBMCs)

Buffy coat preparations were purchased from German Red Cross (Dresden, Germany). Blood samples were taken with the approval of the ethics committee of the Charité–Universitätsmedizin Berlin. Written informed consent was obtained from all donors.

The delivery to our laboratory took place within 24 hours; storage and transport were carried out at room or vehicle temperature. The blood was transferred from the preserved blood bag to sterile falcon tubes and diluted 1: 3 with PBS. Subsequently, the diluted blood was pipetted in a ratio of 3:2 onto a layer of Ficoll-Paque (Roti®-Sep 1077 human); a 50 ml sterile falcon tube thus contained 30 ml of blood to 20 ml of Ficoll-Paque.

In order to avoid a mixing of the layers, particularly slow and careful overlapping was necessary. This was followed by centrifugation at 400 x g in a swinging-bucket rotor for 30 min at RT without brake. After centrifugation, the supernatant portion of plasma was carefully pipetted off about 1 cm above the ring of mononuclear cells without mixing the layers. The cells were then carefully removed with a sterile Pasteur pipette and transferred to a 50 ml sterile falcon tube. Then the falcon tube was afterwards filled up to a total volume of 45 ml with PBS. The cells were afterwards centrifuged at 300 x g for 10 min at room temperature. The supernatant was pipetted off and the cell pellet resuspended in the residual volume. For erythrocyte lysis, 3 ml of RBC lysis buffer was added and incubated for 5 min on ice. Afterwards, the reaction was stopped by adding PBS to a total volume of 45 ml. It was again centrifuged at 300 x g for 10 min. The supernatant was removed, and the cells washed twice with PBS. Finally, the cells pellet was resuspended in 10 ml PBS. The cells were counted after appropriate dilution (see Section 2.2.3.6), and then cultured in RPMI 1640 medium.

2.2.3.6 Cell counting

Cells to be counted should be in suspension. For vital staining trypan blue was used; trypan blue penetrates defective cell membranes and stains corresponding cells dark blue. Living cells and cells with an intact cell membrane remain bright. The staining was carried out in a ratio of 1:1, for example 100 µl trypan blue (0.4% initial concentration) to 100 µl cell suspension. Subsequently, the cell suspension was applied to a Neubauer counting chamber.

At high cell concentrations, a dilution was first required. At high cell numbers (> 200 cells per corner square), the count is no longer possible due to the high cell density. The living unstained

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cells in 4 large corner squares were counted under the microscope and an average value was formed division by 4. Cells touching the left or top lines were counted, but cells at the bottom or right margins were not counted. The microscopy work was performed at the microscope workstation outside the sterile bench. To determine the number of cells per μl , the average of the counted cells was multiplied by the chamber factor and the dilution factor.

The chamber factor results from the reciprocal of the volume of a corner square (0.1 μl) and is thus 10. In order to determine the total cell number of the cell suspension, the number of cells per μl was multiplied by the total volume of the suspension.

2.2.3.7 Surface expression of HLA-A2 molecules

Cell surface expression of HLA-A2 molecules was measured by FACS as previously described using PE-conjugated anti- HLA-A2 antibody clone BB7.2 [249].

2.2.4 Virological methods

2.2.4.1 Stable mammalian cells transfection by electroporation

U251 cells were used in this study for stable plasmid transfection by electroporation. Cells were trypsinized from cell culture plate and washed once with PBS and counted (see Section 2.2.3.6). About 2×10^6 cells were transferred to another 15 ml sterile falcon tube and centrifuged for 5 min at 300 x g, RT and resuspended in 150 μl in hypo-osmolar buffer for eukaryotic cells electroporation in 1.5 μl eppendorf. About 3 μg of plasmid (pcDNAE6/E7Kpn-I) was diluted in 50 μl iso-osmolar buffer and then added to the cells and mixed gently by tipping and left for 30 min incubation at RT. The cells mixture was then transferred to 2 mm cuvette and was pulsed at 240 V for time constant (τ) 40 μsec using the Multiporator device. After the pulse the cells were kept standing in the cuvette for 5 to 10 minutes at RT and then transferred to the culture medium.

After 48 hours, the culture medium was changed, and the cells were fed by fresh medium containing the selection antibiotic (G418) at final concentration of 1 mg/ml. The medium was changed every 3 days with keeping the selective antibiotic at the same concentration.

After two weeks of positive selection, different cell clones were isolated using the cloning rings as described previously [250]. Different clones were allowed to expand in cell culture in separate cell culture wells and plates for further investigations.

2. Materials and Methods

2.2.4.2 HCMV reconstitution from BACs

All modified HCMV BACs in this study were reconstituted in Fi301 cells using Basic Nucleofector™ kit for primary mammalian fibroblasts following the manufacturer's instructions.

Briefly, about 3 µg of the purified DNA BACs (see Section 2.2.2.4) was used for HCMV reconstitution, the cells were given a pulse using program A-024 of the Nucleofector™ 2b device.

On the next day, culture media was changed, and more fibroblasts were added to the transfected cells to keep cell confluency 70-90%. The culture was kept for two weeks with medium change every 4 days. The typical cytopathic effect (CPE) of HCMV appears in 7-14 days after transfection, the infected cells were mixed with fresh non-infected Fi301 in a T-75 cell culture flask and kept for another 7-10 days till were fully infected. The infected cells were then passaged to infect two T-175 flasks of Fi301 cells.

2.2.4.3 HCMV stock generation

To generate high titer of HCMV stocks, Fi301 cells were used in T-175 cell culture flasks with 70% confluency. The cells were infected at multiplicity of infection (MOI) 0.01-0.02 and the infected cells were kept in culture till the cells were fully infected. The supernatant was collected, and the cells were scraped by cell culture scrapers and then resuspended in a minimum residual volume (about 2 ml) and cell were afterwards transferred to a 15 ml sterile falcon tube and kept for -80°C overnight. The cells were then handled for two freezing-thawing cycles with a vortex in between and then cell debris were removed by centrifugation at 200 x g for 5 min, RT. The cell lysate was collected in another 15ml sterile falcon tube.

The collected supernatant was handled separately; the supernatant was ultra-centrifuged for 1 hour at 20,000 rpm, 4 °C. The virus pellets were then combined to the same falcon tube containing HCMV from the cell lysate and aliquoted in 1.5 eppendorf tubes and kept in -80 °C.

2.2.4.4 HCMV infection of cells

Cells were infected with HCMV using different volumes from the virus stock based on the tissue culture infection dose 50 per ml (TCID₅₀/ml) of a virus stock. The TCID₅₀/ml describes

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the amount of a virus dilution needed to infect 50 % of all cells. To determine the volume of virus stock needed to infect cells at a given MOI the following equation was used:

$$\frac{\text{Number of cells} * \text{MOI}}{\text{TCID}_{50}/\text{ml}} = \text{volume of virus stock in ml}$$

The required volume from virus stock was used to infect cells by adding this volume to the attached cells. The culture plates were afterwards rocked gently to ensure even cell infection and then kept in cell culture incubator. After 30 min the plates were rocked gently once again and returned back to the incubator.

2.2.4.5 HCMV titration and growth kinetics

Virus titers of virus stocks and multi-step growth kinetics were quantified by TCID₅₀ assay on Fi301 cells. The TCID₅₀ values were calculated using the method of Reed and Muench [251].

2.2.4.6 HLA ligandome analysis

HLA-A2 Fi301 cells were left uninfected or infected with HCMV (MOI 1). The cells were harvested after 48 hours, washed twice with cold PBS, and stored frozen at -80 °C. The cells were sent to Prof. Stefan Stevanovic (Department of Immunology, Institute for Cell Biology, University of Tübingen, Tübingen) where the cells were subjected to subsequent isolation of HLA peptides using standard immunoaffinity purification and mass spectroscopy, as described previously [252, 253].

Peptides-source proteins were annotated using Uniprot and NetMHCpan 4.0 [254] and were classified according to subcellular locations and biological functions using human protein reference database [255].

2.2.5 HPV oncoprotein assay

2.2.5.1 HPV16-encoded E7 protein assay

Expression of the oncoprotein E7 was detected by recomWell HPV16/18/45 ELISA kit. All steps were done at RT. Cells were trypsinized, centrifuged at 600 x g for 15 min, supernatant discarded, and the pellet resuspended in 250 µl of lysis solution A by vortexing for 3 sec. After an incubation for 30 minutes 250 µl of lysis solution B was added and vortexed for 3 sec. A 100 µl aliquot of the lysate was applied to one well of the ELISA plate and incubated for one

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hour. The plate was washed three times with 300 µl washing buffer and tapping it firmly. The detection antibody (100 µl/well) was added and incubated for one hour. The plate was washed three times. streptavidin conjugate (100 µl/well) was added und incubated for one hour. The plate was washed six times and the substrate solution (100 µl/well) was added and incubated 30 min in the dark. Stop solution (100 µl/well) was added and the absorbance was read at 450 nm in the Multiskan FC microplate photometer.

2.2.5.2 HPV16-encoded E6 protein assay

OncoE6 Cervical Test™ kit (Arbor Vita, USA) was used to detect expression of the oncoprotein HPV16 E6. All steps were done at RT. The trypsinized 1×10^6 cells were centrifuged at 10,000 x g for 5 min and supernatant was discarded. A volume of 930 µl rinse solution was added to the tube containing the cell pellet, the tube was shaken vigorously and centrifuged at 10,000 x g for five minutes. After discarding the supernatant, a volume of 186 µl of lysis solution was added to the cell pellet. The tube was shaken followed by rotating at 8 rpm for 15 min. After a centrifugation at 10,000 x g for 15 sec, 17.5 µl conditioning solution was added to the tube. The tube was shaken and rotated at 8 rpm for 15 min. After centrifugation at 10,000 x g for 15 sec, a 200 µl aliquot of the lysate was filled into the antibody-coated detector vial and slowly tilted for antibody resolution. The test unit was inserted into the lysate and incubated for 55 min, then transferred into a vial containing 200 µl washing solution and incubated for 12 min, and then transferred into a vial containing 650 µl development solution and incubated for 15 min. The test unit was immediately placed onto a reading guide for result interpretation. Appearance of a test line indicated positive detection of oncoprotein E6. Different expression levels could be distinguished by the color intensities of the test lines.

2.2.6 Functional T cell assay

In collaboration with Prof. Gerald Willimsky (Institute of Immunology, Charite Universitätsmedizin Berlin, Berlin), PBMC isolated from healthy donors (see Section 2.2.3.5) and Jurkat cells were transduced by retrovirus encoding specific TCRs specific for HLA-A2-restricted HPV16 E6 or HCMV pp65 peptides.

2. Materials and Methods

2.2.6.1 Generation of TCR expression vectors

For HPV E6₂₉₋₃₆-specific TCR [256], transgene cassettes were codon-optimized for human expression and synthesized by GeneArt (Life Technologies, Darmstadt, Germany). TCR- α/β chains with human TCR constant regions replaced by their murine counterparts were linked via 2A “self-cleaving” peptide sequence from *Porcine teschovirus-1* (P2A) and cloned in the configuration TCR β -P2A-TCR α into pMP71-PRE using *NotI* and *EcoRI* restriction sites as described before [257], the plasmid named as pMP71-PRE-E6. The CMV-specific TCR (NLV-3) was used in its original configuration as described previously (MP71TCR α and MP71TCR β) [247].

2.2.6.2 TCR gene transfer

TCR gene transfer was carried out as described previously [246]. In brief, HEK-293T cells stably expressing GALV-env and MLV-gag/pol were grown to approximately 80% confluence and transfected with 3 μ g of pMP71-TCR vectors in the presence of 10 μ g Lipofectamine2000 (Life Technologies, Germany). A 3 ml of retrovirus containing supernatant were harvested 48 hours and 72 hours after transfection. 1×10^6 from Jurkat cells or frozen human PBMCs, isolated as described previously (see Section 2.2.3.5), were stimulated with 5 μ g/ml anti-CD3 (OKT3) and 1 μ g/ml anti-CD28 (CD28.2) coated plates in the presence of 300 U/ml recombinant human interleukin 2 (hIL-2). Transductions at 48 hours and 72 hours after stimulation were performed by addition of retrovirus containing supernatant and 4 μ g/ml protamine sulfate (Sigma-Aldrich, Steinheim, Germany) followed by spinoculation for 90 min at 800 x g and 32 °C (1st transduction). For 2nd transduction retrovirus was preloaded onto retronectin- coated plates followed by spinoculation for 30 min at 800 x g and 32 °C. Transduced T cells were kept in the presence of 300 U/ml hIL-2 for a total of 2 weeks. At least 2 days prior experiments, PBMCs to be transduced were cultured in the presence of 30 U/ml hIL-2.

2.2.6.3 Functional assays using transduced PBMCs

Interferon- γ (IFN- γ) production was measured by ELISA using the standard protocol after 16 hours co-culture of 1×10^5 TCR-transduced T cells with 1×10^5 target cells (HCMV-infected or peptide-loaded cells). Stimulation with phorbol myristate acetate and ionomycin (P+I) was used as a positive control.

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2.2.6.4 Functional assays using transduced Jurkat cells

For detection of nuclear factor of activated T-cells (NFAT) activation, a previously described cellular platform for analysis of TCRs was used [258, 259]. In the human lymphoma T cell line Jurkat 76 (J76), the response elements of transcription factor NFAT drive the expression of EGFP [258]. The J76 cell line is a subline of cell line Jurkat E6.1 (JE6.1), which lacks expression of the TCR alpha and beta chains [260]. The J76 cell line was transduced with a retroviral vector encoding HLA-A2-restricted HPV E6₂₉₋₃₆-specific TCR [256]. Moreover, J76 cells were co-transduced to express an HLA-A2-restricted HCMV pp65-specific TCR (NLVPMVATV; aa 495-503) and CD8 [259].

For measuring of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) activation a single T cell reporter cell line was used, in which the responsive element for NF-κB controls EGFP expression [261]. This single reporter cell line was transduced with retroviral vector encoding HLA-A2-restricted HPV E6₂₉₋₃₆-specific TCR [256] or with retroviral vector encoding the CMV-specific TCR (NLV3), which recognizes an HLA-A2-restricted epitope derived from pp65 (NLVPMVATV; aa 495-503) [247].

2.2.6.5 Acid wash of MHC-I associated peptides

U251 stably transfected cells with pcDNAE6/E7Kpn-I (pcDNAE6/E7Kpn-I U251 cells) as previously described in (see Section 2.2.4.1) were left uninfected or infected with RVTB40ΔUS11 for 3–24 hours at different MOIs. RVTB40ΔUS11 lacks all known HCMV-encoded immune evasion genes (US2, US3, US6, and US11) that target MHC-I presentation and does not downregulate MHC-I molecules. On uninfected and infected pcDNAE6/E7Kpn-I U251 cells, the existing peptide-MHC-I complexes on U251 cells were removed by acid wash as previously described [262]. Briefly, 1×10^6 cells were harvested, washed with PBS and subsequently washed with ice-cold citric acid buffer (pH 3) for 2–3 min. Afterwards, stripped pcDNAE6/E7Kpn-I U251 cells were pelleted, washed twice with EMEM medium, resuspended in RPMI 1640 medium and subsequently co-cultured for 18 hours with the HPV E6₂₉₋₃₆-specific reporter cell line, in which the responsive element for NF-κB controls EGFP expression [263]. Finally, EGFP expression of reporter cells was determined by FACS analysis. In parallel, the maximal peptide stimulation was always determined by pulsing a cell aliquot with the E6 peptide (1 μg/ml) during co-culture with the E6-specific reporter cell line.

3. Results

3.1 Deletion of MHC-I downregulating genes from HCMV

3.1.1 Deletion of MHC-I downregulating genes from the genome of HCMV strain Merlin

In order to optimize HCMV as a vaccine vector, MHC-I downregulating genes were deleted to potentiate CTL-mediated immune response. We initially used the Merlin strain as this is the closest to wild-type HCMV [245].

Although different BACs of HCMV strain Merlin have been constructed, only one of these BACs was used in this study (RCMV1161). This BAC had been repaired for RL13 and UL128 regions which were previously lost due to virus adaption during passaging in fibroblasts. It also encodes for EGFP cassette under the control of HCMV IE2 [245].

MHC-I downregulating genes (US2, US3, US6 and US11) were deleted sequentially using specific primers (see Section 2.1.11). The En passant mutagenesis steps were carried out as previously described (see Section 2.2.2.6).

Each mutagenesis process was carried out in two steps. Firstly, the gene to be deleted was replaced by the kanamycin cassette. Secondly, excision (resolution) of the kanamycin cassette was performed (Merlin1161 Δ US2-BAC, Merlin1161 Δ US2 Δ 3-BAC, Merlin1161 Δ US2 Δ 3 Δ 6-BAC and Merlin1161 Δ US2 Δ 3 Δ 6 Δ 11-BAC) as shown in Figure 9.

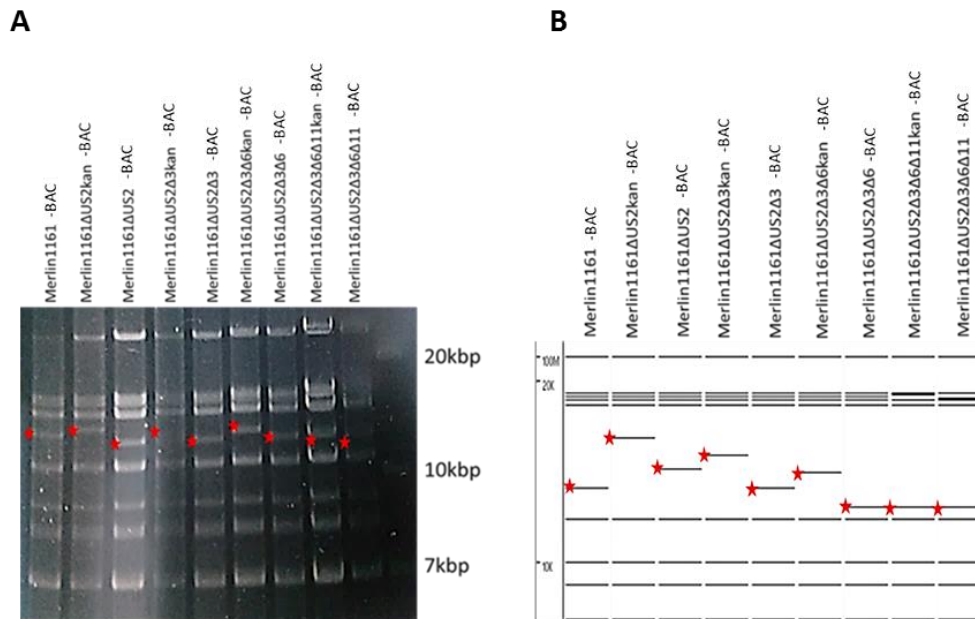


Figure 9. RFLP patterns for RCMV1161 HCMV mutants with MHC-I downregulating genes deletion. (A) Patterns obtained by restriction enzyme *EcoRI*. The DNA was run through an 0.8% agarose gels overnight at 65 V. (B) Vector NTI 9.1 prediction of RFLP patterns are shown with size markers from 7 to 20 kb.

All mutants were sequenced for the modified regions confirming the deletion of these genes (data not shown). However, none of these mutants could be successfully reconstituted by nucleofection (see Section 2.2.4.2). Although transfected cells were kept in culture over three weeks, only single green cells were detected confirming that the mutated (unrepaired) forms of UL128 and RL13 are essential for virus propagation in fibroblasts [245] as shown in Figure 10.



Figure 10. Reconstitution of Merlin HCMV (RCMV1161). Fi301 cells were transfected by RCMV1161 using nucleofection. The image shows EGFP-Fi301 cells using fluorescent microscopy. Only single green cells could be detected without any characteristic CPE of HCMV.

3. Results

3.1.2 Deletion of MHC-I downregulating genes from HCMV (TB40-BAC4)

Another HCMV strain was also included in our study to develop vaccine vectors. TB40/E (TB40-BAC4) is promising as a vector because it is adapted to cell culture and hence is easier than HCMV strain Merlin (RCMV1161) to reconstitute and expand. Moreover, it can infect endothelial cells and DCs while the laboratory strain AD169 cannot, making it a good candidate to drive a CTL-mediated inflammatory response *in vivo* [264].

The mini-F sequence (the BAC backbone) has replaced the region for US2-US6 in TB40-BAC4. For this reason, US11 was the only remaining MHC-I modulating gene that had to be deleted in order to fully optimize TB40-BAC4 by *En passant* mutagenesis (see Section 2.2.2.6). This optimized vector was named as RVTB40 Δ US11 as shown in Figure 11.

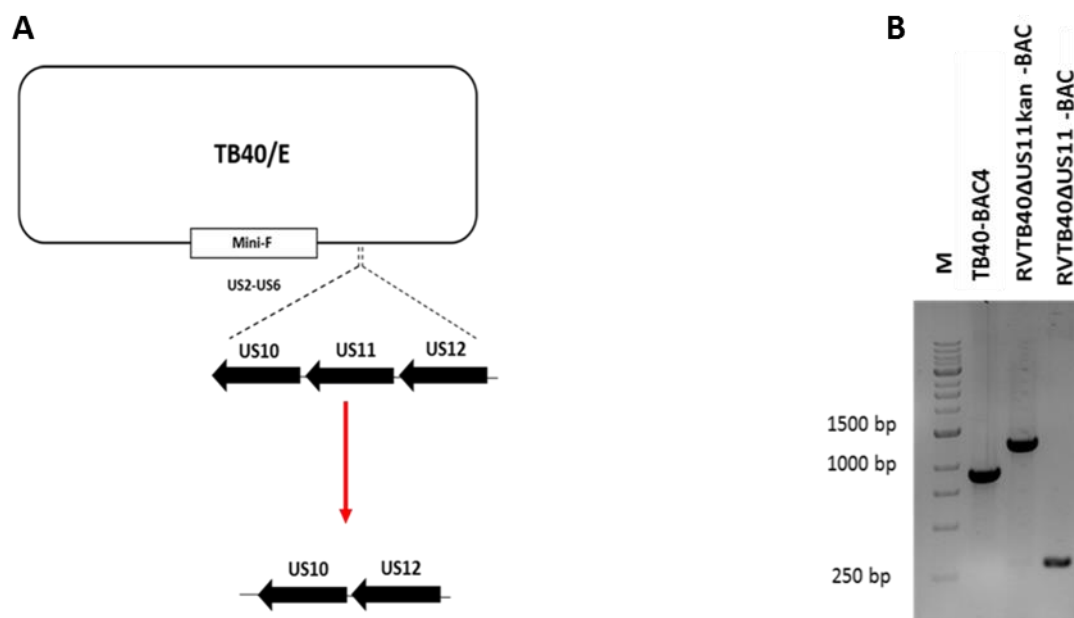


Figure 11. Deletion of US11 gene from HCMV TB40-BAC4. (A) Schematic diagram of TB40-BAC4 showing the deletion US11 (RVTB40 Δ US11-BAC). (B) PCR from up and down-stream of US11 (primers SeqUS11F and R) confirming the insertion and the excision of the kanamycin cassette from TB40-BAC4 using 1 kb DNA ladder GeneSTA (M). The fragment amplified from RVTB40 Δ US11-BAC was sequenced using the same primers and the results confirmed the deletion of US11 gene (data not shown).

RVTB40 Δ US11-BAC was successfully reconstituted as previously described (see Section 2.2.4.2) and the virus growth kinetics were analyzed by TCID₅₀ determination as previously described (see Section 2.2.4.4). The results showed that RVTB40 Δ US11 can grow in fibroblasts like TB40/E WT virus as shown in Figure 12.

3. Results

In order to investigate the effect of all MHC-I downregulating genes (US2, US3, US6 and US11) on the expression and presentation of MHC-I (HLA-A2) molecules, Fi301 infected with TB40/E WT or RVTB40 Δ US11 (MOI 0.5) were stained for HLA-A2 molecules as described (see Section 2.2.3.6) and shown in Figure 12.

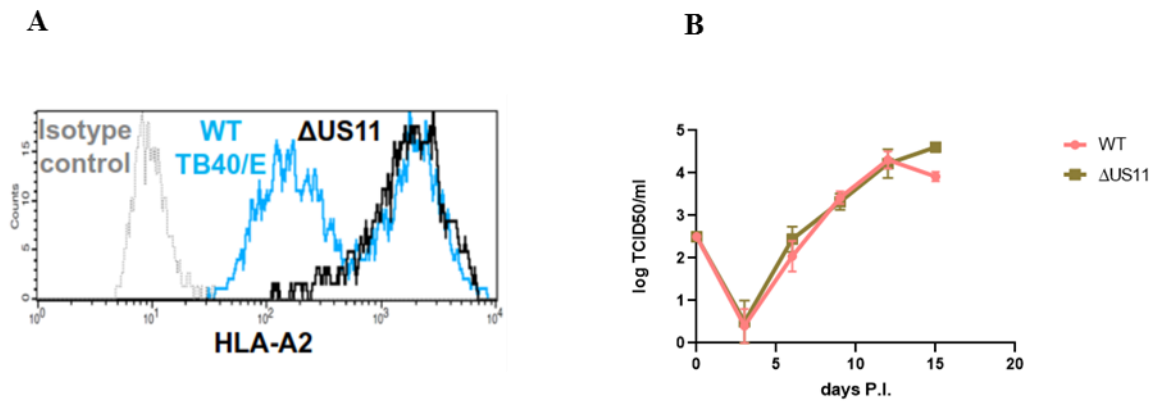


Figure 12. Characteristics of RVTB40 Δ US11 mutant. (A) Fi301 cells were infected with WT TB40/E or RVTB40 Δ US11(Δ US11) at MOI of 0.5. After 2 days cells were stained with HLA-A2 specific antibody or isotype control and analysed by flow cytometry. (B) Multi-step growth kinetics of TB40/E WT (WT) or RVTB40 Δ US11(Δ US11). Fi301 cell were infected with HCMV at MOI 0.01 and the supernatant was collected at different days post infection (p.i.) and was titrated for HCMV as described previously (see Section 2.2.4.5). Results are derived from three independent experiments; error bars represent the mean \pm SEM.

3.2 Infection of GBM cell lines by HCMV

In order to investigate the HLA-A2 GBM selected in this study for their susceptibility to HCMV, GBM cell lines (LN18, U343 or U251 cells) were infected by RV-TB40-BAC_{KL7}-SE-EGFP HCMV (MOI 0.3). At different time points post infection, the percentage of EGFP-expressing GBM cells was measured by FACS and they were all permissive to HCMV at different levels (Figure 13).

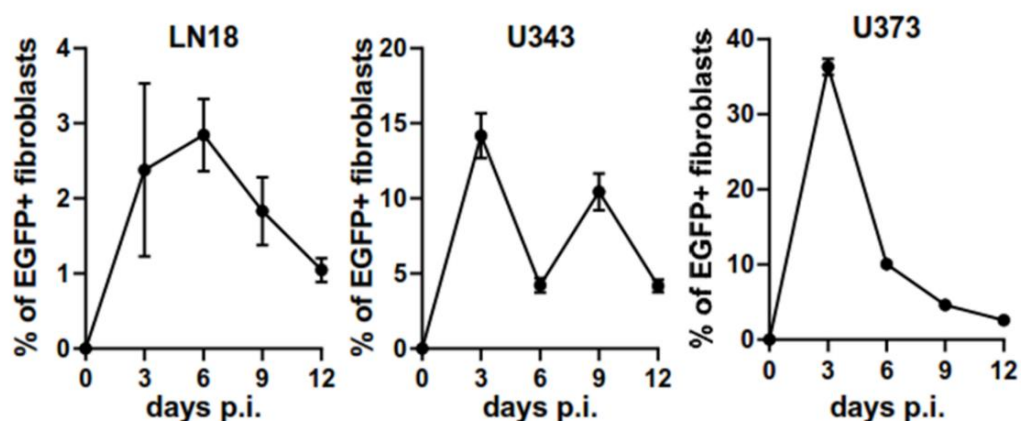


Figure 13. Permissiveness of GBM to RV-TB40-BAC_{KL7}-SE-EGFP HCMV. GBM cells were analyzed by FACS for EGFP expression at different time points p.i. Results are derived from three technical replicates; error bars represent mean \pm SEM.

3. Results

The supernatant of the infected GBM cell line was also analyzed for the presence of cell-free HCMV. The supernatant was collected at the same time points and used to infect Fi301 cells which were further analyzed by FACS after two days for EGFP expression. The results showed that RV-TB40-BAC_{KL7}-SE-EGFP HCMV was highly cell-associated in GBM cell lines (Figure 14).

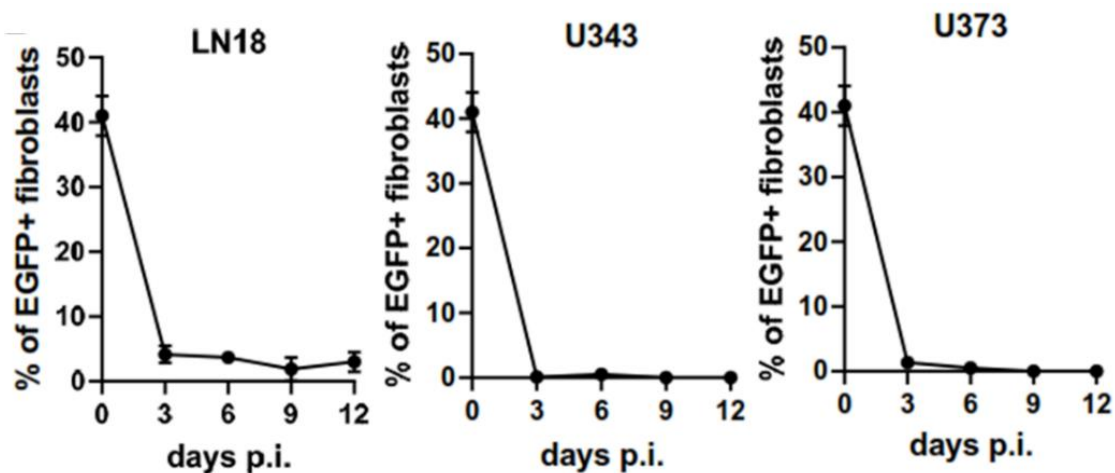


Figure 14. RV-TB40-BAC_{KL7}-SE-EGFP HCMV detection in the supernatant of the infected GBM cells. The results show the percentage of the Fi301 cells infected by the supernatants collected from RV-TB40-BAC_{KL7}-SE-EGFP-infected GBM cell lines. Results are derived from three technical replicates; error bars represent the mean \pm SEM.

3.3 Construction of HCMV-based vaccines

The modified HCMV lacking all MHC-I downregulating genes (RVTB40 Δ US11-BAC) was used to construct all HCMV-based vaccines.

3.3.1 Optimized HCMV-based vaccines expressing fused HPV16 E6/E7 protein

3.3.1.1 Construction of HCMV-based vaccines with E6/E7 expression under control of endogenous or exogenous promoter

In order to investigate the capacity of recombinant HCMV-based vaccines to stimulate T cells, four different vaccines were constructed by *En passant* mutagenesis (see Section 2.2.2.6). All these viruses were constructed using a shuttle plasmid (pEF6E6/E7EcoRI), which encodes the E6/E7 under the control of EF-1 promoter. The kanamycin cassette was also cloned into this vector inside the E6/E7 sequence (see Section 2.2.1.4).

Three viruses were constructed with E6/E7 expression under the control of endogenous HCMV promoters. The first strategy was to express the E6/E7 protein under control of a viral early

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promoter with minimum change of the viral sequence. In this virus, the E6/E7 sequence replaced the sequence of US11. The E6/E7 sequence with kanamycin cassette was amplified (using US11ConE6E7F and R primers) and the amplified PCR product was used to generate recombinant HCMV; this BAC was named as RVTB40E6/E7intoUS11-BAC (Figure 15).

We also sought to investigate the expression of the E6/E7 protein combined with interruption of other immune evasion genes. The amplified E6/E7 sequence (using IL-10FusedE6/E7F and R primers) was used to insert the E6/E7 sequence into UL111A, which is expressed in both lytic and latent infection. This BAC was named RVTB40 Δ US11_E6/E7intoUL111A-BAC (Figure 15). Another vaccine candidate was also constructed by replacing UL83 (using UL83FusedE6/E7F and R primers); this BAC was named RVTB40 Δ US11_E6/E7intoUL83-BAC (Figure 15).

The expression of E6 and E7 under exogenous promoter control was investigated by the amplification of the E6/E7 sequence together with EF-1 promoter and were subsequently inserted into RVTB40 Δ US11-BAC downstream of the mini-F sequence. To construct this virus, the primers EF-1E6/E7F and R were used, and the generated BAC was named RVTB40 Δ US11_EF-1E6/E7-BAC (Figure 15).

To confirm the construction of recombinant HCMV-based vaccines, PCR using primers flanking the target site was carried out (Figure 16) and the amplified fragments from final mutants were sequenced using the same primers (date not shown).

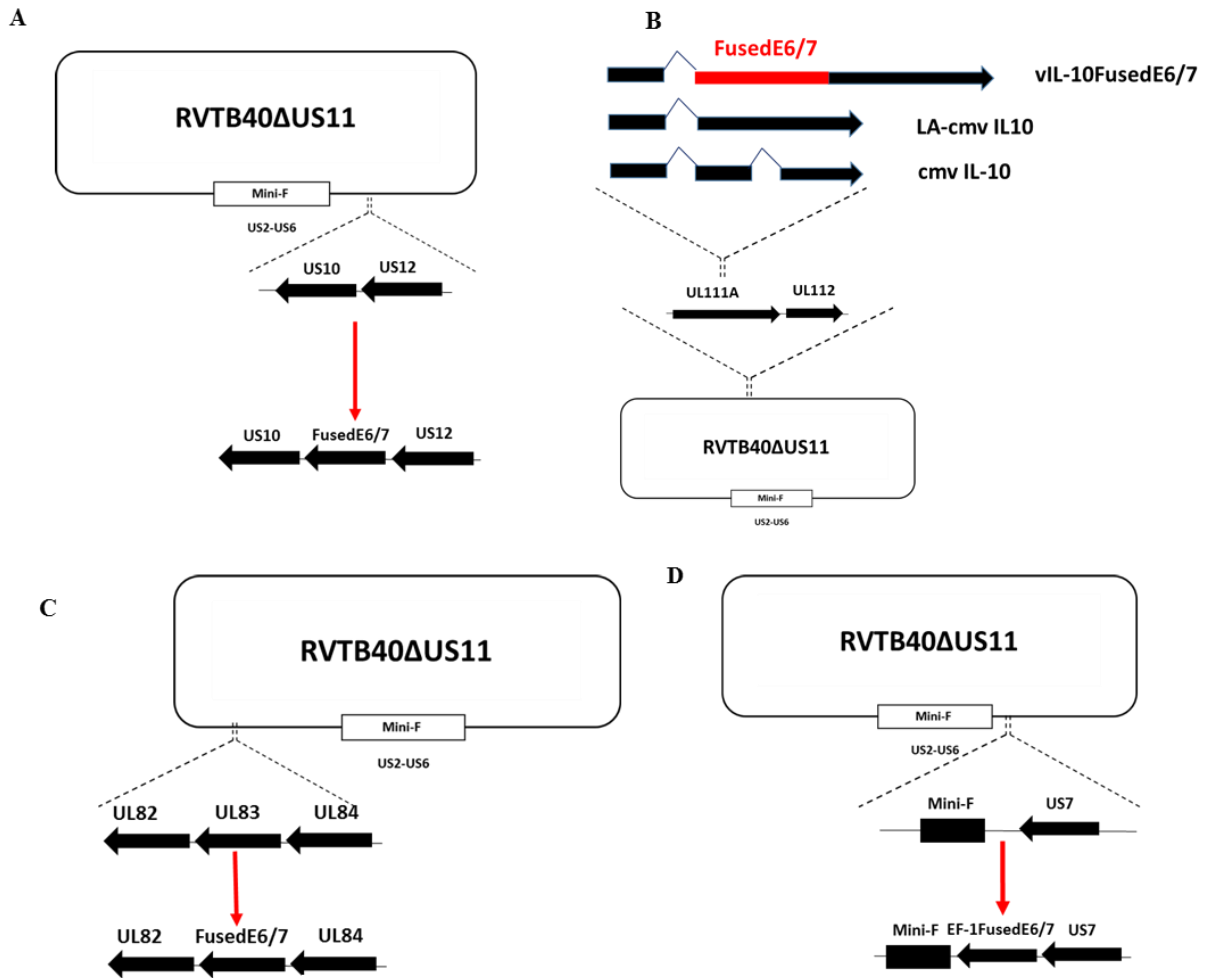


Figure 15. Construction of HCMV-based vaccines expressing the full E6/E7 protein. (A) RVTB40_E6/E7intoUS11-BAC with E6/E7 was expressed under the control of the endogenous US11 promoter. (B) UL111A has alternative splicing in lytic (cmvIL-10) and latency associated form (LA-cmvIL-10). RVTB40ΔUS11_E6/E7intoUL111A-BAC was constructed by inserting the E6/E7 fusion sequence into the second exon. This construction allows the expression of E6/E7 protein in lytic and latent HCMV infection (C) RVTB40ΔUS11_E6/E7intoUL83-BAC. The E6/E7 protein was expressed under the control of the UL83 promoter. (D) RVTB40ΔUS11_EF-1E6/E7-BAC. The E6/E7 protein was expressed under the control of exogenous EF-1 promoter, which was inserted downstream of mini-F sequence.

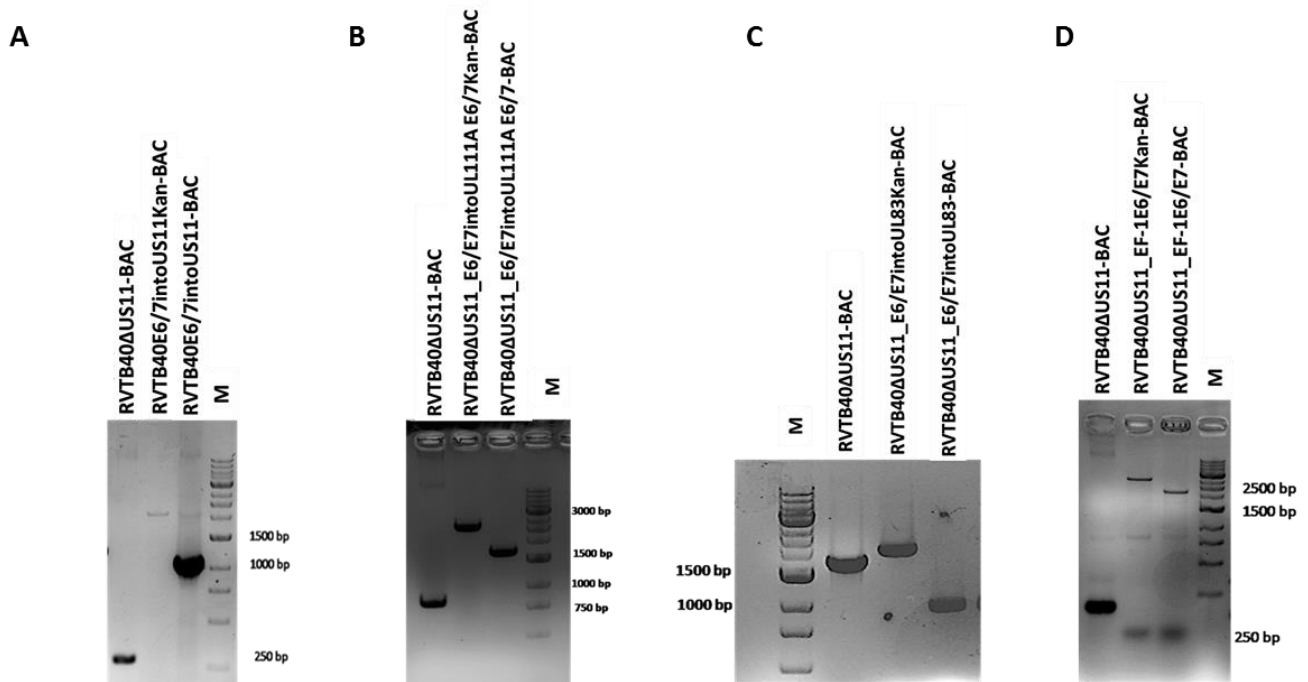


Figure 16. Confirmatory PCR for HCMV-based vaccine expressing the E6/E7 protein. PCR was carried out using primers flanking the target regions. PCR using primers SeqUS11F and R, SeqUL111F and R, SeqUL83F and R, and seqEF-1E6/E7 showing the insertion of the E6/E7 sequence in RVTB40_E6/E7intoUS11-BAC (A) RVTB40ΔUS11_E6/E7intoUL111A-BAC (B), RVTB40ΔUS11_E6/E7intoUL83-BAC (C), and RVTB40ΔUS11_EF-1E6/E7-BAC (D), respectively. The 1 kb DNA ladder GeneSTA was used (M). The amplified DNA fragments from the final mutants were sequenced using the same primers (data not shown).

3.3.1.2 Growth kinetics of HCMV-based vaccines expressing E6/E7 protein

Multi-step growth kinetics were carried out for all recombinant viruses in Fi301 cells as previously described (see Section 2.2.4.5). TCID₅₀ was determined at different time points p.i. and the results are summarized in Figure 17.

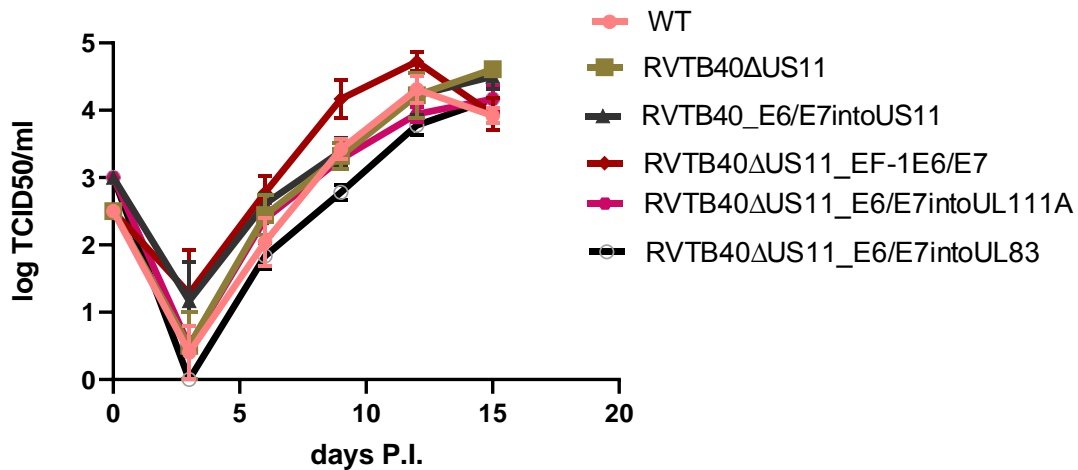


Figure 17. Growth curve kinetics of E6/E7 expressing vaccines. Fi301 cells were infected at MOI of 0.01. Supernatant was collected at different time points after infection and titrated on Fi301 cells to calculate the TCID₅₀. Results are derived from three independent experiments; error bars represent the mean \pm SEM. Results were analyzed using Two-way ANOVA and there was no significant difference among the growth curves (P value 0.1313).

3.3.1.3 Expression of E6 and E7 proteins by HCMV-based vaccines

3.3.1.3.1 Transcription analysis of E6 and E7 genes using RT-PCR

In order to confirm the transcription of E6 and E7, Fi301 cells were infected with RVTB40ΔUS11 or HCMV-based vaccines expressing E6/E7 protein (MOI=1). RNA isolation and RT-PCR were carried out as previously described (see Section 2.2.1.10). All recombinant viruses showed expression of E6 and E7. There was no expression of cmvIL-10 in RVTB40ΔUS11_E6/E7intoUL111A and UL83 in RVTB40ΔUS11_E6/E7intoUL83 as these genes have been modified or deleted in these recombinant viruses (Figure 18).

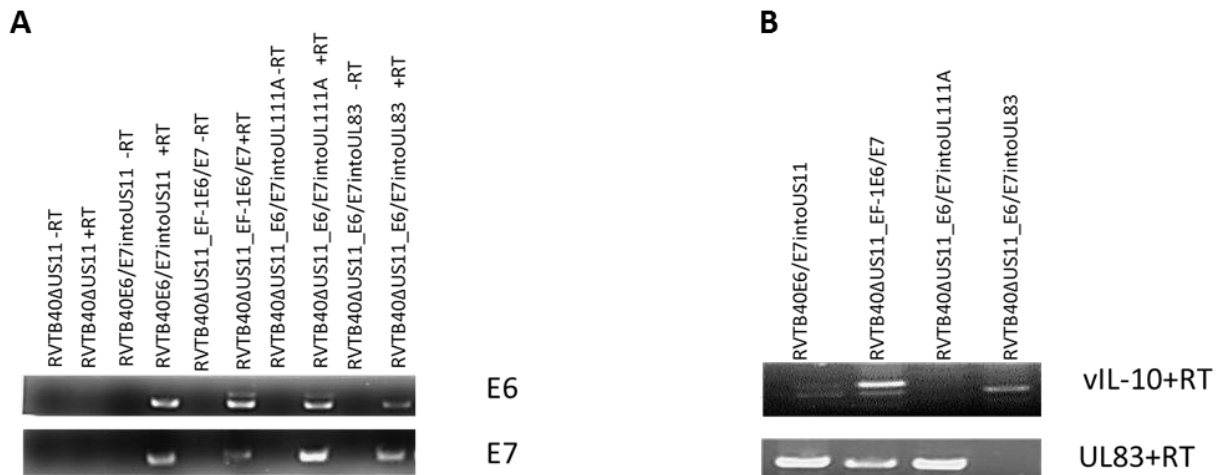


Figure 18. RT-PCR for HCMV-based vaccines expressing E6/E7 protein. 1×10^6 Fi301 cells were infected at MOI 1 and RNA was extracted after 18 hours. (A) PCR was done for E6 and E7 without reverse transcriptase (-RT) or after reverse transcriptase (+RT) using primers (E6RT F and R) for E6 and (ER7T F and R) for E7. (B) PCR was done on +RT samples for UL111A (vIL-10) using primers SeqUL111 F and R or UL83 using primers UL83RT F and R.

3.3.1.3.2 Expression analysis of E6 and E7 proteins

3.3.1.3.2.1 Detection of HPV16 E6 protein

For analyzing oncoprotein expression from HCMV mutants and pcDNAE6/E7Kpn-I U251 cells, OncoE6 Cervical Test™ kit was used to detect expression of oncoprotein E6 as previously described (see Section 2.2.5.2). Appearance of a test line indicated positive detection of oncoprotein E6.

All cells infected with recombinant viruses (MOI 1) as well as pcDNAE6/E7Kpn-I U251 cells showed high band intensity for E6 protein. Different expression levels could be distinguished by the color intensities of the test lines. The band intensity decreases in accordance with the decrease of the cell number (Figure 19).

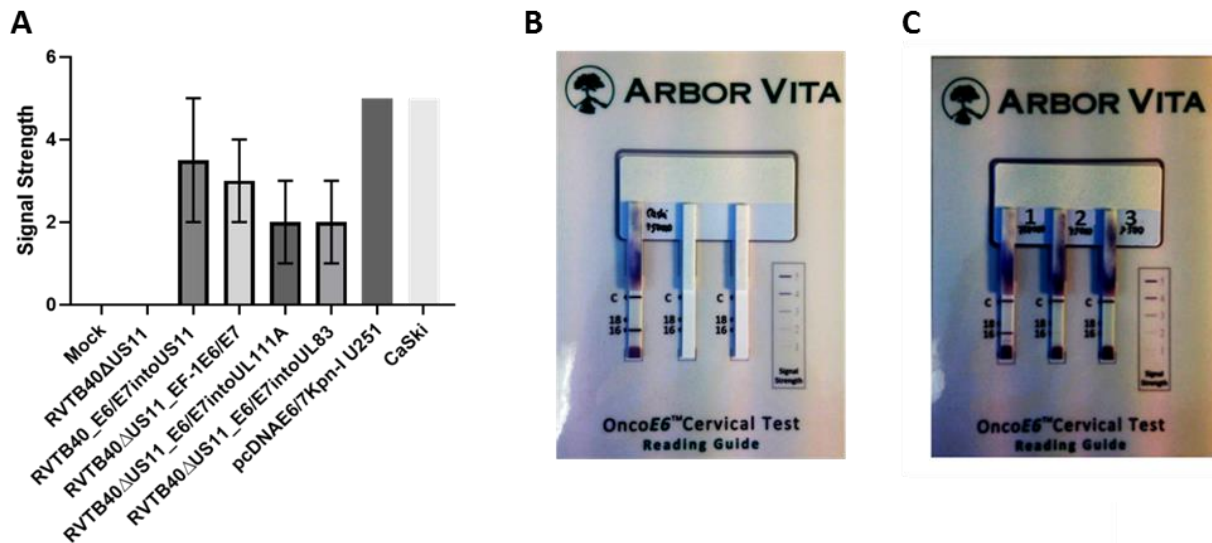


Figure 19. OncoE6 Cervical Test for HPV16 E6 detection. (A) E6 was detected in different infected Fi301 (MOI 1) or pcDNAE6/E7Kpn-I U251 (750.000 cells) with different signal strengths. The bars represent the mean of two independent experiments \pm SD. (B) the read out for the test shows the signal for same number of CaSki cells as positive control. (C) Test read-out showing different signal strength detected upon with different cells number infected with RVTB40_E6/E7intoUS11 which are 750.000 cells (lane 1 with signal strength 5), 75.000 cells (lane 2 with signal strength 1), and 7500 cells (lane 3 without detection of E6).

3.3.1.3.2.2 Expression analysis of HPV16 E7protein

In order to analyze the expression of E7 protein in recombinant HCMV or in pcDNAE6/E7Kpn-I U251 cells, recomWell HPV16/18/45 ELISA kit was used as previously described (see Section 2.2.5.1). All recombinant HCMV-expressing E6/E7 protein showed higher expression of E7 protein compared to CaSki cells which were considered the positive control, as they have integrated HPV16 genome. The results showed that recomWell HPV16/18/45 ELISA kit could be used in research to assess quantitatively the expression of HPV16 E7. The E7 ELISA results were summarized in Figure 20.

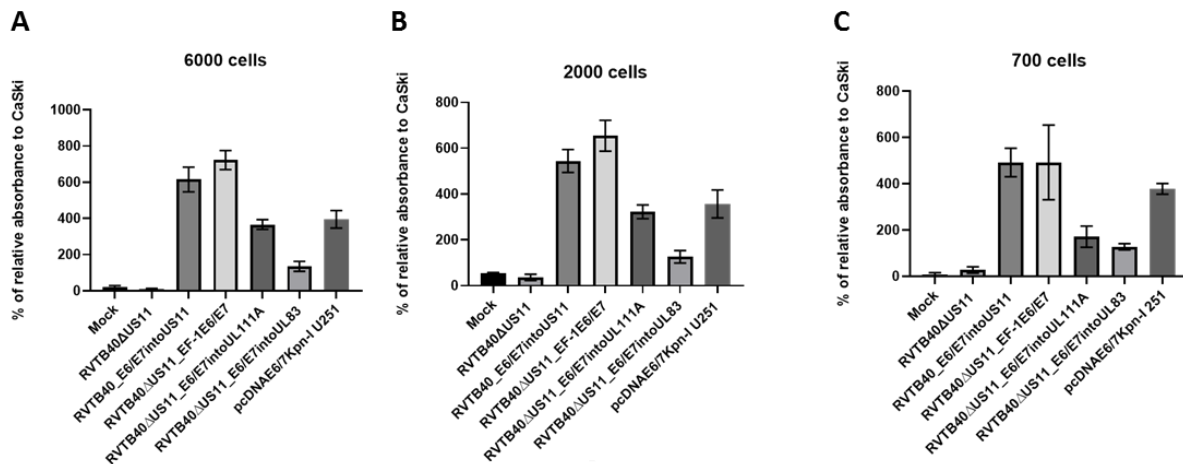


Figure 20. Quantification of HPV16 E7 protein expression by ELISA. In this assay 6×10^3 (A), 2×10^3 (B), and 0.7×10^3 (C) cells were used for each experimental group. For detection of E7 in recombinant viruses, Fi301 cells were left uninfected (Mock) or infected with HCMV (MOI 1). Afterwards, cells were collected after 48 p.i. The same cell number was also used for pcDNAE6/E7Kpn-I U251 cells. The absorbances detected for experimental probes were expressed relative to the absorbance measured for the positive control (the same number of CaSki cells). Bars represent the mean of three independent experiments \pm SEM.

3.3.2 Optimized HCMV-based vaccines expressing HPV16 E6-derived peptide

3.3.2.1 Construction of HCMV-based vaccines with C-terminus fusion of HPV16 E6-derived peptide

In order to investigate the optimum peptide presentation using HCMV-based therapeutic vaccines, the HLA-A2-binding peptide E6₂₉₋₃₈ (TIHDIILECV) derived from the E6 protein of HPV16 [265] was fused with a double Alanine (AA-) linker (AATI₂TIHDIILECV) to the C-terminus of HCMV proteins such as IE1 or UL83. These regions were selected because they have consistent expression and strong T cell recognition in lytic infection, and they have an inflammatory response in latency [14, 125].

This peptide (TIHDIILECV) was chosen as we have an established immunological assay to analyze presentation by TCR-based testing. This allows us to validate our strategy in the development of HCMV-based vaccines (see Section 3.3.4).

Insertion of E6-derived peptide was carried out by En passant mutagenesis (see Section 2.2.2.6). the DNA-encoding sequence for this peptide was already included in the mutagenesis primers, that is why no shuttle plasmids were needed to generate the PCR fragments for mutagenesis.

For this purpose, three viruses were constructed. The RVTB40ΔUS11 was utilized to construct two HCMV-based vaccines. In one of them, the peptide (AATI₂TIHDIILECV) was fused to C-

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terminus of IE1 (using primers E6PepIE1F and R); the generated BAC was named RVTB40 Δ US11_E6peptideIE1-BAC. In the other one, the same peptide was fused to C-terminus of UL83 using primers E6PepUL83F and R, and the generated BAC was named as RVTB40 Δ US11_E6peptideUL83-BAC (Figure 21).

Another BAC (RVTB40 Δ US11_E6/E7intoUL83_E6peptideIE1-BAC) was generated by fusing the E6-driven peptide to C-terminus of IE1 in RVTB40 Δ US11_E6/E7intoUL83; which had been generated on the background RVTB40 Δ US11-BAC (see Section 3.3.1.1). However, this virus is deficient for UL83 which is considered as an immunodominant protein and also plays a role in immune evasion (see Section 1.2.2) (Figure 21).

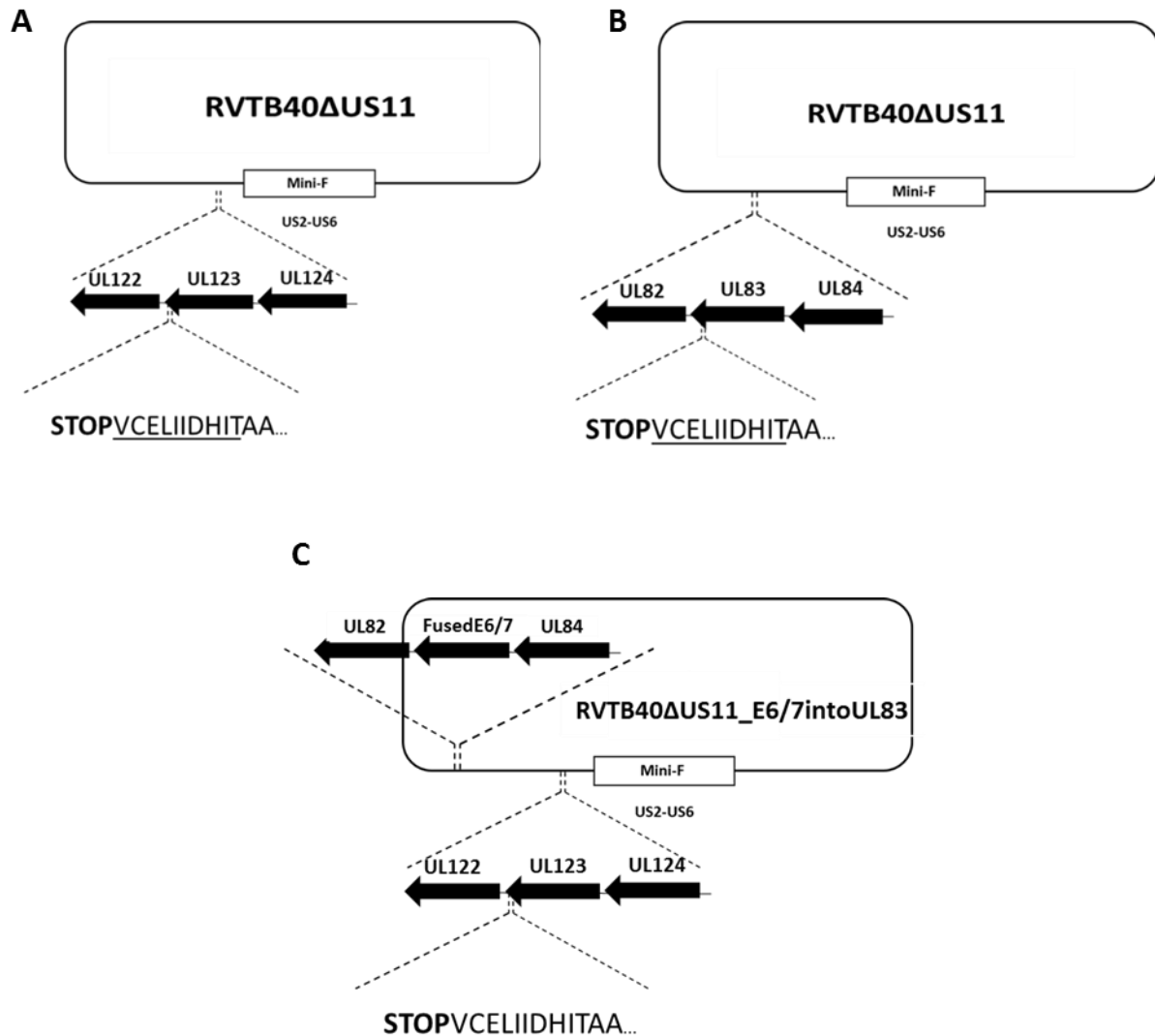


Figure 21. Construction of HCMV-based vaccines expressing E6-derived peptide. The HPV16-E6 peptide (underlined) with AA- linker was fused to C-terminus of HCMV proteins. (A) RVTB40ΔUS11_E6peptideIE1-BAC with E6 peptide fused to UL123 (IE1). (B) RVTB40ΔUS11_E6peptideUL83-BAC with E6 peptide fused to UL83 (pp65). (C) RVTB40ΔUS11_E6/E7intoUL83_E6peptideIE1-BAC with E6/E7 sequence replaced UL83(pp65) and E6 peptide fused to UL123 (IE1).

To confirm the construction of recombinant HCMV-based vaccines, PCR with primers flanking the target site was carried out (Figure 22) and the amplified fragments from final mutants were sequenced using the same primers (date not shown).

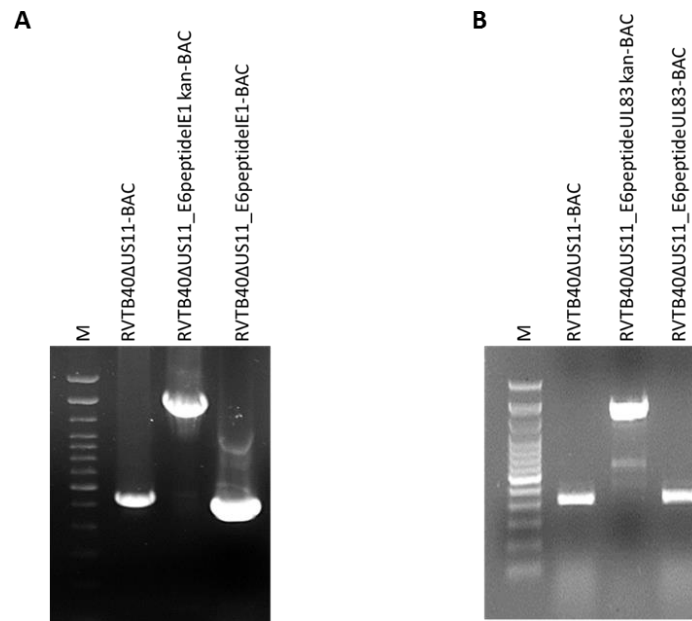


Figure 22. PCR for HCMV-based vaccine expressing E6-derived peptide. PCR was carried out using primers flanking the target regions. (A) PCR using primers SeqIE1F and R, showing the insertion of HPV16 E6-derived peptide with kanamycin cassette (RVTB40ΔUS11_E6peptideIE1 kan-BAC) and the resolution of kanamycin to get the final mutant (RVTB40ΔUS11_E6peptideIE1-BAC). (B) PCR using primers SeqUL83C-terF and R and the bands show the construction of the intermediate mutant with kanamycin (RVTB40ΔUS11_E6peptideUL83fusion kan-BAC) and the resolution of kanamycin cassette (RVTB40ΔUS11_E6peptideUL83-BAC). The 100 bp DNA ladder (Thermo Scientific) was used (M). The amplified DNA fragments from the final mutants were sequenced using the same primers (data not shown).

3.3.2.2 Growth kinetics of HCMV-based vaccines C-terminus fusion of HPV16 E6-derived peptide

Multi-step growth kinetics were carried out for all peptide-expressing viruses in Fi301 cells. The experiment was carried as previously described for E6/E7-expressing viruses (see Section 3.3.1.2) (Figure 23).

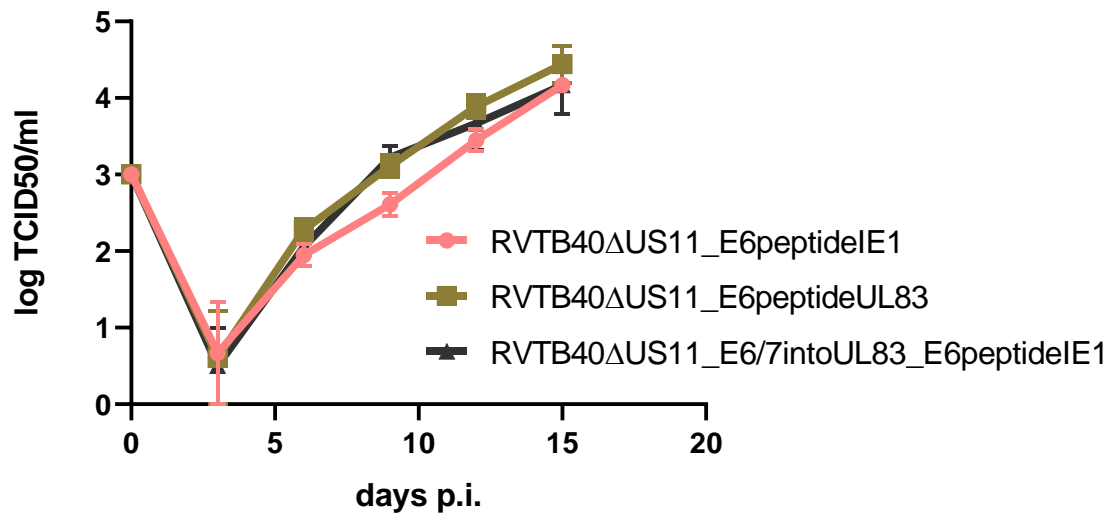


Figure 23. Growth curve kinetics of HCMV-based vaccines with C-terminus fusion of HPV16 E6-derived peptide. Fi301 cells were infected at MOI of 0.01. Supernatant was collected at different time points after infection and titrated on Fi301 cells to calculate the TCID₅₀. Results are derived from three independent experiments; error bars represent the mean \pm SEM. Results were analyzed using Two-way ANOVA and there was no significant difference among the growth curves (P value 0.9807).

3.3.3 Immuno-peptidome analysis of HCMV-based vaccines

In order to investigate the heterogeneous antigen presentation of HCMV-based vaccines, we analyzed the peptides processed and presented by MHC-I and MHC-II molecules as previously described (see Section 2.2.4.6). To gain insight into this, Fi301 cells were left uninfected or infected with HCMV-based vaccine candidates (RVTB40ΔUS11_EF-1E6/E7 and RVTB40ΔUS11_E6/E7intoUL83_E6peptideIE1) at MOI 1 (Figure 24).

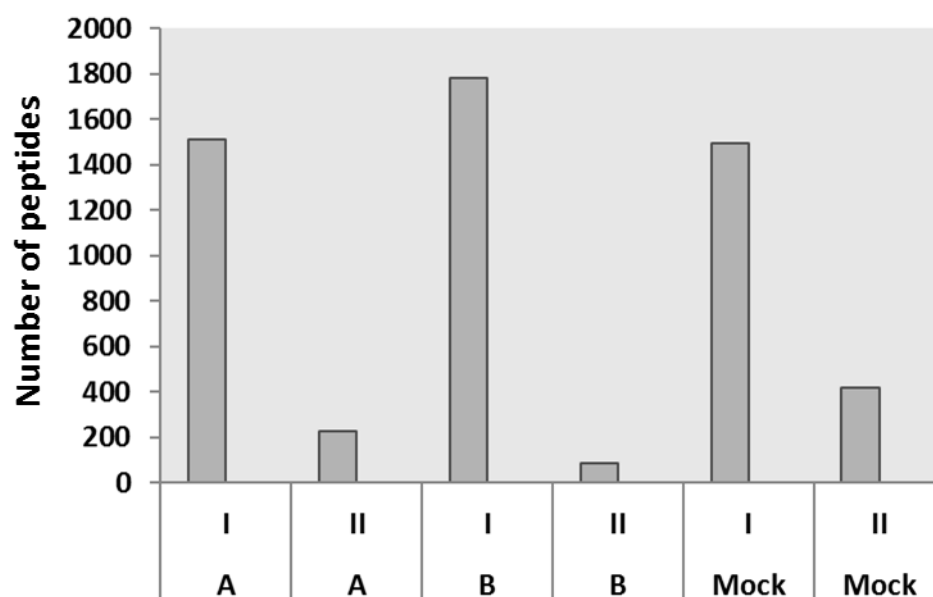


Figure 24. Overview of the identified peptides upon HCMV-based vaccine infection. The number of the derived peptides for MHC-I (I) and MHC-II (II) were analyzed by mass spectroscopy. Fi301 cells were left uninfected (Mock) or infected with RVTB40ΔUS11_EF-1E6/E7 (A) or RVTB40ΔUS11_E6/E7intoUL83_E6peptideIE1 (B). The bars represent the mean of three technical replicates.

Interestingly, the HCMV-derived MHC-I peptides of Fi301 cell were much higher in number upon infection with RVTB40ΔUS11_EF-1E6/E7 than RVTB40ΔUS11_E6/E7intoUL83_E6peptideIE1. However, in both cases no HPV16 peptides could be detected. The identified viral peptides were analyzed as previously described (see Section 2.2.4.6) and summarized in the Table 22.

RVTB40ΔUS11_EF-1E6/E7			
Sequence	Protein group accessions	Best NetMHC allele rank	Gene name
QYDPVAALF	P06725	A2402	UL83 (pp65)
LLIDPTSGLLGA	P16789	A0201	UL98
TLINGVWVV	P09717	A0201	US16
GLLAHIPAL	P09717	A0201	US16
YPRPPGSGSL	P16757	B0702	UL16
ALFNQLVFTA	P16812	A0201	UL34
FQIGHTDSV	P16812	A0201	UL34
TILDKILNV	P16729	A0201	Major capsid protein
LYILLPTL	P09728	C0702	US10
QYAEGLRQL	P16725	C0702	UL76
LLINTGITV	P69334	A0201	US18
YLLEQIQNL	P16732	A0201	Tripartite terminase subunit 3
YVLPDIDVGV	P09700	A0201	US24
AYEYVDYLF	P06473	A2402	gB
ALFSFGVQV	P09729	A0201	US9
YLFEGQYSTI	P17149	A0201	UL70

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SPRFSSPAEF	P17147	B0702	Major DNA-binding protein
SLYADPFFL	P16736	A0201	DNA replication helicase
TRLFLSHVEV	P16736	B2705	DNA replication helicase
VYSPVVESL	P16781	A2402	UL43
LLDDVPPHV	Q7M6R1	A0201	UL21A
FTDNVRFVS	P16724	A0201	Tripartite terminase subunit 1
LYSTNFLTL	P16849	A2402	UL33
RLLDLTQMV	P16784	A0201	UL47
SPSRDRFVQL	P16793	B0702	UL52
RVTB40ΔUS11_E6/E7intoUL83_E6peptideIE1			
Sequence	Protein group accessions	Best NetMHC allele rank	Gene name
VLAELVKQI	P13202	A0201	UL123 (IE1)
YPRPPGSGL	P16757	B0702	UL16
LYILLPTL	P09728	C0702	US10
VYLPKDAFF	P16753	A2402	UL80
LLIDPTSGLLGA	P16789	A0201	UL98
LLINTGITV	P69334	A0201	US18
SRLKLVLSF	P16776	B2705	UL5

Table 22. Immunopeptidomic analysis of HCMV-based vaccines of infected Fi301 cells. HPV16 E6- or E7-derived peptides were not detected from infected Fi301 cells with RVTB40ΔUS11_EF-1E6/E7 or RVTB40ΔUS11_E6/E7intoUL83_E6peptideIE1.

3.3.4 Immunological assays

In order to establish the platform for HCMV-based vaccine development, two different immunological assays were carried out based on specific TCR stimulation for HLA-A2 peptides (E6₂₉₋₃₈: TIHDIILECV and pp65₄₉₅₋₅₀₃: NLVPMVATV). Infection of Fi301 cells and stimulation of PBMCs transduced with specific TCRs could simulate the eventual clinical application of these vaccines for treatment of HPV16-induced cancer. Furthermore, infection of GBM cells with these vaccines could show whether these vaccines would be useful for the treatment of GBM by delivering neoantigens (such as E6 and pp65 peptides) which could render these tumor-driving cells vulnerable to cytotoxic attack by specific CTLs.

3.3.4.1 IFN- γ release in response to E6 and pp65 peptide for Fi301 infected with HCMV-based vaccines

Human PBMCs isolated, as previously described (see Section 2.2.3.5), were transduced with either retroviral vector encoding E6-specific TCR (pMP71-PRE-E6) or retroviral vector encoding pp65-specific TCR (MP71TCR α and MP71TCR β) as previously described (see Section 2.2.6.2). Untransduced PBMCs were included as a negative control and treated in the same way. In order to analyze the T cell stimulation, IFN- γ was measured as previously described (see Section 2.2.6.2). Vaccine-infected Fi301 cells loaded exogenously with the

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corresponding E6-derived or pp65-derived peptides were also co-cultured with the transduced PBMCs as a positive control to give the maximum stimulation for IFN- γ release. Although infected Fi301 with E6/E7 expressing HCMV mutants could stimulate specific T cells for pp65 (except for RVTB40 Δ US11_E6/E7intoUL83 that lacks pp65), none of HCMV-based vaccines expressing E6/E7 protein could stimulate E6-specific T cells. Only RVTB40 Δ US11_EF-1E6/E7-infected Fi301 showed slight stimulation for E6-specific T cells (Figure 25).

Interestingly, all HCMV-based vaccines expressing HPV16 E6-derived peptide could strongly stimulate E6-specific T cells for IFN- γ release. Moreover, all of them could also stimulate pp65-specific T cells for IFN- γ release, except for RVTB40 Δ US11_E6/E7intoUL83_E6peptideIE1 which is pp65-deficient mutant (Figure 25).

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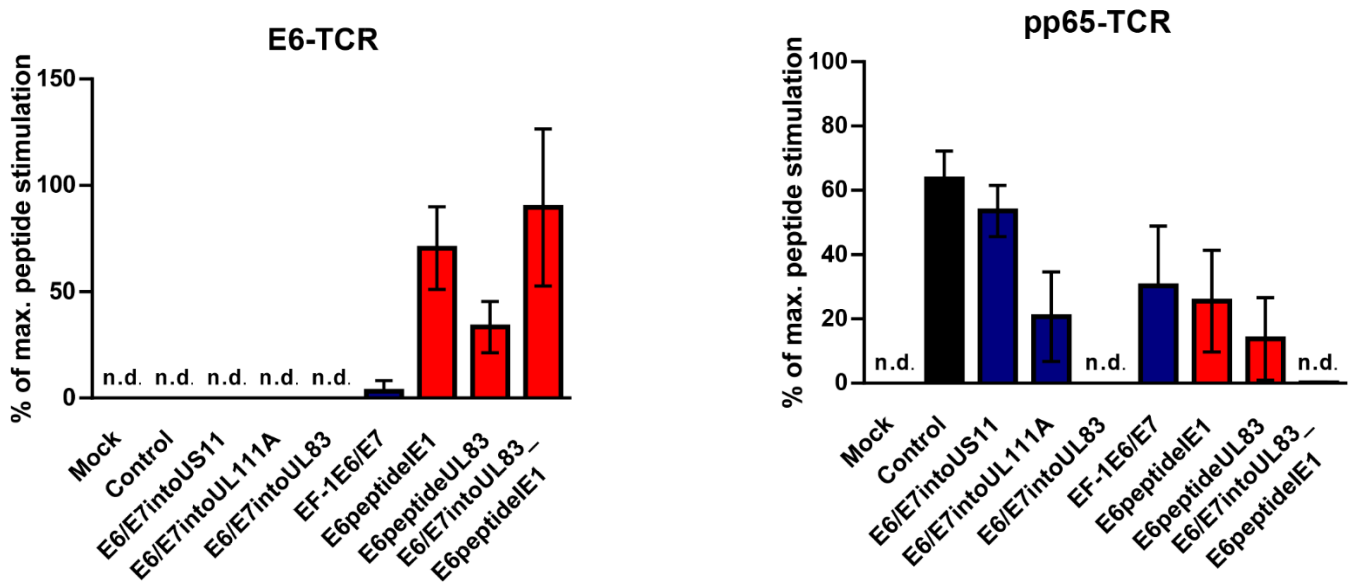


Figure 25 Release of IFN- γ by TCR transduced PBMCs after stimulation with infected cells. 1×10^5 human PBMCs, transduced with HPV E6-specific TCR and HCMV pp65-specific TCR, respectively, or non-transduced (Background), were co-cultured for 16 hours with 1×10^5 Fi301 cells that had been infected for 48 hours with HCMV-based vaccines. PBMCs as described above were also co-cultured with infected Fi301 cells that had been additionally pulsed with the corresponding peptide (maximal peptide stimulation). Subsequently, IFN- γ production was measured by ELISA. Release of IFN- γ is shown as percentage of maximal peptide stimulation after subtraction of the background. Uninfected cells (Mock) and cells infected with RVTB40 Δ US11 (Control) were also included in this type of analysis. Results are derived from three experiments; error bars represent the mean \pm SEM; n.d., not detectable.

3.3.4.2 Stimulation of transduced Jurkat cell with specific TCRs

Three HLA-A2 GBM cell lines were included in this study to analyze the role of HCMV-based vaccine in rendering the GBM susceptible for recognition and attack by specific CTLs. As previously described (see Section 2.2.4.4), the cell lines (U343, LN18 and U251) were infected with HCMV-based vaccines at MOI 5 and reporter Jurkat cell lines were used as a read out of T cell activation.

3.3.4.2.1 Detection of NF- κ B activation upon Jurkat cell line stimulation

Jurkat cell line (Jurkat E6-1) with EGFP expression under the control of NF- κ B [261] was transduced with retroviral vector encoding HLA-A2-restricted HPV E6₂₉₋₃₆-specific TCR or with retroviral vector encoding the HCMV-specific TCR recognizing an HLA-A2-restricted epitope derived from pp65 (pp65₄₉₅₋₅₀₃) as previously described (see Section 2.2.6.2). All HCMV-based vaccines expressing pp65 showed pp65-specific Jurkat cells stimulation, whereas a HCMV-based vaccine lacking pp65 could not (Figure 26). However, only the

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vaccines expressing the E6-derived peptide in fusion to viral protein could significantly stimulate E6-specific Jurkat cell lines (Figure 26).

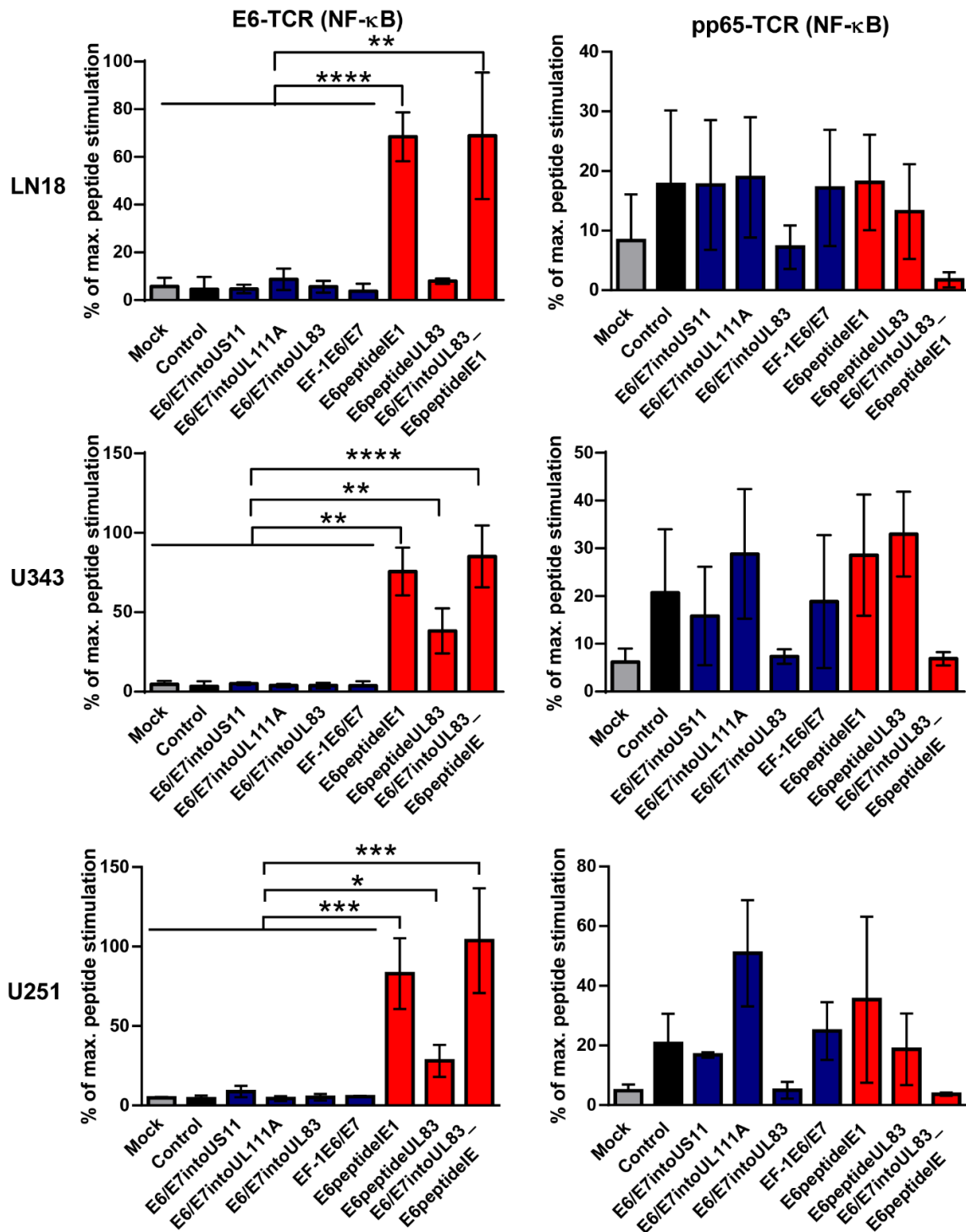


Figure 26. NF-κB-driven EGFP expression in reporter cell lines stimulated by infected GBM cells. 5×10^4 GBM cells (LN18, U343 or U251 cells) were infected with HCMV-based vaccines (MOI of 5). After 2 days and 4 days, respectively, infected cells were co-cultured with HPV E6-specific reporter cells (left graphs) and HCMV pp65-specific reporter cells (right graphs), respectively, for 24 hours at a ratio 2:1. Subsequently, EGFP expression of reporter cells was determined by FACS analysis. Uninfected cells (Mock) and cells infected with RVTB40ΔUS11 (Control) were also included in this type of analysis. Stimulation of reporter cells is given as percentage of maximal peptide stimulation, i.e. stimulation of reporter cells incubated with peptide pulsed cells. Results are derived from three technical replicates; error bars represent the mean \pm SEM. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, one-way ANOVA test.

3.3.4.2.2 Detection of NFAT activation upon Jurkat cell line stimulation

The J76 cell with NFAT-driven EGFP [259] was transduced with a retroviral vector encoding HLA-A2-restricted HPV E6₂₉₋₃₆-specific TCR (pMP71-PRE-E6) or HLA-A2-restricted HCMV pp65₄₉₅₋₅₀₃-specific TCR (MP71TCR α and MP71TCR β) [259] as previously described (see Section 2.2.6.2). Infected GBM cell lines were infected and handled as described for NF- κ B assay (see Section 3.3.4.2.1).

Although the detection of NFAT activation was less sensitive than NF- κ B [258], similar results were obtained. Only HCMV-based vaccine expressing E6-derived peptide fused to viral protein stimulated E6-specific Jurkat cell lines (Figure 27).

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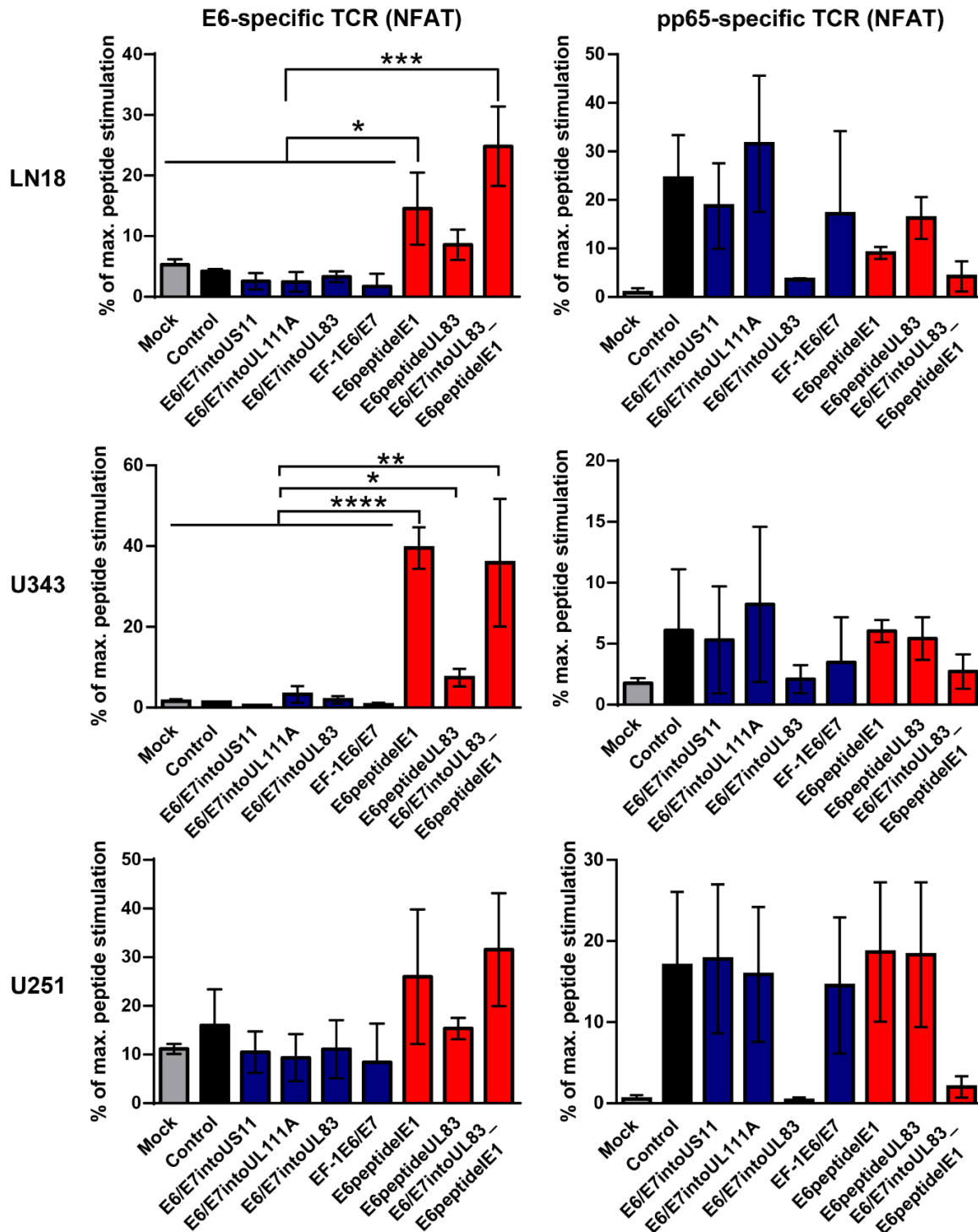


Figure 27. NFAT-driven EGFP expression in reporter cell lines stimulated by infected GBM cells. 5×10^4 GBM cells (LN18, U343 or U251 cells) were infected with HCMV-based vaccines (MOI of 5). After 2 days and 4 days, respectively, infected cells were co-cultured with HPV E6-specific reporter cells (left graphs) and HCMV pp65-specific reporter cells (right graphs), respectively, for 24 hours at a ratio 2:1. Subsequently, EGFP expression of reporter cells was determined by FACS analysis. Uninfected cells (Mock) and cells infected with RVTB40 Δ US11 (Control) were also included in this type of analysis. Stimulation of reporter cells is given as percentage of maximal peptide stimulation, i.e. stimulation of reporter cells incubated with peptide pulsed cells. Results are derived from three technical replicates; error bars represent the mean \pm SEM. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, one-way ANOVA test.

3.3.4.2.3 A novel HCMV-encoded block of MHC-I presentation

The GBM-infected cells with E6/E7-expressing recombinant HCMV vaccine candidate failed to stimulate E6-specific T cells despite abundant E6/E7 protein expression was surprising. To investigate this mechanism, aliquots of pcDNAE6/E7Kpn-I U251 cells, which stably express the E6/E7 protein, were left uninfected or RVTB40ΔUS11-infected at different MOIs with the HCMV-vector. Thereafter, cells were acid washed as described previously (see Section 2.2.6.5). Subsequently, cells were co-cultured with HPV E6₂₉₋₃₆-specific reporter cells, which express EGFP under the control of NF-κB responsive elements [263]. Figure 28 shows that pcDNAE6/E7Kpn-I U251 cells (positive control) but not untransfected U251 cells (negative control) stimulated E6 peptide-specific reporter cells. Interestingly, acid washed pcDNAE6/E7Kpn-I U251 cells that had been infected with different MOIs of the HCMV-vector showed a significantly reduced capacity to stimulate E6-specific reporter cells as compared to acid washed uninfected pcDNAE6/E7Kpn-I U251 cells (Figure 28). After additional pulsing with exogenous E6 peptide, however, acid washed pcDNAE6/E7Kpn-I U251 cells that had been infected could stimulate E6-specific reporter cells to a similar extent as acid washed uninfected pcDNAE6/E7Kpn-I U251 cells (Figure 28). Strikingly, the block of MHC-I antigen presentation induced by the HCMV-vector was more than 50% (Figure 28). Taken together, these results showed a previously unsuspected HCMV-encoded block of MHC-I presentation.

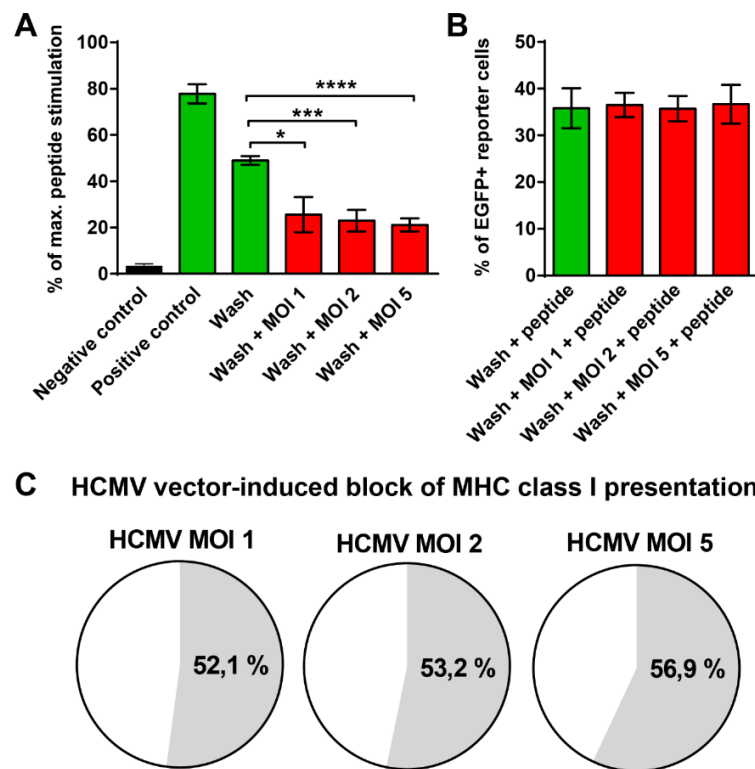


Figure 28. Block of MHC-I presentation induced by immune evasions-deficient HCMV. U251 cells stably expressing the E6/E7 protein (pcDNAE6/E7Kpn-I U251 cells) were left uninfected or infected with RVTB40ΔUS11, a mutant HCMV lacking all known viral MHC-I blockers, at the indicated MOIs for 3 to 24 hours. Subsequently, cells were harvested, washed with PBS and subsequently washed with ice-cold citric acid, to remove all preexisting peptide-MHC complexes and co-cultured at a ratio of 2:1 with HPV E6-specific reporter cells, in which NF-κB drives EGFP. After 18 hours EGFP expression was assessed by FACS analysis. Unwashed U251 cells (Negative control) and unwashed pcDNAE6/E7Kpn-I U251 cells (Positive control) were also co-cultured with HPV E6-specific reporter cells. In parallel, maximal peptide stimulation was determined for each experimental group by pulsing cells additionally with E6 peptide (1 μg/ml) before co-culture with HPV E6-specific reporter cells and subsequent FACS analysis. (A) The stimulation in each experimental group is given as percentage of maximal peptide stimulation. (B) The % of EGFP+ reporter cells after pulsing with E6 peptide (maximal peptide stimulation) is shown for washed pcDNAE6/E7Kpn-I U251 cells left uninfected and washed pcDNAE6/E7Kpn-I U251 cells infected with mutant HCMV at the indicated MOIs. (C). The block of MHC class I presentation after infection with mutant HCMV at the indicated MOIs is given as a percentage. The results shown are derived from three independent experiments. Error bars represent the mean ± SEM (****, P < 0.0001; ***, P < 0.001; *, P < 0.05; unpaired t-test).

4. Discussion

4.1 Optimization of HCMV for vaccine vector construction

The ability of CMV to elicit and maintain high frequencies of effector memory CTLs, makes CMV-based vectors promising to develop novel prophylactic and therapeutic vaccines against infectious diseases as well as cancer [266]. This study initially aimed to generate an optimized HCMV vector that can be eventually used as a platform for generating therapeutic vaccines that induce strong CTL-mediated immunity against a target antigen derived from a pathogen or tumor.

4.1.1 RL13 and UL128 mutations are crucial for HCMV reconstitution and virus propagation in fibroblasts

The most promising HCMV strains for the generation of vaccine vectors should be able to infect DCs and establish latency *in vivo* in order to elicit and maintain potent memory inflation. Simultaneously, these vectors should be safe and amenable to the genetic modifications essential for the insertion of heterologous antigens [266].

The circular mini-F BAC plasmids allow HCMV or other herpesviruses genomes to be stably maintained at low copy number and manipulated in *E. coli* and then reconstituted as infectious virus by transfection of eukaryotic cells [267]. So far, most HCMV research is based on the laboratory strains, i.e. AD169 and Towne, which have major disadvantages [268]. It has been reported, that these HCMV strains show many mutations due to extensive passaging in fibroblasts. The main genomic regions affected are the RL13 and UL128 locus [245, 269]. These mutations can limit the ability to infect endothelial cells and DCs and hence restrict the memory inflation of CTLs [264]. With this in mind, the BAC of the clinical HCMV strain Merlin was selected initially to generate the vaccine vector. Merlin was derived from the urine of a congenitally infected infant and sequenced after 3 passages in human fibroblasts [245, 270].

During the construction of Merlin strain BAC, mutations that affect HCMV coding regions were repaired by mutagenesis and recombineering. The virus recovered from BACs by DNA transfection harbors only a residue of single *loxP* and *NheI* sites (40 bp) located between US28 and US29. However, the presence of this sequence does not show any effect on the expression of the flanking genes [245].

The HCMV Merlin BAC (RCMV1161-BAC), which had been repaired for both RL13 and UL128 was used. The acquisition of mutations in both RL13 and UL128 may have resulted

either from sequential mutations in the same template or as a result of recombination between independent mutants [245]. Several mutants were constructed using RCMV1161-BAC to delete all MHC-I downregulating genes (US2, US3, US6, and US11). However, none of these mutants could be reconstituted and propagated in fibroblasts. These findings were in accordance with a previous study showing that RL13 and UL128 restrict either HCMV cell-to-cell transmission or production of cell-free virus in fibroblasts [245].

4.1.2 Deletion of HCMV-encoded MHC-I downregulating genes improves antigen presentation

The failure to reconstitute RCMV1161-BAC prompted us to use HCMV TB40/E strain which combines the high endothelial cell and DCs tropism of a clinical isolate with the high titer growth of a cell culture adapted strain [23, 271]. Interestingly, the mini-F sequence of the corresponding BAC (TB40-BAC4) was inserted into to the viral genome, replacing the US2-US6 region [271]. In a previous study, DCs infected with RVTB40/E Δ US11 stimulated T cells to produce more IFN- γ than DCs infected with TB40/E WT although both infected DCs equally well [272].

Although HCMV strain TB40/E also shows mutations in both RL13 and UL128L these stable mutations did not prevent the virus from being propagated in fibroblasts while retaining epithelial cell tropism [268, 273]. These results suggested that TB40-BAC4 is well suited for generation of vaccine vectors. For further optimization, the US11 gene was deleted and the BAC generated (RVTB40 Δ US11-BAC) was successfully reconstituted in fibroblasts. Although US11-deleted HCMV has been generated previously [272], we aimed to generate this mutant in our laboratory and to characterize it for further clinical applications. The resulting mutant lacks US2-6 and US11, which account for the MHC-I downregulation observed in HCMV-infected cells. However, a previous study showed that these genes do not alter the expression levels of other immunologically relevant molecules such as MHC-II, CD40, CD80, CD86 and CD83 [272]. Although fibroblasts infection by TB40/E WT downregulate MHC-I molecules, the analysis of MHC-I expression in RVTB40 Δ US11-infected Fi301 cells showed that the downregulation effect mediated by US2, US3, US6, and US11 was abolished upon deletion of these genes.

The HCMV mutant RVTB40 Δ US11 could replicate in fibroblasts and showed growth kinetics similar to TB40/E WT. This finding demonstrates that US2-6 and US11 are not essential for viral reconstitution and propagation in cell culture and further confirm the findings of the previous studies [272].

The CMV-encoded MHC-I downregulating genes could totally abrogate the development of specific CTL response against heterogenous antigens in rhesus macaques using RhCMV-based vaccines against Ebola [162]. However, this can be also due to another CMV-encoded block of MHC-I antigen presentation (see Section 4.2.1).

4.2 Construction of HCMV-based vaccine against HPV16-induced cancer

It is estimated that HPV-related cancers account for 7-8 % of all human cancers [181]. Although several prophylactic vaccines against HPV have been generated and are being used worldwide [274], there is no approved therapeutic vaccine against HPV-induced cancer [275]. In the last few years, several immunotherapeutic approaches have been explored, however, therapeutic vaccines are very promising as a treatment option for HPV-induced cancer [276]. In this work, we decided to generate a therapeutic vaccine against HPV-induced cancer using our optimized HCMV vector (RVTB40 Δ US11).

4.2.1 HCMV-based vaccine expressing E6/E7 protein does not present peptides derived from E6/E7

As a DNA virus, HPV is relatively stable [277]. Only a limited number of genotypes are implicated in HPV-induced cancers, HPV16 being the most prevalent [278, 279]. HPV-encoded E6 and E7 are the main conserved oncogenes responsible for cell transformation and carcinogenesis. After HPV-induced cell transformation, the cancer express HPV-derived E6 and E7 oncogenes that are both of low diversity, making them ideal targets for immunotherapy. [280, 281]. A fused DNA consensus sequence encoding non-transforming fusion protein of HPV16 E6/E7 that covers all relevant antigenic peptides was synthesized following the published sequence [282]. In a previous study, the mice vaccinated with this DNA vaccine encoding this fused protein showed both CD4⁺ T cells and CTL responses against the encoded antigens with prophylactic and therapeutic effects [282].

Several HCMV BACs encoding the E6/E7 protein were constructed during this study. However, only four of them could reconstitute virus and were thus examined further. The resulting HCMV-based vaccines differ in the expression characteristics of E6/E7 protein due to different promoter activities that drive E6/E7 expression. For example, the RVTB40E6/E7intoUS11 and RVTB40 Δ US11_EF-1E6/E7 mutants, expressed high level of E6/E7 protein under the control of endogenous and exogenous promoters, respectively. Some other major immune evasion genes were interrupted or deleted in other vaccine candidates, such as RVTB40 Δ US11_E6/E7intoUL111A and RVTB40 Δ US11_E6/E7intoUL83.

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The HCMV RVTB40_E6/E7intoUS11 mutant shows a minimal change to the parent RVTB40 Δ US11 genome by replacing US11 with the E6/E7 sequence. This virus can express the E6/E7 protein under the control of the US11 promoter, which is activated in the early phase of infection [283]. In fact, several mammalian promoters are commonly used in cell culture to generate stable and potent expression of genes. One of these promoters, the EF-1 α promoter, is constitutively active in a broad range of cell types [284, 285]. Moreover, several studies showed that EF-1 α promoter is more resistant to silencing than other viral promoters [286-288]. The HCMV RVTB40 Δ US11_EF-1E6/E7 mutant was constructed to express E6 and E7 under the control of exogenous promoter EF-1 α [286-288]. Our results showed that the TB40-BAC4 can tolerate insertion of large DNA fragments up to 2200 bp (the EF-1 α promoter and fused E6/E7 genes) with successful reconstitution of these modified BACs.

During HCMV latency, only a subset of viral genes are transcriptionally active [109, 289-292], including HCMV UL111A (cmvIL-10), which encodes a protein that is a homolog of the potent immunomodulatory cytokine hIL-10. UL111A is transcriptionally active during both productive and latent HCMV infection and encodes several viral IL-10 proteins according to alternative splicing of the transcribed RNA [107, 109, 110, 116]. UL111A-encoded protein mediates different immunomodulatory functions that support viral latency, including inhibition of cytokine synthesis, MHC-I molecule modulation in infected cells, suppression of B cell stimulation during latency, and hindering DCs maturation and cytotrophoblast function [112, 117, 293, 294]. It has been shown that cmvIL-10 expression during viral latency in primary human myeloid progenitor cells is implicated in modulation of MHC-II levels which results in inefficient recognition of the infected cells by allogeneic or autologous CD4⁺ T cells [117]. The HCMV RVTB40 Δ US11_E6/E7intoUL111A mutant was generated through insertion of the E6/E7 sequence in frame with the second exon, making it translationally active in both lytic and latency phases [116, 295].

HCMV UL83-encoded protein (pp65) is one of the most abundant tegument proteins, however it is not essential for viral growth and production of infectious virions *in vitro* [296]. Moreover, it is implicated in several immunomodulatory features in infected individuals that counteract both innate and adaptive immune responses against HCMV infection [54, 297, 298]. We have successfully generated a pp65-deficient vaccine candidate, RVTB40 Δ US11_E6/E7intoUL83. In this mutant, UL83 was replaced in frame by the E6/E7 sequence. UL83 has an early-late expression profile in lytic infection cycles and it is expressed in latency as well. It has also an

4. Discussion

immune evasion action by blocking antigen presentation [21, 298], modulating NK cells functions [299] and suppressing several antiviral cytokines [300-302].

For HPV16 infection diagnosis, previous studies have shown that western blot (WB) as well as ELISA can be used [303]. However, WB of E6 and E7 proteins showed either a low sensitivity or specificity when compared to other diagnostic tools such as Hybrid Capture 2 (HC2) test and ThinPrep cytological test (TCT), respectively [304]. In this study, the expression of both HPV16 E6 and E7 was analyzed for all recombinant viruses after the infection of Fi301 cells. In addition, these tests were used to determine expression of the E6/E7 protein in stably transfected cells with E6/E7 protein expression plasmid (pcDNAE6/E7Kpn-I U251 cells) as a positive control. Our results showed that all these mutant viruses can express the E6/E7 protein with different expression levels. As shown in results, the recomWell HPV16/18/45 ELISA kit can be used in research to assess quantitatively the expression of HPV16 E7. On the other side, OncoE6 Cervical Test™ kit can be used for semi-quantitative detection of HPV16 E6 in research assays. Taken together, these assays showed sensitivity to E6 and E7 despite low cell numbers and could be a useful tool for *in vitro* evaluation of HPV16 E6 and E7 protein expression.

Interestingly, none of the fused HPV16 E6/E7-expressing HCMV mutants stimulated Jurkat or PBMCs transduced by E6₂₉₋₃₆-specific TCR. In contrast, all pp65-encoding HCMV mutants stimulated pp65₄₉₅₋₅₀₃-specific TCR transduced Jurkat or PBMCs. As a positive control, uninfected pcDNAE6/E7Kpn-I U251 cells stimulated E6₂₉₋₃₆-specific reporter Jurkat cells.

In a previous study exploring MCMV-based therapeutic vaccine against HPV-induced cancer, it was proposed that the exact position of the immunogenic peptide in the viral protein is crucial for peptide processing and presentation [205]. Our results showed that pcDNAE6 /E7Kpn-I U251 cells stimulated the specific Jurkat cells while HCMV-expressing the same form of fused E6 /E7 did not. We conclude that HCMV impairs presentation of heterologous (recombinant, non-HCMV) proteins in a selective way. This mechanism is independent on MHC-I downregulation because all MHC-I downregulating genes were deleted from our vector. The underlying mechanism, however, is unknown and needs to be researched. We also assume that this blocking mechanism is involved in the failure of RhCMV-based vaccine expressing Ebola virus glycoprotein, a heterologous protein, to drive Ebola-specific CTLs stimulation [162].

4.2.2 HCMV-based vaccines expressing E6 peptide fused to viral protein stimulate E6-specific T cells

In previous studies, MCMV-based vaccines were able to stimulate CTLs against heterologous antigens (such as influenza and HPV16 antigens) with the antigenic peptides were fused to viral proteins, resulting in protective and therapeutic responses when animals were vaccinated and challenged with pathogenic viruses expressing the same antigens [158, 205]. Although these vaccine models were based on MCMV, we constructed recombinant HCMV-based vaccines using the same strategy. We generated three HCMV-based vaccines expressing the antigenic peptide fused to C-terminus of viral proteins (IE1 and pp65) with a double Alanine (AA-) linker that facilitates cleavage and processing of the immunogenic peptide.

As shown in results, the fusion of the HPV16 E6-derived peptide to the C-terminus of HCMV IE1 stimulated specific CTLs to secrete higher levels of IFN- γ than HCMV with peptide fused to C-terminus of pp65. However, replacing UL83 with fused E6/E7 protein and fusing the HPV16 E6-derived peptide to the C-terminus of IE1 (RVTB40 Δ US11_E6/E7intoUL83_E6peptideIE1) did not show better stimulation than by just fusing the HPV16 E6-derived peptide to C-terminus of IE1 (RVTB40 Δ US11_E6peptideIE1). We conclude that expressing the immunogenic peptide at the C-terminus of HCMV proteins such as IE1 and pp65 is essential for efficient MHC-I presentation, and this is not improved by additional expression of the full-length protein (fused E6/E7).

We also performed a ligandome analysis for HCMV-infected Fi301 cells with RVTB40 Δ US11_EF-1E6/E7 and RVTB40 Δ US11_E6/E7intoUL83_E6peptideIE1 mutants, which showed several HCMV-derived peptides. However, peptides derived from the E6/E7 fusion protein were not detected for either mutant viruses although Fi301 cells infected with RVTB40 Δ US11_E6/E7intoUL83_E6peptideIE1 stimulated E6-specific T cell. This finding demonstrates the higher sensitivity of reporter T cells as compared to detection of mass spectrometry, although it could also be that the HPV16 E6-derived peptide (TIHDIILECV), a cysteine-containing 10mer peptide, may be difficult to be detected by mass spectrometry.

4.3 GBM infection by HCMV could be a promising therapeutic option

GBM is the most aggressive malignant primary brain tumor and is highly lethal with inevitable relapse after standard treatment [208]. The current therapeutic options for GBM treatment are radiotherapy, chemotherapy (temozolomide), and surgery if possible [221]. However, none of the current therapeutic options are efficient. GBM patients have a median survival of only 14–

15 months after GBM diagnosis [221, 305]. Thus, there is an urgent need to develop novel approaches for GBM treatment.

4.3.1 Virotherapy can be a novel approach for GBM treatment

One of the novel treatment approaches is the clinical use of oncolytic viruses, which can selectively replicate in cancer cells. The results of recent phase I/II clinical trials showed that this approach could be highly promising as a treatment option [306, 307]. Virotherapy based on utilizing oncolytic replicating virus can induce specific killing of the infected cancer cells. Different mechanisms are hypothesized to elucidate killing of cancer cells by these viruses, most of these mechanisms are immunogenic [308]. Recently, the use of oncolytic viruses in combination with other treatment options such as radiation therapy or chemotherapy has been shown to have a synergistic activity [309-311]. This indicates the feasibility of new GBM treatment protocols based on the combination of virotherapy with approved forms of GBM therapy; especially that GBM and glioma stem-like cells are radio- and chemo-resistant in many cases resulting in progression and recurrence of tumor cells. [312, 313]. Interestingly, GBM infection by HCMV does not increase the resistance of GBM cells to chemotherapy, however, it significantly reduces tumor cell viability [238].

4.3.2 GBM infection by HCMV produces cell-associated virus rather than free virions

The detection of HCMV DNA and proteins was associated with GBM in some studies [314, 315], however, other studies showed contradicting results that HCMV genomes and/or proteins were present in neglected proportion in GBM [316-319]. However, GBM cell lines are permissive for HCMV which encouraged us to use our generated HCMV-based vaccine as a treatment option for GBM.

The permissiveness of GBM cells to HCMV is not consistent among all cell lines. In a previous study using different HCMV strains, all tested HCMV strains have been shown to infect GBM cells [238]. In this study, we chose three different GBM cell lines for further experiments (LN18, U343 and U251) due to their expression of HLA-A2 haplotype. This makes it possible to analyze the presentation of peptides binding to HLA-A2 by using our T cells recognizing E6-peptide in the context of HLA-A2.

Upon GBM infection with EGFP-expressing TB40/E HCMV at intermediate infection level (MOI 0.3), the GBM cells showed different levels of permissiveness and EGFP expression. Interestingly, free HCMV virions were undetectable in the supernatant. That would also be

advantageous in our treatment approach, limiting the spread of HCMV to healthy tissues distal to the tumor. However, further studies using different MOIs were not done.

4.3.3 HCMV-based vaccines expressing E6 peptide fused to viral protein render GBM cells susceptible for recognition and attack by T cells

We aimed to use HCMV-based vaccines as an adjuvant for the presentation of high levels of vector-encoded neoantigens and repurposing HCMV-specific CTLs to fight the GBM tumors. This strategy was supported by our finding that pcDNAE6/E7Kpn-I U251 cells, which were stably transfected with E6/E7-expressing plasmid, could stimulate specific TCR-transduced T cells. The E6/E7 fusion protein is normally expressed neither in healthy tissue nor in GBM cells. After *in situ* vaccination of GBM with E6/E7-expressing HCMV vaccine, E6/E7 could act as a neoantigen that render GBMs susceptible for specific T cell attack.

We established immunological assays based on Jurkat cells (J E6-1 and J76) that were transduced with retroviral vectors encoding TCRs specific for HPV16 E6- or HCMV pp65-derived peptides. These Jurkat cells were modified for NF- κ B or NFAT-driven EGFP expression. This assay facilitates the analysis of T cells activation. The cooperation between NFAT and NF- κ B proteins induces maximal transcription of the IFN- γ gene [320]. Moreover, NFAT is required for expression of the cytokine IL-2 [321].

Our established immunological assays showed that GBM cell lines infected with HCMV-based vaccine expressing the entire E6/E7 protein separately from any viral protein did not stimulate E6-specific Jurkat cells. They were, however, highly stimulatory for viral pp65-specific Jurkat cells when using pp65-encoding viruses. In contrast, GBM cells infected with HCMV-based vaccines expressing the HPV16 E6-derived peptide fused to viral proteins could efficiently stimulate E6-specific Jurkat cells as well as pp65-specific Jurkat cells (with the exception of mutant HCMV lacking pp65). These results are in accordance with our finding of TCR-transduced PBMCs stimulation and confirm that fusing the antigenic peptides derived from heterologous proteins to the C-terminus of HCMV proteins (such as IE1 and pp65) is highly efficient for processing and presentation of the non-HCMV antigenic peptides.

In conclusion, HCMV-based therapeutic vaccines are very promising as a novel therapeutic approach for GBM and HPV-induced cancer therapy. Further studies using animal models are recommended to investigate the therapeutic potential of our vaccines. More studies are needed for constructing other recombinant HCMV-based vaccines based on our prototype to develop more therapeutic and prophylactic vaccines using different neoantigens. For clinical use, our

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group are exploring the attenuated HCMV-based vaccines to undergo one only round of infection. In addition, we are currently investigating the mechanism underlying HCMV-induced block of MHC-I presentation which remains elusive.

5. Summary

Human papilloma virus (HPV), which belongs to the most common sexually transmitted human pathogens, is linked to several cancers including cervical and oropharyngeal cancer. The most important HPV genotypes in this respect are HPV16 and HPV18. Expression of the highly conserved HPV-encoded E6 and E7 oncoproteins is required for the initiation and progression of HPV-associated malignancies. So far, no approved therapeutic vaccine against HPV-associated cancer is available. Another important type of cancer is glioblastoma multiforme (GBM), the most aggressive primary brain tumor. GBM patients have a very short median survival time despite standard treatment involving surgery, chemotherapy, and radiation. Thus, novel therapeutic strategies are urgently needed to improve the poor prognosis of GBM patients.

The aim of this study was to develop a human cytomegalovirus (HCMV)-based platform for generation of therapeutic vaccines against cancer. Malignant cells are killed by cytotoxic CD8⁺ T lymphocytes (CTLs) that recognize tumor neoantigens presented by MHC class I molecules (MHC-I). Thus, therapeutic cancer vaccines should induce a high frequency of CTLs that can recognize tumor neoantigens. In this regard, HCMV is a very promising starting point for a vaccine platform. HCMV, a member of the *β-herpesvirinae* affecting 60-80% of the population in the developed countries, causes latent asymptomatic infection in immunocompetent individuals. The persistent HCMV infection is associated with a uniquely high frequency of HCMV-specific CTLs with an effector-memory phenotype representing 10-20% of all circulating T cells, a phenomenon called memory inflation. Accordingly, a tumor neoantigen vectored by HCMV should induce strong antitumor responses. As a proof of principle, we developed a HCMV based therapeutic vaccine that can induce CTLs recognizing HPV16-encoded E6 as a neoantigen. Intriguingly, these HCMV-based vaccines can also infect GBM cells. This finding paves the way for altering the immunogenicity of GBM by driving the presentation of E6 as a neoantigen, thus rendering GBM susceptible to CTL attack, e.g. after adoptive transfer of E6-specific autologous T cells.

In order to optimize antigen presentation to CTLs, HCMV-encoded MHC-I downregulating genes (US2-US11) were deleted in the HCMV vector. This modified vector was used to express a non-transforming HPV16 E6/E7 fusion protein that covers all the relevant antigenic peptides. Further HCMV modifications such as insertion of exogenous promoter or interruption of additional immune evasion genes (such as UL111A) were introduced to optimize the CTL response. In addition, different types of HCMV-based vaccine candidates were constructed by linking a dominant antigenic peptide derived from HPV16 E6 to HCMV proteins. Surprisingly,

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only the latter could efficiently stimulate E6 peptide-specific T cells. To explore the mechanism that interferes with processing and presentation of the HPV16 E6/E7 fusion protein, cells were stably transfected with a plasmid expressing the HPV16 E6/E7 fusion protein. Intriguingly, these E6/E7 expressing cells were able to stimulate E6 specific T cells in the absence of HCMV. However, this stimulation was significantly inhibited when E6/E7-expressing cells were additionally infected with HCMV, although the HCMV used no longer possessed any MHC-I-downregulating genes. This finding shows a novel HCMV-encoded block of MHC-I antigen presentation that is independent of the already known MHC-I downregulating HCMV genes.

This is the first study describing the construction of HCMV-based vaccines expressing tumor neoepitopes and provides the preclinical basis for development of HCMV-based therapeutic vaccines against cancers. Further investigations using different cell lines *in vitro* as well as *in vivo* studies exploring the safety and therapeutic effect of HCMV-based vaccines are warranted.

6. Zusammenfassung

Das humane Papillomvirus (HPV), welches zu den häufigsten sexuell übertragbaren menschlichen Krankheitserreger gehört, ist mit verschiedenen Krebsarten einschließlich Cervixkarzinom und Oropharynxkarzinom assoziiert. Die wichtigsten HPV-Genotypen in dieser Hinsicht sind HPV16 und HPV18. Die Expression der hochkonservierten HPV-kodierten E6- und E7-Onkoproteine ist für die Initiierung und das Fortschreiten von HPV-assoziierten Malignomen erforderlich. Bislang ist kein zugelassener therapeutischer Impfstoff gegen HPV-assoziierten Krebs verfügbar. Eine weitere wichtige Krebsart ist das Glioblastoma multiforme (GBM), der aggressivste primäre Hirntumor. GBM-Patienten haben trotz der Standardbehandlung mit Operation, Chemotherapie und Bestrahlung eine sehr kurze mediane Überlebenszeit. Daher werden dringend neue therapeutische Strategien benötigt, um die schlechte Prognose von GBM-Patienten zu verbessern.

Das Ziel dieser Studie war die Entwicklung einer auf dem humanen Cytomegalievirus (HCMV) basierenden Plattform für die Herstellung therapeutischer Impfstoffe gegen Krebs. Bösartige Zellen werden von zytotoxischen CD8⁺ T-Lymphozyten (CTLs), die von MHC-Klasse-I-Molekülen (MHC-I) präsentierte Tumor-Neoantigene erkennen, abgetötet. Daher sollten therapeutische Krebsimpfstoffe eine hohe Frequenz von CTLs induzieren, die Tumor-Neoantigene erkennen können. In dieser Hinsicht ist das HCMV ein sehr vielversprechender Ausgangspunkt für eine Impfstoffplattform. HCMV, ein Mitglied der *β-Herpesvirinae*, das 60-80% der Bevölkerung in den entwickelten Ländern betrifft, verursacht eine latente asymptomatische Infektion bei immunkompetenten Personen. Die persistierende HCMV-Infektion ist mit einer einzigartig hohen Frequenz von HCMV-spezifischen CTLs mit einem Effektor-Gedächtnis-Phänotyp verbunden, der 10-20% aller zirkulierenden T-Zellen ausmacht, ein Phänomen, das als „Memory inflation“ bezeichnet wird. Dementsprechend sollte ein durch HCMV exprimiertes Tumor-Neoantigen starke Immunreaktionen gegen den Tumor auslösen. Als „Proof of principle“ haben wir einen therapeutischen Impfstoff auf der Basis von HCMV entwickelt, der CTLs induzieren kann, die HPV16-kodiertes E6 als Neoantigen erkennen. Interessanterweise können diese Impfstoffe auf HCMV-Basis auch GBM-Zellen infizieren. Diese Erkenntnis ebnet den Weg für eine Veränderung der Immunogenität von GBM, indem sie die Präsentation von E6 als Neoantigen ermöglicht und damit GBM anfällig für einen CTL-Angriff macht, z.B. nach adoptivem Transfer von E6-spezifischen autologen T-Zellen.

6. Zusammenfassung

Um die Antigenpräsentation für CTLs zu optimieren, wurden HCMV-kodierte MHC-I herunterregulierende Gene (US2-US11) im HCMV-Vektor deletiert. Dieser modifizierte Vektor wurde zur Expression eines nicht-transformierenden HPV16 E6/E7-Fusionsproteins verwendet, das alle relevanten antigenen Peptide abdeckt. Weitere HCMV-Modifikationen wie die Einfügung eines exogenen Promotors oder die Unterbrechung zusätzlicher Immunevasionsgene (wie UL111A) wurden eingeführt, um die CTL-Antwort zu optimieren. Darüber hinaus wurden andere Impfstoffkandidaten auf HCMV-Basis konstruiert, die ein dominantes antigenes Peptid des HPV E6 Proteins verknüpft mit HCMV-Proteinen exprimieren. Überraschenderweise konnten nur letztere effizient E6-Peptid-spezifische T-Zellen stimulieren. Um den Mechanismus zu untersuchen, der die Prozessierung und Präsentation des HPV16 E6/E7-Fusionsproteins stört, wurden Zellen stabil mit einem Plasmid transfiziert, welches das HPV16 E6/E7-Fusionsprotein exprimiert. Interessanterweise konnten diese E6/E7-exprimierenden Zellen E6-spezifische T-Zellen stimulieren – in Abwesenheit von HCMV. Diese Stimulation wurde jedoch bei einer zusätzlichen Infektion E6/E7-exprimierenden Zellen mit HCMV signifikant gehemmt, obwohl das verwendete HCMV keine MHC-I-herunterregulierenden Genen mehr besaß. Dieser Befund zeigt einen neuartigen HCMV-kodierten Block der MHC-I-Antigenpräsentation, der unabhängig von den bereits bekannten MHC-I herunterregulierenden HCMV-Genen ist.

Dies ist die erste Studie, welche die Herstellung von Tumor-Neoepitop exprimierenden HCMV-basierten Impfstoffen beschreibt. Sie ist damit die präklinische Grundlage für die Entwicklung von therapeutischen Impfstoffen gegen Krebs auf HCMV-Basis. Weitere Untersuchungen mit verschiedenen Zelllinien *in vitro* und *in vivo*, die die Sicherheit und die therapeutische Wirkung von Impfstoffen auf HCMV-Basis untersuchen, sind gerechtfertigt.

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8. Publications and presentations

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Original articles:

1. Abdelaziz MO, Ossmann S, Kaufmann AM, Leitner J, Steinberger P, Willimsky G, et al. Development of a Human Cytomegalovirus (HCMV)-Based Therapeutic Cancer Vaccine Uncovers a Previously Unsuspected Viral Block of MHC Class I Antigen Presentation. *Front Immunol.* 2019;10:1776.
2. Raftery MJ, Abdelaziz MO, Hofmann J, Schonrich G. Hantavirus-Driven PD-L1/PD-L2 Upregulation: An Imperfect Viral Immune Evasion Mechanism. *Front Immunol.* 2018;9:2560.

Reviews:

1. Schonrich G, Abdelaziz MO, Raftery MJ. Herpesviral capture of immunomodulatory host genes. *Virus Genes.* 2017;53(6):762-73.

Talks:

Human Cytomegalovirus-based Vaccine for Cancer Therapy. The 12th Mini-Herpesvirus Workshop, September 22, 2017 in Berlin, Germany.

Posters:

Human Cytomegalovirus-based Therapeutic Vaccine for Human Papillomavirus-induced Cancer. The 26th Annual Meeting of the Society for Virology, April 06 - 09, 2016 in Muenster, Germany.

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10. Selbständigkeitserklärung

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Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Mohammed Omar Abdelaziz Yassen

2020, Berlin

