



Molecular analysis of the human mammary epithelial cells infected by human cytomegalovirus.

Fatima Al Moussawi

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THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE FRANCHE-COMTE

PREPAREE A EPILAB 4266 et l'Université Libanaise

Ecole doctorale n°554

Environnement-Santé

Doctorat de Virologie

Par

Mlle.AL MOUSSAWI Fatima

Analyse moléculaire des cellules épithéliales mammaires humaines infectées par le cytomégalovirus humain

Thèse présentée et soutenue à « Hadat-Liban », le « 26 Octobre 2018»

Composition du Jury :

M.LIAGRE Bertrand	Professeur, Université de Limoges	Président
M. SCHWARTZ Christian	Professeur, Université de Strasbourg	Rapporteur
M.ZARAKET Hasan	Professeur Assistant, American University of Beirut	Rapporteur
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M ^{me} . DIABASSAF Mona	Professeur, Université Libanaise	Codirectrice de thèse



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List of abbreviations:

ABL1	Abelson murine leukemia viral oncogene homolog 1
AIDS	Acquired Immunodeficiency Syndrome
AP	Assembly protein
ATM	Ataxia telangiectasia mutated
ATF	Activating transcription factor
Bak	Bcl-2 homologous antagonist killer
Bax	BCL2-Associated X Protein
BBC	Basal-like breast cancer
BCCL	Breast cancer cell line
BCL-2	B-cell lymphoma 2
BCR	B-cell receptor
bFGF	Basic fibroblast growth factor
BMT	Bone marrow transplant
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CCN	Cyclin
CCPH	Complement control proteinhomolog
CD	Cluster of differentiation
CDH	Cadherin 1, type 1, E-cadherin (epithelial)
CDK	Cyclin-dependent kinase
CDKN	Cyclin-dependent. Kinase inhibitor
CLDN	Claudin
CMV	Cytomegalovirus
CNS	Central nervous system
COX-2	Cyclooxygenase-2

CREB	cAMP response element binding
CSF	Colony Stimulating Factor
CSF-R	Colony Stimulating Factor Receptor
CTL	Cytotoxic T-cell
CTNNB1	Catenin (cadherin associated protein) beta 1
CTSD	Cathepsin D
DBs	Dense bodies
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferase
dsDNA	Double stranded Deoxyribonucleic acid
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EED	Embryonic ectoderm development
EGF	Epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
eIF4A1	Eukaryotic Translation Initiation Factor 4A1
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial–mesenchymal transition
ER-a	Estrogen receptor alpha
ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
EV	Epidermodysplasia verruciformis
FHIT	Fragile histidine triad gene
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
FOXD3	Forkhead box D3
GATA3	GATA binding protein 3

GBM	Glioblastoma Multiforme
GCV	Ganciclovir
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBV	Hepatitis B virus
HCC	Hepatocellular Carcinoma
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HDAC	Histone Deacetylase
HER-2	Human epidermal growth factor receptor 2
HHV	Human herpesvirus
HIC	Hypermethylated in cancer
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte antigen
HMEC	Human mammary epithelial cell
HPV	Human papillomavirus
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
HSP	Heat shock proteins
HSV	Herpes simplex virus
hTERT	Human Telomerase Reverse Transcriptase
HTLV-1	Human T-lymphotropic virus type 1
IBC	Inflammatory breast cancer
ID2	Inhibitor of DNA binding 2
IDC	Invasive ductal carcinoma
IE genes	Immediate early genes
IFN	Interferon
IGF1R	Insulin-like growth factor 2 receptor

IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
ITGA6	Integrin alpha 6
JAK/STAT	Janus-activated kinase/ Signal transducer and activator of transcription
JNK	Jun N-terminal Kinase
JUN	Jun oncogene
KITLG	KIT ligand
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
KRT	Keratin
KS	Kaposi sarcoma
KSHV	Kaposi's sarcoma associated herpesvirus
L genes	Late genes
LANA	Latency-Associated Nuclear Antigen
LCL	Lymphoblastoid cell lines
LIR-1	Leukocyte Ig-like receptor
LMP	Latent membrane protein
LncRNA	Long non coding RNA
LT	Large T antigen
MAD1	Mitotic arrest defective protein
MAPK	Mitogen activated protein kinase
MCC	Merkel cell carcinoma
MCL	Myeloid cell leukemia
MCMV	Murine cytomegalovirus
MCP	Monocyte Chemoattractant Protein
MCV	Merkel cell polyomavirus
MET	Met proto-oncogene (hepatocyte growth factor receptor)

MHC	Major histocompatibility complex
MICB	MHC Class I-related chain B
MIEP	Major immediate early promoter
MIG	Monokine induced by γ -interferon
MiK67	Antigen identified by monoclonal antibody Ki-67
MIP	Macrophage inflammatory protein
MLH1	Mutl homolog 1
MOS	Molony murine sarcoma viral oncogene homolog
mRNA	Messenger Ribonucleic Acid
MRP	Multidrug resistance protein
mTOR	Mammalian target of rapamycin
MYB	Myeloblastosis viral oncogene
MYC	Myelocytomatosis viral oncogene
MYD88	Myeloid differentiation primary response protein
NAP	Nucleosome Assembly Protein.
NFKB	Nuclear factor kappa B
NFKBIA	Nuclear factor kappa B inhibitor alpha
NIEPs	Non-infectious enveloped particles
NK	Natural killer
NGK2D	Natural killer group 2D
NMSC	Non-melanoma skin cancer
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
ORF	Open Reading Frame
Orilyt	Origin of lytic replication
PBL	Peripheral Blood Leukocyte
PBMCs	Peripheral Blood Mononuclear Cells

PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PIK3C2A	Phosphatidylinositol- 3-Kinase, class 2, Alpha polypeptide
PIK3CA	Phosphatidylinositol- 3-Kinase Catalytic Alpha polypeptide
PI3k	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PML	Promyelocytic leukemia
PR	Progesterone receptor
pRb	Retinoblastoma protein
PRC2	Polycomb repressive complex 2
PRKCA	Protein kinase C alpha
PTEN	Phosphatase and tensin homolog
PVs	Papillomoviruses
RAF	V-raf-1 murine leukemia viral oncogene homolog
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RARA	Retinoic Acid Receptor Alpha
RASSF1A	Ras-association domain family
REL	Reticuloendotheliosis viral oncogene homolog
RET	Ret proto-oncogene
ROS	Reactive oxygen species
ROS-1	C-ros oncogene 1, receptor tyrosine kinase
RUNX1	Runt-related transcription factor 1
S100A4	S100 calcium binding protein A4
SAPK	Stress-activated protein kinase
SERPINE1	Serpin peptidase inhibitor

SPT6	Transcription elongation factor SPT6
sT	Small T antigen
STAT1	Signal transducer and activator of transcription 1
STK11	Serine/threonine kinase 11
SUZ12	Suppressor of zeste 12 homolog
SV40	Simian virus 40
Syk	Spleen tyrosine kinase
TAM	Tumor-Associated Macrophage
TAP	Transporter associated with antigen processing
TBP	TATA box binding protein
TFF1	Trefoil Factor 1
TGF	Transforming growth factor
Th-1	T Helper Cell Type 1.
THBS	Thrombospondine
TLR	Toll Like Receptor
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor
TOP2A	Topoisomerase II alpha
TRAFs	Tumor necrosis factor receptor associated factors
TSC	Tuberous sclerosis
TSG	Tumor suppressor genes
TSP	Thrombospondin
UL	Unique long
US	Unique short
VEGF	Vascular endothelial growth factor
vFLIP	Viral FLICE inhibitory protein

VHL	Von Hippel-Lindau tumor suppressor
vICA	Viral inhibitor of caspase activation
vIRF	Viral interferon regulatory factor
vMIA	Viral mitochondria-localized inhibitor of apoptosis
VG	Valganciclovir
VZV	Varicella zoster virus
WHO	World health organization

Résumé :

Depuis plusieurs années, le rôle joué par le cytomégalo virus humain (HCMV) dans le développement des maladies inflammatoires et du cancer a été étudié par plusieurs groupes de recherche. Divers tissus tumoraux, notamment dans le cancer du colon, du foie, de la prostate, du cerveau (glioblastome, médulloblastome) et du sein, ont montré la présence d'antigènes ou d'ADN du HCMV. Cette accumulation de preuves de l'implication de l'infection par le HCMV dans les maladies malignes de diverses entités cancéreuses a conduit au développement du concept d'«oncomodulation», qui est expliqué par la capacité du HCMV à contribuer au processus d'oncogenèse, sans toutefois aucun potentiel de transformation directe. HCMV-DB (KT959235) est un isolat clinique provenant d'un échantillon de col de l'utérus d'une femme enceinte de 30 ans, préalablement isolé dans notre laboratoire. Cette souche virale a montré sa capacité à infecter les macrophages primaires et a montré une réplication productive dans les cellules épithéliales mammaires humaines (HMECs). Les HMECs infectées entraînaient l'établissement d'un environnement cellulaire pro-oncogène avec une hyperphosphorylation de Rb et une activité fonctionnelle réduite de p53, une régulation positive de c-Myc, une surexpression de l'activité télomérase et de STAT3, et une régulation positive de la cycline D1, provoquant une prolifération cellulaire accrue. En outre, HCMV-DB a montré son potentiel pour transformer les HMECs primaires par test de formation de colonies sur gélose molle, un test connu pour l'observation de la transformation cellulaire. De manière intéressante, les HMECs infectées par HCMV-DB en culture ont montré l'émergence d'amas de cellules sphéroïdes, qui ont été désignées cellules CTH (HMECs transformées par le CMV). Dans notre thèse, nous avons caractérisé le profil génomique de la souche HCMV-DB et nous l'avons comparé à des souches soit cliniques soit de laboratoire. HCMV-DB a été caractérisée comme proche des génomes des souches Toledo et JP, et cette dernière est une souche clinique isolée à partir d'un tissu glandulaire, la prostate. Nous avons également comparé les gènes impliqués dans l'entrée virale par des analyses phylogénétiques et nous avons observé la proximité de HCMV-DB avec la souche prototypique du HCMV, Merlin. En étudiant le profil transcriptomique des HMECs infectées par HCMV-DB, nous avons trouvé qu'elles présentent un phénotype basal-like triple négatif, ER-/PR-/HER2-, ainsi que des caractéristiques oncogéniques, incluant une up-régulation de l'expression de plusieurs oncogènes, de gènes pro-survie (avec down-régulation de la caspase 8), et de marqueurs de la prolifération, du caractère souche des cellules et de la transition épithélio-mésenchymateuse (EMT). Le profil transcriptomique des HMECs infectées par HCMV-DB a également montré des modifications variées dans la signalisation cellulaire, l'angiogenèse et la protéolyse. Au niveau de la chromatine, les HMECs infectées par HCMV-DB ont révélé une hypométhylation globale. En cherchant la présence du génome de HCMV-DB dans les cellules CTH formées, nous avons détecté une signature du génome de HCMV-DB, à savoir le lncRNA4.9. Globalement, nos données ont montré que le transcriptome des HMECs infectées par HCMV-DB révèle clairement des traits pro-oncogéniques et la détection d'une partie du génome de HCMV-DB suggère que cette partie du génome viral peut être responsable de la transformation cellulaire obtenue.

Mots clés : HCMV-DB – cancer du sein- HMEC- Transcriptome- génome.

Abstract

Since several years, the role played by human cytomegalovirus (HCMV) in the development of inflammatory diseases and cancer has been extensively studied and addressed by different research groups. Various tumor tissues originating from colon, liver, prostate, brain (glioblastoma, medulloblastoma) and breast cancer have shown to harbor either the antigen or the DNA of HCMV. These growing evidences about the implication of HCMV infection in malignant entities had led to the emergence of the concept of “Oncomodulation”. This is explained by the ability of the virus to contribute to the oncogenic processes, however without any direct transformatory potential. HCMV-DB (KT959235) is a clinical isolate obtained from a cervical swab specimen of a 30-year-old pregnant woman previously isolated in our laboratory. This viral strain had shown its ability to infect the primary macrophages and to replicate productively in the human mammary epithelial cells (HMECs). In fact, HMECs infected by HCMV-DB resulted in the establishment of a pro-oncogenic cellular environment characterized by retinoblastoma (Rb) hyperphosphorylation and a decreased p53 functional activity, enhanced telomerase activity, upregulation of c-Myc, activation of Akt and STAT3, and upregulation of cyclin D1 causing an enhanced cellular proliferation. Furthermore, HCMV-DB had shown its potential to transform the primary HMECs by colony formation on soft agar, a well know assay to perceive transformation. Interestingly, HCMV-DB infected HMECs in culture showed the emergence of clusters of spheroid cells that were named CTH cells (CMV Transformed HMECs). In our thesis we characterized the genomic profile of HCMV-DB strain and compared it to either clinical or laboratory strains. HCMV-DB was shown to be close to the genomes of Toledo and JP strains where the JP strain is a clinical strain that was isolated from a glandular tissue, the prostate. We also compared the genes that are involved in virus entry using phylogenetic analyses and we observed that HCMV-DB is close to the prototypic HCMV strain, Merlin. By studying the transcriptomic profile of HMECs infected with HCMV-DB, we found that it displays a triple negative basal-like phenotype, ER–/PR–/HER2–, and presents oncogenic characteristics with upregulated expression of several oncogenes, pro-survival genes (with a down-regulation of caspase 8), proliferation markers, stemcellness and epithelial mesenchymal transition (EMT). The transcriptomic profile of HMECs infected with HCMV-DB also displays variant modifications in cell signaling, angiogenesis and proteolysis. At the chromatin level, HMECs infected with HCMV-DB reveals a global hypomethylation state. By screening for the presence of HCMV-DB genome in the formed CTH cells, we detected a signature of the HCMV-DB genome, namely a lncRNA4.9. Taken together, our data showed that the transcriptome of HMECs infected with HCMV-DB clearly reveals a pro-oncogenic traits and the detection of part of the HCMV-DB genome suggests that this part of the viral genome might be responsible for the obtained cellular transformation.

Keywords: HCMV-DB- Breast cancer- HMEC- transcriptome- genome.

HUMAN CYTOMEGALOVIRUS (HCMV)

1- The discovery of HCMV

In 1881, a German pathologist named Hugo Ribbert was the first who observed what is believed to be cytomegalovirus (CMV). He noticed enlarged kidney cells in babies without being able to explain the cause of that condition. By the year 1904 Jesionek and Kiolemenoglou described similar enlarged kidney cells of a still-born infant as 'protozoan-like'. In the same year Jesionek reported the presence of similar 'protozoan-like' cells in the liver, lungs, and kidneys of prematurely born fetuses. In 1907 Charles Lowenstein mentioned cytoplasmic and nuclear inclusions, surrounded by a clear zone in the described protozoal-like cells ¹⁻³. During the same year these abnormal cells were described by the term 'cytomegalia' by Goodpasture and Talbot. They disagreed that a protozoan had caused the inclusions without being clear over the cause of that cytopathology. By 1925 Von Glahn and Pappenheimer observed inclusion bodies in cells infected with herpesviruses and they suggested that the cytomegalic cells were caused by a virus rather than protozoa. Later on, Cole's findings indicated that the cause of inclusion bodies formation was most likely to be viruses infections ⁴. This yet unknown viral disease was termed as 'generalised cytomegalic inclusion disease' ⁴. In the mid of the 1950s, a virus was isolated from tissue cultures of human adenoid and salivary gland by three different laboratories ^{5,6}. It was called first the 'salivary gland virus'; thereafter Thomas Weller and collaborators named it 'cytomegalovirus' (CMV) ⁷. Virus isolation and propagation in cultures had enabled further understanding of its structure, its life cycle, and the molecular understanding of its pathogenicity.

2- *Herpesviridae* and its subfamilies

Herpesviridae, one of the largest viruses' families, consists of viruses that are highly prevalent in nature. Most animal species have been infected with at least one herpesvirus during their lifetime. Viruses of the *Herpesviridae* family share similar biological properties upon infection, they have similar virion architecture features, and conserved genomic segments ⁸. Recent classification of new viruses is mainly based on data obtained from sequence similarities of nucleotides and amino acids ⁹.

Herpesviridae family has some general characteristics. All its members have a linear double-stranded DNA (dsDNA) genome which ranges from 120-240 kbp in size, and is enclosed by an icosahedral capsid composed of 162 capsomers (12 pentons and 150 hexons). The capsid is surrounded by an asymmetric and amorphous protein layer, named the tegument, which is surrounded by a host-derived lipid bilayer membrane called the envelope. The envelope surface is studded with viral transmembrane proteins used for binding and entry into the target cells ⁸.

Although all herpesviruses have similar features, the number of open reading frames (ORF) in their genomes varies considerably ranging from 70 in VZV to more than 750 in CMV.

Depending on the primary variations in the biological properties of viruses such as: replication cycle, host cell tropism, latency features, and differences in the clinical manifestations, herpesviridae family is further classified into three major subfamilies: i) *Alphaherpesvirinae* ii) *Betaherpesvirinae* and iii) *Gammapherpesvirinae*. Viruses of each subfamily have variable hosts including humans. Among the known herpesviruses, eight are known to primarily infect humans. These eight human herpesviruses belong to the three subfamilies as described below ¹⁰.

i- *Alphaherpesvirinae*: Three alpha herpesviruses are known to infect humans: herpes simplex virus type 1 (HSV-1 or HHV-1), herpes simplex virus type 2 (HSV-2 or HHV-2), and varicella zoster virus (VZV). All three viruses establish latency in sensory ganglia. They have short replication cycles, about 12-18 h, and can either results in a lytic infection or be latent in cells for years.

i- *Betaherpesvirinae*: Three human viruses belong to this family: human herpesvirus-5 or HHV-5 which is also named human cytomegalovirus or HCMV, human herpesvirus-6 or HHV-6 (two subtypes A and B), and human herpesvirus-7 or HHV-7. Latent infection is established in secretory glands, lymphoreticular cells, and bone marrow cells. Frequently, the infected cells become enlarged in size (cytomegalic cells) and in general, members of this subfamily have long replication cycles (> 24 h). Infections with these viruses progress slowly and result either in cell lysis or in persistent infection.

ii- *Gammaherpesvirinae*: Two human viruses are in this subfamily, both of them are considered to be oncogenic viruses: the Epstein-Barr virus (EBV) or human herpesvirus-4 (HHV-4), and the Kaposi' sarcoma associated herpesvirus (KSHV) or human herpesvirus-8 (HHV8). Both viruses infect, replicate and establish their latency in lymphoblastoid cells such as T- and B- lymphocytes. They also infect and can cause a lytic infection in epithelioid/fibroblast cells. KSHV is associated with Kaposi sarcoma in AIDS patients and EBV is associated with several types of lymphomas.

3- HCMV structure

As mentioned in the *Herpesviridae* section above, the HCMV virion architecture is similar to that of other herpesviruses members. The mature virion particle of HCMV is 150-200 nm in diameter. It consists of a lipid bilayer envelope composed of a large number of viral glycoproteins surrounding a proteinaceous layer known as the matrix or the tegument, which in turn, surrounds an icosahedral nucleocapsid (100-nm in diameter) containing the 230-kbp double stranded linear DNA genome ¹¹ (fig. 1).

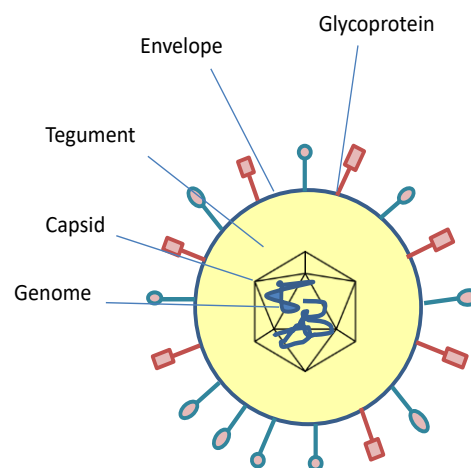


Figure 1: HCMV structure

Cell cultures infected with the virus had shown the production of infectious virions, non infectious enveloped particles (NIEP) and dense bodies (DBs). The non infectious enveloped particles are viral particles with defects. They are composed of enveloped

immature capsids (type B) where they lack the DNA and contain the viral scaffolding/assembly protein (AP) which is normally not found in the fully mature nucleocapsids (C-capsids). Dense bodies are also enveloped particles and contain several tegument proteins but they lack the assembled nucleocapsid and the viral DNA. Depending on the number of passages in cell culture and the viral strain, the relative amounts of the three viral forms vary ¹² (fig. 2).

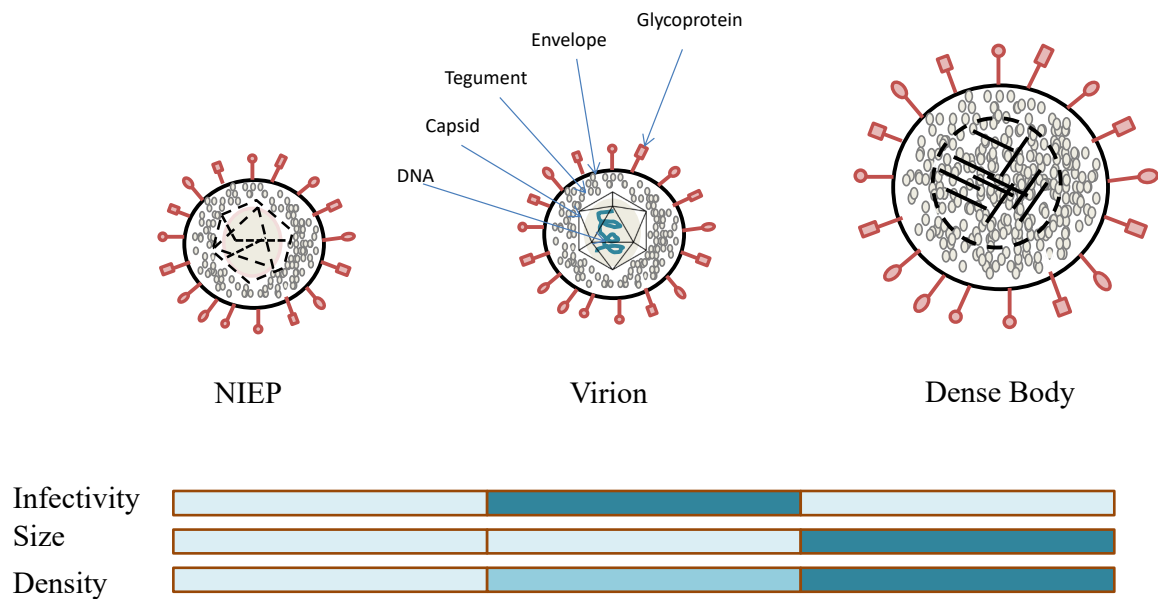


Figure 2. HCMV infection is producing, virions, dense bodies (DBs) and Non-infectious enveloped particles (NIEPs). Virions and NIEPs are similar in their size, about 250 nm, whereas DBs are bigger in size, about 250-600 nm. Virions are the fully mature infectious particles, however NIEPs and DBs are considered as non-infectious. The density of DBs is higher than that of virions and NIEPs (adapted and updated from: <http://www.researchgate.net/publication/317039508>).
 legend: The higher the intensity of blue color, the higher the density, infectivity and size of the particle is.

3.1 Envelope

The envelope is a lipid bilayer surrounding the tegument layer and is responsible for keeping the entire virion intact. It is highly involved in the virus attachment and entry by its interaction with the host cell membrane on the target cells. Six virus encoded glycoproteins: gpUL55 (gB), gpUL73 (gN), gpUL74 (gO), gpUL75 (gH), gpUL100 (gM), and gpUL115 (gL) are present in the phospholipid envelope. These glycoproteins are important for virion maturation, viral entry into host cells and cell-to-cell viral spread ¹³. Disruptions in gH, gB, gL, and gM open reading frames (ORFs) detected by mutational

analysis result in the failure in producing infectious progeny. Such disruptions underscore the important role of these proteins for a productive replication ¹⁴.

3.2 Tegument

The tegument is the layer that closely surrounds the capsid. It is the widest layer within the virion structure and it contains the largest amount of proteins constituting the entire virion. Using electron microscopy studies, the tegument was shown as an amorphous and not well structured layer. The most well studied tegument proteins so far are pp65/pUL83, pp71/pUL82¹⁵, and pUL69. They may have a role in the progeny virion maturation and additionally, they may be involved in the early stages of infection. In antigenemia assays, pp65 is the target antigen used for rapid diagnosis of HCMV-sustained clinical infections because of its presence in large amounts. pUL69 is a multifunctional regulatory protein. It is a member of the ICP27 family, which is a group of proteins having similar functions and that have homologues in all the sequenced herpesviruses ¹⁶. pUL69 is directly released into the host cell upon infection^{17,18}. pUL69 has several roles either in the transcription of viral genes or in the regulation of its translation, in addition to its effect on the cell cycle progression¹⁹. pUL82 is implicated in the escape from the host immune response and thus it promotes the development of HCMV latency²⁰.

3.3 Capsid

The capsid is the innermost core layer which contains and protects the genome of a virion particle. It is an icosahedral capsid formed of 162 capsomeres (150 hexons and 12 pentons). HCMV capsids are of three different types: A-capsid with only a capsid shell, B-capsid is a capsid shell with assembled proteins, and C-capsid is a mature capsid which contains the viral genome. These three different capsids are the stages of capsid maturation taking place in the nucleus of the infected cells ²¹.

4- HCMV genome.

The HCMV genome is the largest among all herpesviruses. Its size ranges from 220-240 kbp depending on the HCMV strain. It was first isolated over 50 years ago²² and the complete genome of HCMV was published first in 1990^{23,24}. HCMV genome seems to be the most complex among all human herpesviruses genomes. By analyzing different HCMV strains, it was first observed that there is about 165-252 ORFs potentially coding for about 170 proteins^{25,26}. Recently, using ribosomal profiling and transcripts analysis, researchers found 751 unique translated ORFs in HCMV infected cells. Thus, HCMV has much more complex transcriptional and translational capabilities than what was previously believed²⁷.

HCMV genome sequencing analysis has revealed its complex organization. HCMV genome is composed of two major segments; the unique long (UL) and the unique short (US) segments which are joined by the Internal Repeated Long segments (IRL) and Internal Repeated Short segments (IRS). These are flanked with the Terminal Repeated Long segments (TRL) and Terminal Repeated Short segments (TRS). The sequences of TRL and TRS are arranged inversely to IRL and IRS²⁸. Herpesviruses genomes are classified in classes depending on the organization of the genome segments where the HCMV genome is classified as an E genome¹⁰ (fig. 3).

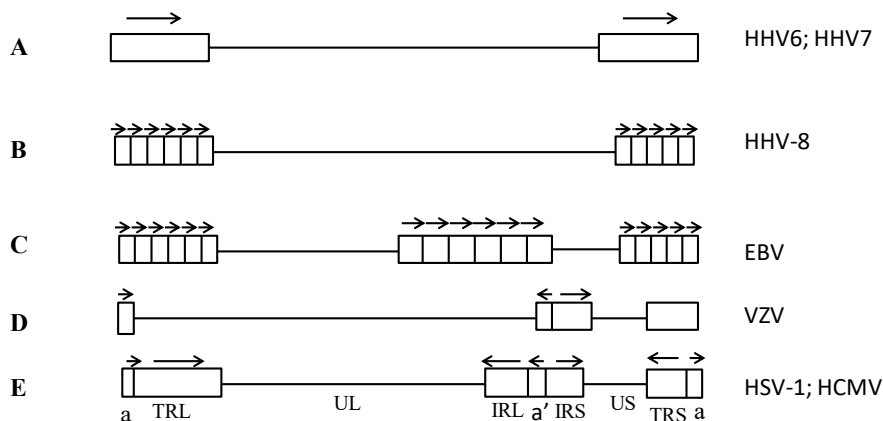


Figure 3: Different classes of herpesvirus genomes. (not to scale) defined by Roizman and Pellett (2001). Arrows: Orientations of repeats; Horizontal lines: Unique regions; Rectangles: Repeat regions. The nomenclature of unique and repeat regions, including the terminal redundancy (*a*) and its internal, inverted copy (*a'*), is indicated for the class E genome.

5- HCMV replication cycle and viral gene expression

5.1 Cell permissivity

HCMV infects a wide range of epithelial tissues as shown by autopsy specimens. The most commonly infected among all is the ductal epithelial cell that develops a typical cytopathology²⁹. During the natural infection it is believed that HCMV has a productive replication in mesenchymal cells, hepatocytes, smooth muscle cells, endothelial cells, epithelial cells, granulocytes and monocyte-derived macrophages^{29–33}. However, *in vitro*, only the human skin or lung fibroblasts are fully permissive cells for laboratory strains replication, whereas clinical isolates replicate both in fibroblast cultures and among others in epithelial or endothelial cell cultures^{30,34}.

5.2 Virus binding and penetration

The virus has a rapid and efficient attachment and penetration either in permissive or in nonpermissive cell types. Since a very restricted range of human cells provide a productive replication for the virus, it is thought that there is a post penetration block to viral gene expression which is responsible for replication restriction in non-permissive cells³⁵.

There are three major steps that are involved in the entry process: i) Attachment to the host cell surface ii) Interaction with the entry receptors present at the host cell membrane iii) Particle internalization or viral envelope fusion with the cell membrane (depending on the cell type)³⁶.

HCMV has two various entry routes in different cell types. In fibroblasts, viral entry is mediated via direct fusion at the plasma membrane by the following viral envelope glycoprotein complexes: gH/gL/gO and gH/gL-gB^{37,38}. However in monocyte/macrophages dendritic cells, epithelial cells and endothelial cells, an essential viral pentameric complex mediates the receptor-mediated endocytosis. This complex involves gH/gL/pUL128/pUL130/pUL131A³⁹. Then, the viral envelope fuses with the endosomal membrane to release its capsid into the cytoplasm. Following entry, tegument dissociation will occur and the capsid will be directed to the nuclear pore in order to deliver the DNA to the nucleus. Many tegument proteins are involved in this process⁴⁰.

5.3 Viral replication

The nucleus of the infected cells is the site where replication, inversion and packaging of the HCMV genome occur. After 16 hours post infection, viral DNA synthesis begins, and it requires the functions of both viral and cellular proteins ¹².

Examination of the CMV genome has revealed that they do not encode the enzymes of deoxyribonucleotide biosynthesis. These enzymes include: dihydrofolate reductase, an active form of ribonucleotide reductase, thymidine kinase, and thymidylate synthase ^{41,42}. Thus, for the virus to be able to replicate its DNA, it must depend on the host cell metabolism to have a sufficient supply of dNTPs. As a result, it does not stop the host macromolecular synthesis but it does stimulate the cellular transcription and translation ⁴³. Cells infected with CMV fail to undergo cellular DNA replication and division. This failure is due to the blockages in cell cycle progression that prevent the DNA replication machinery of the host to compete with the virus for the access to DNA precursors ^{43,44}.

In the genome of HCMV there are six herpesvirus-conserved ORFs responsible for providing the core replication proteins for viral DNA replication. The single-stranded DNA-binding protein pUL57 is one of them. It functions as an inhibitor of the DNA strands reannealing after their unwinding by the helicase-primase complex. This complex is composed of three proteins encoded by *UL70*, *UL102*, and *UL105* genes. Also the DNA polymerase which is encoded by *UL54* gene and the DNA processivity factor pUL44 which prevents the pUL54 dissociation from the template ¹². Other viral proteins are also required to maximize the DNA replication such as pUL84, pUL112/pUL113, and pUL114. The *UL84* gene encodes for a 75-kDa protein that acts as an origin-specific initiator factor, stimulating the viral origin (*oriLyt*)-dependent DNA synthesis ⁴⁵. The *UL112/113* genomic region encodes for phosphoproteins that play a role in regulating the establishment of the so-called replication centers that correspond to subnuclear sites of the synthesis of HCMV DNA. pUL112/pUL113 represent the early precursors of the replication centers and provide the core replication proteins and enzymes ^{46,47}. Finally, *UL114* gene encodes for a protein expressing the activity of a functional uracil DNA glycosylase. This protein appears to be required in post-mitotic cells for efficient viral DNA replication, since the substitution of *UL114* in a mutant virus revealed a defect in the transition to high-level, late-phase replication of the DNA ⁴⁸.

Furthermore, the immediate-early (IE) proteins are required for the transient complementation of *oriLyt*-dependent DNA synthesis. Such IE proteins are those expressed by the *UL36-38* genomic region and the transactivator proteins that are encoded by *UL123(IE1)/UL122(IE2)* and *TRS1/IRS1* genes¹². Newly synthesized genomes mature during the late stages of viral DNA replication. Their maturation occurs through their inversion, cleavage, and packaging⁴⁹. The entire HCMV replication cycle is long and takes approximately 72 h for the complete maturation of new virions to occur and permit the infection of the next cells either by cell-to-cell spreading mechanisms or by release from the infected cells⁵⁰.

5.4 Virion assembly, maturation, and egress

HCMV capsid formation and viral DNA packaging occur in the nucleus. Consequently, the nucleocapsids develop a primary envelopment derived from its inner leaflet by budding at the nuclear membrane. Then, their maturation occurs via a process of de-envelopment/re-envelopment in the cytoplasm^{12,51}, where they cross the lumen to fuse the outer leaflet either of the nuclear membrane or of the ER membrane, and then they lose their primary envelope to pass into the cytoplasm. Moreover, maturation of HCMV virion particles occurs by acquiring their tegument. Then, tegumented capsids bud into vesicles of the Golgi apparatus to receive their definitive envelope⁵². The process of tegumentation is unclear, but because all capsids present in the cytoplasm are tegumented, and none in the nucleus, it is highly probable that tegumentation must be either through or rapidly after nuclear egress⁵³. Multiple specific protein-protein interactions drive the processes of tegumentation and re-envelopment to secure the integrity of the viral particle⁵¹. Mature particles are preserved within the vesicles and then transported via the Golgi network to reach the cell surface. During the late replication stages, Golgi alterations create inclusions around the nucleus resulting in its typical kidney-like appearance. Progeny virus then accumulates in the cytoplasm. The release of infectious virus into the extracellular compartment begins at 72 hours post infection. Mature particles are released by transport of Rab3 secretory vesicles. Mature particles are released either by cell lysis or through fusion of the vesicles with the plasma membrane⁵³ (fig.4).

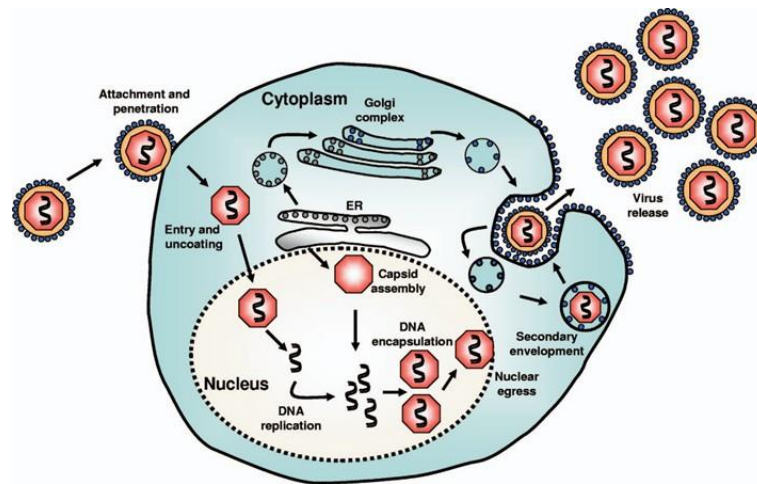


Figure 4. Life cycle of HCMV. First, HCMV enters into the human cells either by direct fusion or by an endocytic pathway. Attachment to the cell occurs through the interactions between viral glycoproteins (e.g. gB and gH) and specific surface receptor(s) (e.g., platelet-derived growth factor). This will be followed by the fusion of the viral envelope with the cellular membrane to release the nucleocapsids into the cytoplasm. Nucleocapsids will then translocate into the nucleus where the release of viral DNA occurs. The expression of IE-1/IE-2 will be initiated. Viral DNA will be encapsidated to form the capsids that will be transported from the nucleus to the cytoplasm. In the cytoplasm, at the endoplasmic reticulum (ER)-Golgi intermediate compartment secondary envelopment occurs. This will be followed by a complex final envelopment of two stages and egress process that leads to the release of virions by exocytosis at the plasma membrane. Figure and figure legends are adapted from (Tania Crough and Rajiv Khanna, 2009).

6- HCMV Latency and Reactivation

Latency can be defined as the phase where the viral genome persists dormant in the infected cells, where the virus can be reactivated again whenever the environment permits that. Latency establishment is considered as one of the key biological properties of herpesviruses. It allows the virus to have a persistent infection, without destroying the cells of the host. An immunocompetent host with primary infection with HCMV is often asymptomatic and will have a latent and persistent infection. HCMV DNA was detected first in peripheral blood mononuclear cells (PBMCs) using the in situ hybridization technique⁵⁴. Later on, several groups used the PCR methods and confirmed the presence of HCMV in blood^{55,56}. Furthermore, it was established that the virus genome is localized as an episome during the latency phase⁵⁷, and that bone marrow derived CD34+ myeloid progenitor cells^{58,59} and their derivative CD14+ monocytes are the hosts of latent infection^{60,61}. However, despite that T-cells and B cells are also both derived from the CD34+ myeloid lineage they cannot maintain the latent infection⁶².

The maintenance of HCMV in a latent state or its entrance into the lytic phase depends on the interplay between the host and viral encoded proteins. The molecular mechanisms of latency of a natural infection start to be elucidated. The major immediate early promoter (MIEP) of HCMV is essential for viral replication and its reactivation from latency since it is a transcription trans-activator of most HCMV encoded genes. During latency, the cellular factors HP1 and Ets2 transcriptionally repress the MIEP promoter⁶³⁻⁶⁶ and thus prevent the entrance of the virus into the lytic cycle phase. Early studies suggested that during latency, there is no transcription of the HCMV genome. In fact there is only a limited set of genes that are transcribed during the latent phase. The pUL138 protein is produced during latency⁶⁷. pUL138 has been shown to upregulate the surface expression of TNFR1, and thus to sensitize the infected cells to TNF- α . Moreover, TNF- α signaling has a positive role on HCMV reactivation. The expression of pUL138 during latency and the positive effect of TNF- α on the reactivation of HCMV indicate that pUL138 may be implicated in the reactivation of HCMV⁶⁸. pUL138 can also impair the HCMV specific immune response, since it causes downregulation of the multidrug resistance protein-1, MRP1⁶⁹ leading to reduced cellular leukotriene C4 export. This may prevent dendritic cells (DCs) from reaching the lymph nodes, and thus affecting the immune response⁷⁰. Furthermore, HCMV pUL111a expression during latency favors immune suppression. The UL111a gene encodes for a functional IL-10 homologue with strong immunosuppressive effects. During latency, the UL111a transcript undergoes alternative splicing. This leads to the expression of a latency associated cmvIL-10 transcript, which is translated into a protein with a function similar to human immunosuppressive cytokine IL-10. This impairs the recognition of infected cells by the immune system and thus avoid their clearance⁷¹. Additionally, pUS28 and pUL144 are also expressed during the latent phase and they play a role either in redirecting the immune response or in blocking the immune recognition⁷²⁻⁷⁴. HCMV infection of differentiated cells allows for viral replication, however HCMV remains in a non-permissive state in some undifferentiated cells⁷⁵. For instance, monocytes infection will not allow for an active infection, but differentiated macrophages do. Latent HCMV will be reactivated only after monocytes differentiation into macrophages or dendritic cells where it can replicate. IFN- γ can promote macrophages differentiation, which could enhance HCMV reactivation and replication⁷¹.

7- Epidemiology of HCMV infection.

HCMV is found in both the developed industrial societies and the isolated aboriginal groups. Worldwide, HCMV seroprevalence ranges from 45-100% depending on the geographical location and/or the socio-economical status ⁷⁶. (Fig 5)

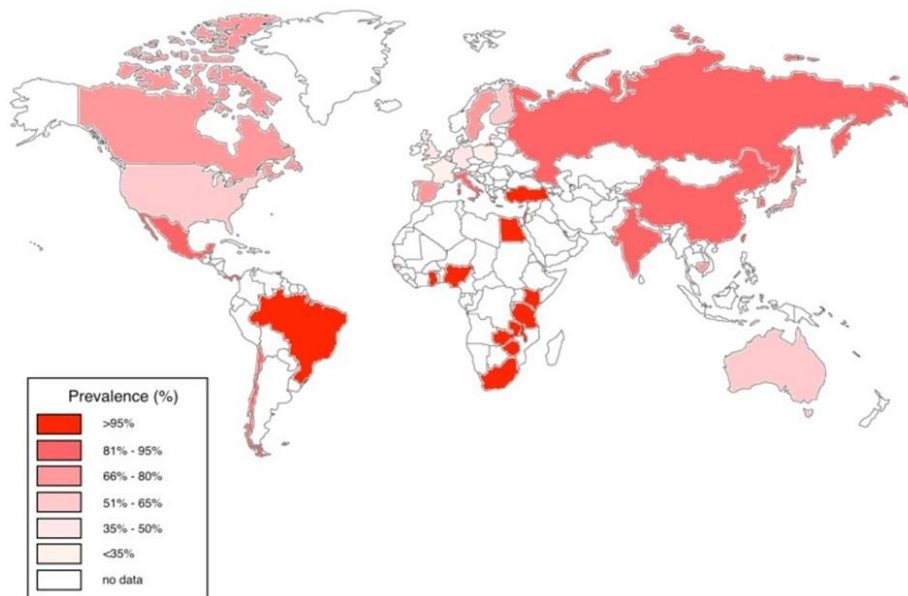


Figure 5. Worldwide HCMV seroprevalence rates in adults. Studies of adults aged 16–50 years published between 2005 and 2015 from Australia, Belgium, Brazil, Canada, Cambodia, Chile, China, Finland, France, Gambia, Germany, Ghana, India, Italy, Japan, Kenya, Mexico, Nigeria, Panama, South Africa, Spain, Sweden, Taiwan, Tanzania, Turkey, UK, USA, Zambia, and Zimbabwe. Figure and Figure legend is adapted from (Adland *et al.*, 2015)

After infection, HCMV is excreted for months to years in almost all the body fluids: urine, tears, milk, semen, saliva, and cervical secretions. Actually, the infection by HCMV is usually mild and subclinical. Unsuspecting host has the ability to transmit the virus to others either horizontally or vertically. The virus can appear following the primary infection, and also after reinfection, or reactivation. Mothers can transmit the infection to their infants through the placenta, during delivery, and by breast feeding ^{77,78}. Either women who are infected for the first time during pregnancy or those infected long time before conception (recurrent infection), can spread the infection via the placenta. Around 30-40% of children are infected with HCMV by the age of one year ⁷⁹. Pregnant women with primary HCMV infections have higher risk of transmitting the virus to the

fetus with substantial risk of birth defects⁸⁰. The congenital infection is asymptomatic at birth in most cases. The prevalence of newborns infection is between 0.2% and 2.5% of all births; only about 10-15% of them have clinical symptoms and only few show congenital abnormalities⁸⁰. Furthermore, the most common route of viral transmission is breast-feeding. Transmission depends on the duration of breast-feeding and the viral load in the milk⁸¹. For instance, it has been shown that no infection occurs in infants nursed for <1 month in comparison with 40% infected infants who nursed longer. In addition, when the virus can be isolated from the milk, up to 69% of infants are infected, whereas when the mother is seropositive and negative for the virus in milk, only 10% of infants are infected. A strong relationship between the presence of viral DNA in the milk and the transmission to infants has been demonstrated by several polymerase chain reaction (PCR) studies⁸². Moreover, preterm infants who are born before the gestational age of 30 weeks and below 1000g birthweight are at higher risk of acquiring an early and symptomatic infection than the infants born at term⁸³. HCMV is the leading cause of permanent hearing loss in those infants⁸⁴.

During childhood, HCMV could be transmitted to children in the nurseries or preschool centers through contaminated toys or by direct contact. Infection increases in people living in crowded, unhygienic conditions, and thus it is most common in countries with disadvantaged socio-economic conditions. In fact, most children who correspond to populations with low socio-economic backgrounds show infection at the onset of puberty, whereas in the industrial countries <40% of adolescents are infected followed by an increase of ~1% per year⁷⁷.

8- Clinical features associated with HCMV infection

8.1 Infection in immunocompetent hosts

In general, HCMV infection is subclinical in immunocompetent hosts, nevertheless 8% of all the cases of mononucleosis result from HCMV infection⁸⁵. Common symptoms may also include: splenomegaly, lymphadenopathy, fever, headache, malaise, lethargy and sore throat⁸⁶.

8.2 Congenital infection

Newborns that are infected congenitally show cytomegalic inclusion disease. Symptoms include intrauterine growth retardation, petechiae, thrombocytopenia, chorioretinitis, hepatosplenomegaly, jaundice and hepatitis, along with the involvement of the CNS in the form of encephalitis, microcephaly, focal neurological signs and seizures, deafness and hearing loss ^{87,88}. In the vast majority of cases, most of the non-CNS (liver and blood-forming organs) manifestations are self-limiting and resolve without therapy. However, the neurological damage is permanent and causes long-term morbidity with poor prognosis of cytomegalovirus inclusion disease ⁸⁷. Prospective studies conducted on long-term indicate that 80% of infants having symptomatic congenital infection will exhibit serious life-long neurological abnormalities. In 11-20% of cases, the damage caused leads to the death of the patient during infancy ⁸⁹.

8.3 Infection in the immunocompromised host

In contrast to the relative mild HCMV infection in healthy people, this virus can cause life-threatening disease in immunocompromised patients. In immunocompromised patients, HCMV is a highly opportunistic pathogen. Patients may have primary infection, reactivation of latent virus, or reinfection, and often the infection is clinically silent whatever its type. Spiking pyrexia is the mark of the onset of infection, however, it may resolve in few days ⁸⁹. The severity of this pyrexia is roughly parallel to the level of immunosuppression, where it is the greatest in AIDS patients having low counts of CD4+ T-cell and in bone marrow transplant (BMT) recipients. Patients receiving immunosuppressive chemotherapy, solid organ transplant recipients, and subjects with congenital immunodeficiencies may also be symptomatic ⁸⁹.

In organ transplant patients, HCMV infection is associated with either acute or chronic transplant rejection, in addition to other post transplant related complications ⁹⁰. Reports mentioned a significant morbidity and mortality in patients undergoing either organ or stem cell transplantation as well as in AIDS patients. HCMV infection can be found in various organs in the body of immunocompromised patients; it can cause pneumonitis, gastrointestinal disease, retinitis, but rarely encephalitis ⁹¹. Increasing evidence also exhibited a significant association between HCMV seropositivity and

atherosclerosis and increased cardiovascular mortality ⁹². Furthermore, HCMV has been correlated also with rheumatoid arthritis ⁹³, systemic lupus erythematosus ⁹⁴, Sjögren's syndrome ⁹⁵ and inflammatory bowel diseases ⁹⁶. Such studies suggest a link between HCMV infection and autoimmune diseases.

9- Infection routes

Although epidemiologic studies have elucidated that blood transfusion is not a common route of viral transmission, it was noted in the mid-1960s that there is primary infection with HCMV through blood transfusions. Culturing attempts of the HCMV from fresh donor blood have scarcely been successful. Thus, it is assumed that for healthy donors, the virus present in their blood cells is latent and is reactivated after transfusion when they encounter an allogeneic stimulus. Attention is being increasingly focused on the monocytes/macrophages as carriers of the latent virus ^{30,61,97}. Moreover HCMV is also a significant post-allograft pathogen. It has been shown by several studies that seronegative patients, who receive an organ from seropositive donors, have high risk of acquiring a primary infection with developing a more severe disease than seropositive recipients of seropositive organs. This indicates that acquired immunity changes the infection prognosis ^{98,99}. Furthermore, as the level of immunosuppression increases, the risk of HCMV reactivation and pathogenicity increase. The source of allograft, the type of immunosuppression, donor and recipient serological status, the type and amount of blood products used and HLA matching of donor and recipient, all are factors contributing to post-transplant infection ^{100,101}.

It is impossible to determine the routes of transmission after a postnatal infection, because of the absence of symptoms. The molecular analysis of CMV isolates has greatly enhanced our understanding of the epidemiology of CMV ¹⁰².

In pregnant women, the primary infection with HCMV does not usually result in clinical illness and therefore is difficult to identify. Intrauterine infection occurs in only one-third of primary infected pregnant women and the biological mechanisms preventing the infection of the fetus are not wellknown, although the macrophages that present in the placenta may form a barrier ¹⁰².

The main perinatal route of infection is through the ingestion of infected maternal genital secretions or breast milk. Because of recurrent maternal infection, copious quantities that contain high virus titers will surround the fetus during delivery where it may result in virus transmission to the newborn ¹⁰³.

In breast milk, virus titers are usually low, but feeding for a long time will result in the build-up of an effective inoculum. Women with infected breast milk, who feed their newborns with formula milk prevent them from infection. When the virus is ingested, it infects the mucosa of the esophagus, oropharynx, or the upper airways ¹⁰³. Premature infants are at greater risk of developing severe symptoms of HCMV infection via maternal milk ¹⁰⁴.

10- Pathogenesis and pathology

Histologically, CMV is recognized by its characteristic intranuclear inclusions “owl eye” with a surrounding halo and margined chromatin. The cytomegalic cells are typically found in the bile duct, salivary gland, islet cells, bronchial and renal tubular epithelium, the capillary endothelium, astrocytes, epithelial cells of the inner ear, and neurons ⁸⁹. Evidence showed that in cases of severe disseminated disease, HCMV involvement can be found in all organs. The involvement of salivary gland is probably chronic, and since it is more frequent in infants and young children and decreases with age, it is probably the outcome of subclinical congenital and perinatal infection.

Moreover, viruria results from the replication in the genitourinary tract and it is constant in all age groups ³⁰. In the kidney, renal infection rarely leads to dysfunction in normal individuals but, it has direct involvement in renal transplant dysfunction and rejection ¹⁰⁵. In immunocompetent individuals, elevated levels of liver enzymes indicate a subclinical hepatitis and can be associated with HCMV infection ¹⁰⁵. Furthermore, HCMV pneumonia is uncommon in immunocompetent individuals, while it is severe in immunosuppressed patients, particularly in BMT and heart-lung recipients ^{106,107}. The damage of the CNS is a frequent feature of congenital infection ¹⁰⁸. Symptoms include seizures, hypotonia, mental retardation, and hearing loss. In histological sections, inclusion-bearing cells that are positive for viral antigen have been seen in neurons, meninges, ependyma, glia, choroid plexus, and vascular endothelium. These cells are also spread in the vestibular membrane, the semicircular canals, and cochleae ⁸⁹.

11- Host defences

The immune system is responsible for controlling the infection by HCMV. First, HCMV infection induces an innate immune response, followed by adaptive and cell-mediated responses. However, HCMV is rarely cleared totally and its genome remains in a latent state from which it can reactivate under immune suppression ¹⁰⁹. Individuals with impaired cell-mediated immunity can be subject to severe infections since the primary anti-HCMV response is provided by cell-mediated immunity. However, we should not overlook the supportive role provided by the humoral system to keep HCMV loads below critical thresholds. The presence of several HCMV strains in the same individual indicates that the immune response does not protect the host from being re-infected, however it may do protect from symptomatic recurrent infections ⁸⁹. In addition, HCMV has developed multiple strategies to evade the host immune system and remain latent for a long period of time.

11.1 HCMV and NK cell response

NK cells play a major role in the innate immune response, mainly in parasitic and viral infections. They also help in driving the adaptive immunity. The recurrent HCMV associated disease observed in patients with NK cells defects, had led to a better understanding of the importance of such cells in immune responses against viruses ¹¹⁰. NK cells can be activated by a balance of several activating and inhibiting cell surface ligands. The TLR2 receptors recognize the HCMV gB and gH and initiate the production of proinflammatory cytokines and interferons. This subsequently results in the activation of NK cells through NF-kB ¹¹¹. Moreover, HCMV infection induces the expression of UL16 binding protein (ULBP1 and 2) ligands, which will activate the NK-cells via the NKG2D receptor.

11.2 Humoral immunity against HCMV

Immunocompetent individuals produce during the primary infection anti-HCMV immunoglobulin M (IgM) antibodies. These will persist for 3-4 months to be followed after few weeks by immunoglobulin G (IgG) antibodies with lifelong persistence. This humoral response is beneficial as shown by experimental and clinical findings where the mice immunization against murine CMV gB was protecting them against a lethal

challenge ¹¹². Also in the same way, pregnant guinea pigs that were immunized against guinea pig CMV envelope glycoprotein provided protection for their fetuses ¹¹³. The immune system is capable of recognizing many proteins of CMV ¹¹⁴. The targets of virus-neutralizing antibodies are mostly the envelope glycoproteins ⁸⁹. As compared with the other components of the envelope, gB is the predominant target because it is immunodominant and expressed at high abundance. Antibodies against gH are potent, complement-independent, but with minor neutralizing activity. Furthermore, viral tegument components trigger an intense antibody response lasting for a long time and providing an indirect measure of the replication of the virus and correlating with the clinical outcome. These tegument components include pp150 (pUL32), pp65 (pUL83) and pp28 (pUL99). However, these antibodies have a limited importance for a protective response since they are unable to react neither with the surface of virions nor with the infected cells ⁸⁹.

11.3 Anti-HCMV Cell mediated immunity

Early studies indicated that recovery from CMV infection requires the response of CMV-specific cytotoxic T lymphocyte (CTL). The suppression of CMV-specific CTLs causes the reactivation and dissemination of CMV infection. Further studies revealed that both CD8⁺ cytotoxic T-cells and natural killer (NK) cells have a primary importance in preventing the viral recurrence ^{99,115,116}. Experiments done on mice with deficiency in perforin and granzyme or Fas-mediated cytotoxicity have demonstrated that NK cells and CD8⁺ cells control the viral replication through the perforin and granzyme-mediated pathways, while the Fas-Fas ligand system is not critical ^{117,118}.

Following CMV infection, 18-58% of CTLs recognize the IE1-expressing target cells, whereas only less than 6% of CTLs are gB-specific. Subsequent studies had shown high frequencies of CTLs specific for pp65 and IE1 proteins demonstrating that they are the chief targets of the immune response mediated by CTLs ⁸⁹. In vivo evidence of the effects of CTLs in controlling HCMV infection has been obtained by the adoptive transfer of virus-specific clones. In addition, after BMT to seronegative recipients of marrow from seropositive donors, the passive administration of autologous HCMV-specific CD8⁺ CTLs at set intervals generated a vigorous CTL response specific to CMV without the onset of viremia or CMV disease ¹¹⁹. Thus these studies demonstrate a critical role for

CMV-specific CTLs in the control of CMV infection, and highlighted new therapeutic perspectives to control CMV infection in transplant recipients.

The immune system can recognize various HCMV peptides, and it is remarkably strong and increases with age, where it may constitute around 50% of the CD8⁺ T-cell repertoire in elderly individuals ¹²⁰. Ex vivo experiments showed that there is a protection from HCMV primary and recurrent infection in patients who received ex vivo expanded CD8⁺ T cells. However, the function of infused HCMV-specific CD8⁺ T cells was dependent on the presence of HCMV-specific CD4⁺ cells, which suggest that CD4⁺ helper T cells might be critical for an optimal CD8⁺ T cell responses ¹²¹. Actually, CD4⁺ T cells have an important role in controlling HCMV infections. They recognize the viral peptides presented on the antigen presenting cells via MHC-II molecules ¹²² and participate to the clearance of CMV-infected cells ^{123,124}. Another minor subset of T cells that also expands during HCMV infection are the gamma delta T-cells. These cells are able to mediate cytotoxicity of infected cells ¹²⁵.

12- Immune evasion by HCMV

HCMV infection is characterized by the persistence of the viral genome at specific anatomical sites in the normal host for months or even years, often at a latent viral state ¹¹⁶. Several factors are involved in allowing the virus to escape from immune clearance. Such factors include: (1) exploiting tissues that are immunologically privileged for replication (i.e., epithelial cells of the salivary glands that express insufficient levels of MHC Class I molecules to trigger the clearance by CD8⁺ cells), (2) inducing a latent state of infection, (3) expressing genes interfering with the immune response ²⁸.

NK cells are an important defense line against virally infected cells. HCMV has several strategies to subvert the NK cell-mediated killing of infected cells (reviewed in ⁶⁰). HCMV pUL18 protein is a MHC-I homologue which triggers leukocyte immunoglobulin-like receptor (LIR-1) to inhibit NK cell responses ¹²⁶. Moreover, the pUL16 protein is binding to some ligands such as MHC class I polypeptide-related sequence B (MICB) and pUL16 binding protein 1 and 2 (ULBP1 and 2). These ligands are recognized by the NKG2D receptor. Therefore pUL16 prevents the NK cell activation

that is mediated by NKG2D. pUL16 also protects the infected cells, by an unknown mechanism, from the effect of cytolytic proteins that are released from NK cells ^{127,128}.

In addition, most of the cells have a surface receptor that deliver an inhibitory signal to NK cells. This receptor has a specific interaction with the nonclassical MHC Class I molecule HLA-E which requires the binding of a specific nonamer peptide. The UL40 gene of HCMV encodes for a nine-amino acid sequence which is exactly similar to the HLA-E binding peptide, thus it will upregulate the HLA-E cell surface expression, and inhibit the NK cell-mediated cytotoxicity ¹²⁹.

In addition, the complement system is also considered as a main line of immunological defence against the invading pathogens. HCMV interferes with the complement activation by inducing the cellular proteins CD35, CD46, and CD55. These induced proteins will inhibit opsonisation by phagocytic cells and thus will prevent the complement-mediated cell lysis ¹³⁰.

There are various HCMV gene products that are known to interfere with the antigen presentation via MHC molecules. MHC peptide presentation is required for the CD8+ cytotoxic killing. HCMV pUS3 favors the MHC-I molecules retention in the endoplasmic reticulum (ER), pUS6 causes the inhibition of peptide translocation, which is mediated by the transporter associated with antigen processing (TAP), into the ER. Also, pUS11 mediates the dislocation into the cytoplasm of MHC-I heavy chain, and pUS2 provides the export of MHC-I heavy chain from the ER. The suppression of antigen presentation will prevent the T-cell mediated killing of cells infected with HCMV (as reviewed by ⁶⁰). Furthermore, the HCMV pUS2 and pUS3 decrease the MHC-II surface expression and thus favor the escape from CD4+T cell recognition ¹³¹.

13- Biological diagnosis

13.1 Isolation of HCMV by cell culture in MRC5 cells

MRC5 are cultured and maintained in MEM until their inoculation with the clinical specimens which could be from various body fluids such as saliva, blood, amniotic fluid, vaginal secretions, and urine. The culture is then monitored for the development of the typical cytopathic effect (CPE) of HCMV infection ¹³².

13.2 Detection of HCMV load by quantitative PCR

The quantitative real-time PCR is a useful technique for the rapid diagnosis of HCMV infection either in the plasma or in other biological samples. The real-time PCR method is based on the use of a fluorogenic probe and an instantaneous laser scanning in order to measure the accumulation of PCR products which will be monitored after each amplification cycle¹³³.

13.3 HCMV serological diagnosis

The detection of HCMV-specific immunoglobulin IgG and IgM is used for the determination of a past or recent HCMV infection. Investigation is performed with ELISA tests¹³⁴. HCMV-specific IgM is a specific and sensitive indicator for the presence of a primary HCMV infection, but can also be present in viral reactivation¹³⁵. Measuring the HCMV-IgG avidity index has been recently shown to be useful to determine the time frame following a primary HCMV infection. The detection of low HCMV-IgG avidity indicates a primary HCMV infection that has just occurred during the last 18-20 weeks, while the detection of high HCMV-IgG avidity will indicate an HCMV infection which started several months ago¹³⁵.

14- HCMV Treatment.

Antiviral drugs available to treat HCMV infection are as follows: ganciclovir, valganciclovir, foscarnet, cidofovir, fomivirsen. Except fomivirsen, all other drugs are directly or indirectly targeting the viral DNA polymerase pUL54. However, all these drugs have side-effects and may result in drug resistance¹³⁶.

14.1 Ganciclovir (GCV) and Valganciclovir (VGV)

GCV is an acyclic nucleoside analogue of 2'-deoxyguanosine, it gets incorporated instead of the nucleotides and inhibits viral DNA synthesis. It has to be phosphorylated by the pUL 97 viral protein kinase in order to become biologically active as a triphosphate form¹³⁷. Patients infected with HCMV strains that contain mutations in the UL97 gene or the DNA polymerase gene (UL54) had shown resistance to GCV¹³⁸. GCV was the first antiviral drug available for HCMV infection treatment, and it is still the drug of choice. It is available as a sustained-release intraocular implant and also in intravenous formulation. VGV is a pro-drug of GCV which is available as enteral formulation, which rapidly metabolize to the active form

in the liver and intestinal wall ¹³⁹. The major side-effects include: hepatic and renal toxicity, haematological abnormalities (primarily anemia, thrombocytopenia, and neutropenia)¹³⁹.

14.2 Foscarnet

Foscarnet is a pyrophosphate analogue, which inhibits viral DNA polymerase and does not require enzyme activation after intake ¹³⁶. It is manufactured as intravenous formulation. Its administration requires extensive pre-hydration and slow infusion. Serum creatinine levels should be monitored during treatment due to its potential nephrotoxicity as side-effect ¹⁴⁰. This drug is used for those who cannot be treated with GCV due to developing resistance to GCV or dose-limiting neutropenia or leucopenia, ¹⁴¹.

14.3 Cidofovir

Cidofovir is an acyclic nucleoside phosphonate analogue which becomes activated by cellular kinases. It causes premature termination in viral DNA synthesis and inhibits the viral DNA polymerase. Cidofovir has low oral bioavailability. It is only produced as intravenous formulation. Due to its side-effects, this drug is used only as a second line therapy ¹⁴². The major side effects are neutropenia, renal toxicity, and electrolyte imbalance. Moreover, preclinical studies had shown it to be teratogenic and carcinogenic ¹⁴³.

14.4 Fomivirsen

This drug acts as an anti-sense RNA inhibitor (5'-GCG TTT GCT CTT CTT CTT GCG-3') with phosphorothioate linkage that protects it from degradation. It binds complementary to HCMV IE86 RNA and inhibits its translation. The pIE86 protein have an important role in the production of early and late proteins. It is also important for viral replication. Fomivirsen drug is available as an intravitreal formulation, used for treating HCMV retinitis in immunocompromised patients, mostly HIV patients. Its half-life is long around 55 hours, and it has no systemic effects during treatment due to its intraocular administration ¹⁴⁴.

15- Prevention of HCMV infection and disease

The risk of primary infection in solid organ transplant recipients could be reduced by matching seronegative donors and recipients, although the scarcity of donor organs hampered this step ¹⁶⁷. The use of antiviral agents and pre-emptive therapy as a prophylaxis has proved their efficiency in transplant patients ¹⁴⁶. To avoid transmission of the virus through blood transfusion to pregnant women, immunocompromised individuals, and premature newborns, the use of HCMV-seronegative, filtered, or leukocyte deprived blood products could be an option. Although applying this method is placing a greater burden on blood transfusion centers, but it shows a significant reduction in HCMV transmission, thus it should be applied whenever possible ^{147,148}. Another way to protect groups at risk from infection is through the administration of an HCMV vaccine to provide pre-exposure immunization but until now, there is no definitive commercial vaccine for HCMV infection.

HCMV and Cancer

Cancer results from an imbalance between cell proliferation and cell death. A number of genetic changes occurring in the cell lead to its transformation. There are many factors involved in the oncogenic process such as: lifestyle, host factors, environment, inheritance and infectious agents. By understanding the etiology of cancer and its risk factors, there will be a useful tool helping in the identification of high-risk populations and the improvement of either the screening procedures or the preventive methods ^{149,150}. Infectious agents could be one of the important preventable causes of cancer. Within this group, the most common agents that are involved in the pathogenesis of human cancer are viruses. Recently, estimates showed that infectious agents attribute to 17.8% of cancer cases, where viral infections correspond to 12.1% of them ¹⁵¹.

Several DNA viruses are known to be associated with neoplasms. Human T-cell Leukemia virus-1 is associated with T-cell leukemia; hepatitis B and hepatitis C viruses with hepatocellular carcinoma; Kaposi's sarcoma associated herpesvirus with Kaposi's sarcoma and primary effusion lymphomas; Epstein-barr virus with B-cell lymphoproliferative diseases and nasopharyngeal carcinoma; human papillomaviruses are associated with cervical carcinoma ¹⁵², and Merkel cell polyomavirus is associated with Merkel cell carcinoma ¹⁵³. All these viruses can cause cancer although they are not belonging to the same family, and they are referred to as human oncoviruses. All these viruses share common features including their ability to infect host cells and persist in the cell. They establish a long term persistent infection by evading the immune system of the host ¹⁵⁴. Usually, the infection by itself is not sufficient to trigger the cancer. Host immunity and chronic inflammation are two factors that play an important role in promoting the conditions for neoplastic cells to proliferate ¹⁵².

1- Human oncoviruses

1.1 Hepatitis B virus (HBV) and hepatitis C virus (HCV)

Around 80-90% of all the hepatocarcinoma (HCCs) arise from a cirrhotic liver. Annually HCC occurs in 1-7% of HCV-infected cirrhotic patients ¹⁵⁵ and 3-8% of HBV-infected cirrhotic patients ¹⁵⁶. Typically the HCC follows several years of chronic viral

infection¹⁵⁷, which lead to the development of cirrhosis in about 20-30% of patients, and subsequently to HCC¹⁵⁸.

HBV has a small circular double-stranded DNA genome and is a member of the Hepadnavirus family. The genomic integration of HBV is present in over than 85-90% of HBV-related HCCs and is usually preceding the development of HCC. HBV is not only found in HCC, but it is also present in non-tumoral tissue of patients with chronic HBV^{159,160}.

In the host genome, the integration of HBV induces a wide range of genetic alterations including genomic instability, production of fusion transcripts, chromosomal translocations, deletions, and amplification of cellular DNA^{161,162}.

The HBx protein is an HBV protein whose sequence is commonly integrated into host DNA in HCC. HBx is responsible for the activation of several cytoplasmic signaling pathways including: stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK), activator protein-1 (AP-1), AP-2, protein kinase C (PKC), JAK/STAT, PI3K, ras-raf-MAPK, Smad and Wnt, nuclear factor- κ B (NF- κ B), and by binding to nuclear transcription factors including Oct-1, TATA box binding protein (TBP), activating transcription factor 2 (ATF-2), and cAMP response element binding (CREB)^{161,163}. HBx also causes a transcriptional repression of the *p53* gene¹⁶⁴ and interacts with p53 leading to an inactivation of several important p53-dependent activities, including cell cycle regulation, DNA repair, apoptosis and tumor suppression¹⁵⁸.

HCV is a single-stranded RNA virus of the flaviviridae family. It is unable to reverse transcribe its genome and thus to integrate it into the host genome. The core protein of HCV is shown to be responsible of a wide range of functions and interacts with many cellular proteins. It has been proposed to be involved in lipid metabolism, transcriptional activation, signal transduction, apoptosis, reactive oxygen species (ROS) formation, transformation and immune modulation^{165,166}. HCV core protein also binds to tumor suppressor proteins, including pRb, p73 and p53^{167,168}. It interacts with p73, causing a nuclear translocation of the core protein and prevents, in a p53-dependent manner, the p73- α -dependent cell growth arrest^{169,170}. HCV core is also involved in the modulation of the expression of the major target of p53, the cyclin-dependent inhibitor

p21WAF1, and thus regulates the activities of cyclin/cyclin-dependent kinase complexes that are involved in cell-cycle control and tumor formation ^{171,172}.

1.2 Human T-cell lymphotropic virus (HTLV-1)

HTLV-1 is a single-stranded RNA virus. It was the first human retrovirus known to be associated with malignancy ¹⁷³. Evidences indicate that the viral protein responsible for the leukocyte-transforming and oncogenic features of HTLV-1 is its regulatory protein p40Tax ^{174–176}. Tax protein had been shown to be necessary and sufficient for the cellular transformation. Moreover, the cellular proliferation is reversibly arrested in G1 phase when Tax transcription is blocked, indicating a critical role for the G1- to S-phase transition in immortalization of T lymphocytes ¹⁷⁷. The singular expression of Tax in transgenic mice revealed the induction of various tumors (including leukemia). Tax is responsible for the alteration of mitotic checkpoints by direct effect on the mitotic arrest defective protein (MAD1), thus causing aneuploidy. Also Tax interferes with the DNA repair mechanisms via an interaction with the chromatid remodeling factor, histone deacetylase 1, or by causing the repression of the activity of DNA β -polymerase. Thus Tax contributes to the instability of the genome and to the malignant transformation ^{178,179}. In addition, Tax promotes anti-apoptotic effects by impairing the p53 function. It also activates the pathways that have particularly potent pro-proliferative effects on lymphocyte, NF- κ B and activator protein1 (AP-1) ¹⁷⁸. Moreover; Tax causes an indirect suppression of pRb, where it activates the cyclin D/CDK4-6 complex, thus leading to cell cycle progression ^{178,180}.

1.3 Kaposi's sarcoma associated herpesvirus (KSHV) or Human herpesvirus 8 (HHV-8)

Human herpesvirus 8, also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is a double-stranded DNA virus that belongs to the gamma herpesvirinae sub-family. HHV-8 is linked to the Kaposi sarcoma (KS) and primary effusion lymphoma ¹⁸¹.

The latently expressed KSHV proteins that play a role in promoting cell survival and proliferation and thus may be involved in cellular transformation include: viral cyclin (v-cyclin, ORF72), viral interferon regulatory factor 1 (vIRF-1), the ORF73–71 locus-encoded latency associated antigen (LANA, ORF73), viral FLICE inhibitory protein

(vFLIP, ORF71), and the Kaposin/K12 gene. LANA induces cellular proliferation and survival^{182,183}, v-cyclin stimulates cell cycle progression¹⁸⁴, vIRF3 and vFLIP mediate the pro-survival signaling^{185,186}, and kaposins promote cytokine expression and cell growth¹⁸⁷.

In addition, KSHV expresses several immune-evasion genes which include: vIRFs, Orf45, MIR1, MIR2, and complement control protein homolog (CCPH). These immune evasion proteins ensure the life-long persistence of the virus in the host, and thus, contributes to the pathogenesis associated with KSHV (reviewed in^{188,189}).

1.4 Epstein - Barr virus (EBV)

EBV is a double stranded DNA virus that belongs to the γ herpesvirus subfamily. More than 95% of the worldwide population are infected with EBV¹⁹⁰. EBV infections occur mainly during the childhood and are usually asymptomatic. However the post-adolescent infection with EBV frequently leads to the self-limiting lymphoproliferative disease called infectious mononucleosis¹⁸¹.

In immunocompromised patients, cells infected with EBV reveal an increase in number and activation of the B-cell growth control pathways leading to transformation and malignancies¹⁹¹. EBV encodes several viral proteins with a transforming potential including EBV nuclear antigen 2 and 3 (EBNA2 and EBNA3) and EBV latent membrane protein 1 and 2 (LMP1 and LMP2). LMP1 interacts with several tumor necrosis factor receptor associated factors (TRAFs), result in the activation of NF- κ B, Jun, and p38 in LMP1-expressing epithelial and B cells^{191,192}. LMP2 is dispensable for naïve B cells transformation but is required for the transformation of post-germinal center B cells. LMP2 interacts with Syk and Lyn to mimic B-cell-receptor (BCR) signaling, which include the activation of the PI3K/AKT survival pathway¹⁹³.

The viral proteins EBNA2 and EBNA3 are also involved in the transformation process. The human peripheral blood B cells infected in vitro with EBV result in long term growth of EBV transformed lymphoblastoid cell lines (LCLs). Removing the EBV signals stops the proliferation of the cells, however by restoring the expression of LMP1 or EBNA2, the cells start to proliferate again¹⁸¹.

1.5 Human Papillomavirus (HPV)

Papillomaviruses (PVs) are small nonenveloped double-stranded DNA viruses of the Papillomaviridae family. They infect the squamous epithelia of different species. Approximately 200 types of human papillomaviruses (HPV) have been identified ¹⁹⁴. HPVs can be classified into two groups: cutaneous and mucosal which are classified further depending on the propensity of the associated lesions for the progression into malignancies of either high or low grade ¹⁸¹. The cutaneous HPVs 5 and 8 can be considered of high-risk. They are associated with epidermodysplasia verruciformis (EV), a rare genetically determined skin disease. These lesions favor the appearance of skin cancers especially in sun-exposed areas of the body. The first HPV cancer type associated with the HPV infections was skin cancer in EV patients ^{77,195–197}. HPV 5 and 8 and the related cutaneous HPVs may be associated with the development of non-melanoma skin cancer (NMSC), especially in the immunocompromised patients ^{198,199}.

Within the mucosal HPVs, the concept of high-risk and low-risk HPVs has been clearly established. HPV6 and 11 are low-risk mucosal HPVs that cause genital warts. In contrast HPV16 and 18 are high-risk mucosal HPVs causing squamous intraepithelial lesions that can develop into invasive squamous cell carcinoma. HPV also contribute to oral and other anogenital malignancies, but it is mainly associated with cervical cancer. Over 99% of cervical cancers are associated with HPV infections of high-risk. Malignant progression often occurs in the presence of other risk factors including: smoking, prolonged use of birth control pills²⁰⁰, and decreased immune function ¹⁸¹. The molecular mechanisms by which high risk HPV develop cervical cancer have been studied extensively, where several viral and host interactions that can be associated with transformation and malignancy have been identified (reviewed in ²⁰¹).

Frequently, the HPV genome integrates into a host cell chromosome, and the viral oncoproteins, E6 and E7, are often the only viral proteins that are expressed in HPV positive cervical carcinomas ²⁰². Viral genome integration often results in mutation and/or deletion of the viral genes. During integration, the expression of the transcriptional repressor E2 is often lost. Thus, the genome integration of HPV is often associated with higher and dysregulated expression of E6/E7 ²⁰³. The sustained expression of E6 and E7 is necessary for the transformed phenotype maintenance of cervical carcinoma cells ^{204,205}.

The transforming potential of the high-risk oncoproteins, E6 and E7 is related to their association with cellular regulatory protein mainly p53 and pRb (reviewed in ^{201,202}). Normally, p53 and pRb control the cellular differentiation, proliferation, and apoptosis. The abrogation of the normal biological activities of p53 and pRb, will place the cell at a risk of malignant progression. Moreover, HPV E6 and E7 expressing cells have a reduced ability to maintain the genomic integrity ²⁰⁶. HPV E7 acts as a mitotic mutator and thus induces multiple forms of mitotic abnormalities. These abnormalities include unaligned or lagging chromosomes, anaphase bridges, and mainly multipolar mitoses ²⁰⁷. The multipolar mitoses are considered as histopathological hallmarks of cervical lesions and cancer associated with high-risk HPV ²⁰⁸.

1.6 Merkel cell polyomavirus (MCV).

Initially MCV was detected by digital transcriptome subtraction in Merkel cell carcinoma (MCC) ¹⁵³. Merkel cell polyomavirus is a non-enveloped, icosahedral virus with a double stranded circular DNA genome of around 5,000 base pairs which belongs to the *Polyomaviridae* family ²⁰⁹. It was discovered first after identifying a fusion transcript between a human receptor, tyrosine phosphatase and undescribed viral T antigen ¹⁵³. MCV is found to be integrated in the tyrosine phosphatase type G receptor gene (PTPRG). Viral infections are often associated with an epigenetic silencing of the tumor suppressor genes (TSG). A frequent promoter hypermethylation of PTPRG, CDKN2A, and RASSF1A was detected in MCC tumors ²¹⁰. The MCV T antigens are expected to have a critical role in the viral life cycle. In vitro studies revealed that the activity of the large T antigen (LT) is required for an efficient DNA replication where it is enhanced by the small T antigen (sT) ²¹¹.

There are several suggested mechanisms of viral-induced transformation. Several groups had showed that the knockdown of pan-T antigen in MCC cell lines favors cell cycle arrest and cell death. These data suggest that the MCC tumor cells rely on the MCV T antigen protein for unchecked cell division ^{212,213}. A specific targeting of sT protein had shown that it exhibited an increased transformation capacity as compared to LT ²¹³. The most widely characterized role of polyomavirus sT antigen is its ability to bind to the cellular protein phosphatase PP2A and reprogram it. This role is highly associated to its

ability to promote the progression of the cell cycle and transformation (Reviewed in 214,215).

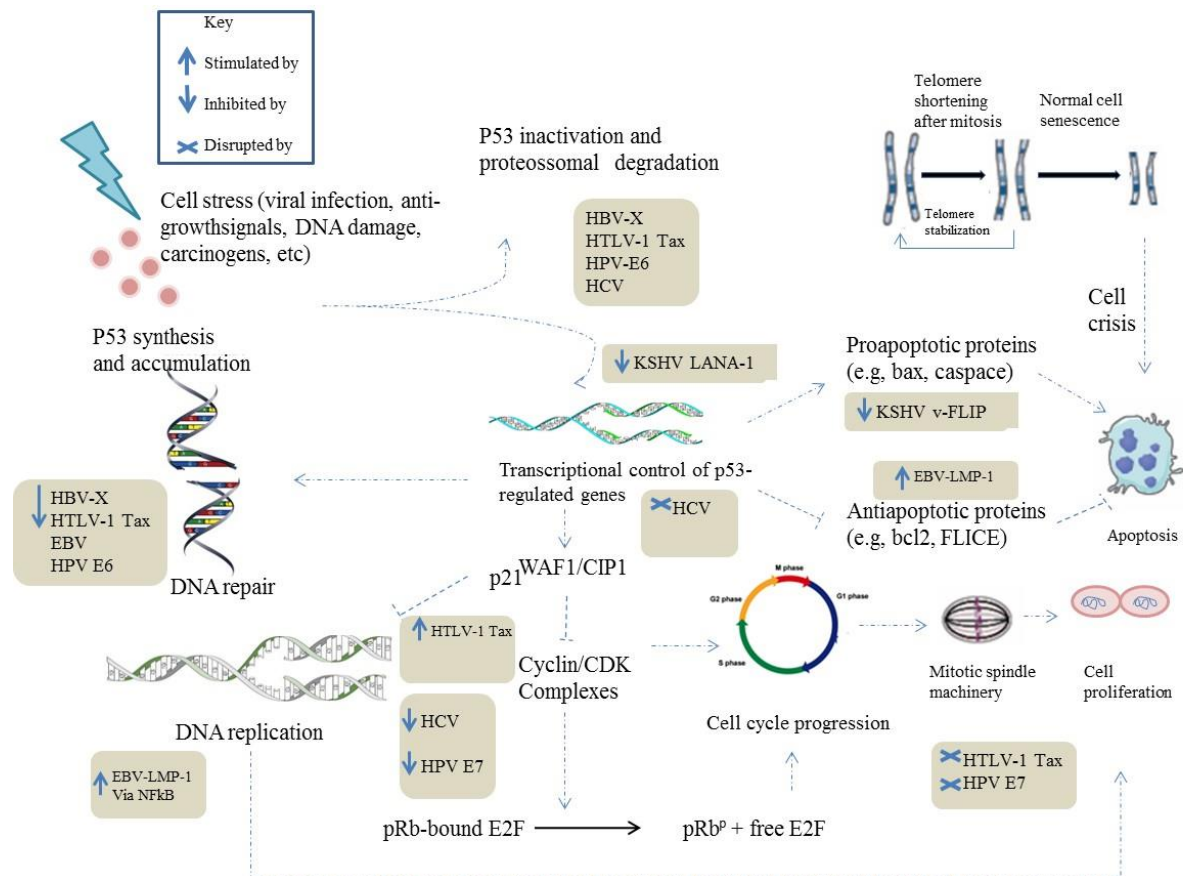


Figure 6. Overview of cellular pathways that are targeted by known tumor viruses. *Modified from (Elgui de Oliveira 2007)*

2- HCMV and cancer hallmarks

Cancer, a term used for diseases in which the abnormal cells divide without any control and can invade the nearby tissues. It can also spread, through the blood and lymph systems, to other parts of the body. Scientists had identified several hallmarks of malignancy. These include: the uncontrolled and sustained cell growth, genomic instability, lack of senescence, resistance to induced cell death, insensitivity to negative growth regulation, angiogenesis and invasion and metastasis (reviewed by ²¹⁶).

Recent work suggests that HCMV encodes for more than 750 proteins which may interfere with several physiological functions in the infected cells. This makes the HCMV far more complex than what was believed before ²⁷. HCMV gene products had shown their ability to alter the cell cycle progression differently in various cells. This may be dependent on the state of the cell, differentiation and/or immortalization, which make it possible for the virus to have a paradoxical role where it blocks growth in non-transformed cells and promotes it in tumorous cells ^{217,218}. The influence of HCMV infection on cancer hallmarks will be described further in this section.

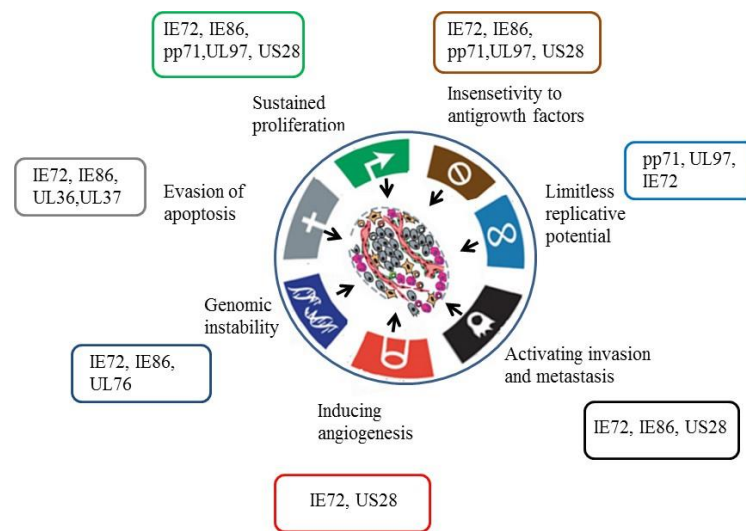


Figure 7. Overview of HCMV genes that interfere with hallmarks of cancer. Adapted and Modified from (Hanahan & Weinberg, 2011).

2.1 Sustained Cellular Proliferation

Proliferation in normal cells involves a coordinated events series, which are often directed by cytokines and growth factors. In cancer cells, the deregulation of signaling networks results in the capability of cancer cells to proliferate endlessly. The two major signaling pathways that are frequently mutated in tumors are the MAPK and the PI3K/AKT signaling pathways. Either mutations or induced activation in these pathways will cause a stimulation of cell growth, survival and proliferation ^{219,220}. In addition, mutations in the feedback mechanisms are also involved in the enhanced proliferative signaling. For instance, defects occurring in the mammalian target of rapamycin (mTOR)

kinase or in PTEN phosphatase will lead to sustained proliferation because of their role in counteracting PI3K pathways in normal conditions ^{221,222}.

It has been shown that HCMV has developed multiple mechanisms to ensure MAPK activation in the infected cells ²²³. In addition, HCMV infection of normal fibroblasts activates rapidly the host cell mitogen pathways, for instance the PI3K pathway ²²⁴. Furthermore, HCMV infection has been shown to have a role in the development of tumors through the pUS28 viral protein which is responsible for the induction of several factors playing an important role in tumor cell migration, inflammation, angiogenesis, and tumor progression. These factors include IL-6, cyclooxygenase-2 (COX-2), STAT-3, and vascular endothelial growth factor (VEGF) ^{225,226}. Additionally, several HCMV proteins are involved in the inactivation of Rb family of proteins, and thus promote the entry of the cells into the S phase including the tegument protein pp71 ²²⁷, IE-72, IE-86, ²²⁸, and pUL97 ²²⁹. IE-72 and IE-86 can also interact with the p53 suppressor proteins causing deregulation of the cell cycle checkpoints controls ²²⁸.

2.2 Genomic instability

Genomic instability is considered as one of the most important requirements for cellular transformation and oncogenesis. The deregulation of DNA repair pathways leads to genetic instability. The p53 protein has a critical role in the DNA repair, and mutations in p53 could result in an uncontrolled DNA repair mechanism. In addition, telomerase activity is highly important for the maintenance of the genome integrity, and the loss of telomeric DNA will lead to karyotypic instability and the associated deletions and amplifications of chromosomal segments ²³⁰.

Various studies indicated that HCMV can induce genetic damage ²³¹ mainly in chromosome 1, where DNA strand breaks at positions 1q42, 1q21 and 1q23.3 in HCMV infected cells were observed, especially when the infection occurs while cells are in the S phase of the cell cycle ^{232,233}. Deletion of 1q42 chromosome had been related to the development of glioblastoma ²³⁴. Moreover, some tumor suppressor genes are proposed to be located at the positions 1q21-31 in patients with breast cancer ²³⁵. In addition the HCMV protein pUL76 has been shown to induce micronuclei, chromosomes

misalignment, bridging and lagging. This suggests that HCMV can induce the DNA damage and can cause an accumulation of chromosome aberrations ²³⁶. Shen et al. found in their study that HCMV IE-72 and IE-86 gene products can transform, in cooperation with adenovirus E1A protein, the primary baby rat kidney cells. They also found that in these transformed cells, p53 gene was mutated and thus this mutation could be one of the mechanisms involved in the contribution of IE proteins to transformation ²³⁷.

2.3 Limitless Replicative Potential

Senescence is a non-dividing state reached by the normal cells after their division for a limited period of time. It is a natural process that is controlled by several molecular mechanisms. Blocking the tumor suppressor proteins p53 and pRb will lead to the circumvention of senescence and thus causing the continuous division and multiplication of cells. This process is known as immortalization ²³⁸. Immortalization is a characteristic acquired by tumor cells during the transformation process and it is very typical for tumor cells in culture ²³⁹. The HCMV proteins pp71 and pUL97 are involved in the phosphorylation and inactivation of the Rb tumor suppressor family of proteins, thus they affect the proliferation and survival of the cells similar to what is done by typical human oncogenic viruses ^{227,229}. Moreover, Telomerase is highly expressed by cancer cells. It is an enzyme responsible for the protection of telomeres during each cell division from shortening, and thus preventing senescence and favoring a sustained cellular division ²⁴⁰. Oncogenic viruses commonly induce high telomerase activity. Strååt et al. showed that HCMV stimulates hTERT transcription and increases the telomerase activity. They also observed the co-expression of hTERT and HCMV IE-72 proteins in glioblastoma (GBM) tissue samples ²⁴¹.

2.4 Apoptosis blockade

Apoptosis or programmed cell death is an essential mechanism for the maintenance of tissue hemostasis. Apoptosis is activated by two separated pathways, the intrinsic and the extrinsic pathways. Both pathways lead to the activation of caspases which in turn induce cell death. The intrinsic pathway is activated by signals from DNA damage, stress signals, and loss of cell survival factors. It is a mitochondrial pathway that is regulated by the Bcl-2 family of proteins. Also, the p53 and Rb tumor suppressor proteins are involved in this pathway. The extrinsic pathway is activated from outside the cell. It is triggered

by cell death receptors such as the TNF- α receptor and the FAS ligand receptor (reviewed in ²⁴²).

HCMV, and other viruses, inhibit apoptosis through multiple strategies ²⁴³. They interact with the anti-apoptotic proteins either directly or indirectly. HCMV IE-72 and IE-86 proteins are involved in blocking apoptosis that is mediated by TNF- α ²⁴⁴. Moreover, HCMV IE-86 binds to p53 and inhibits the p53-mediated apoptosis ²⁴⁵. In addition, HCMV pUL36, also named viral inhibitor of caspase activation (vICA), and pUL37, also named viral mitochondria-localized inhibitor of apoptosis (vMIA) inhibit apoptosis through the inhibition of the activation of FAS mediated caspase ²⁴⁶ and the blockade of the pro-apoptotic proteins Bax and Bak activity, respectively ²⁴⁷. HCMV can also block the apoptotic machinery either directly through activating prosurvival pathways, e.g Wnt/ β -catenin and PI3K/AKT ²²⁶, or indirectly as a result of the induction of tumor promoting cytokines ²⁴⁸. Moreover, HCMV proteins had been shown to induce the expression of the anti-apoptotic protein Bcl-2 in neuroblastoma cells and colon cancer cells. The enhanced Bcl-2 upregulation results in acquired resistance to the cytotoxic drugs, which is reversed after treating the cells with HCMV antiviral drugs ^{249,250}.

2.5 Insensitivity to antigrowth signals

Under normal physiological conditions, the action of anti-proliferative signals is an essential mechanism for the maintenance of tissue homeostasis, in addition to the role of apoptosis ²⁵¹. Normal cells should be able to circumvent the powerful programs that regulate cell proliferation negatively in order to acquire the oncogenic capability. pRb and p53 are two important players in the cellular regulatory circuit in which they are responsible for deciding the cell fate, whether it should proliferate or be senescent. Rb protein receives many signals either from the intracellular or from the extracellular sources, and decides, in response, if the cell should proceed with its growth cycle and division or not. Cells with defected Rb will lose this service where the disruption of the pRb pathway will lead to the release of E2Fs and thus permits cells proliferation, and make the cells insensitive to the released anti-growth factors ²⁵¹. P53 is receiving abnormality sensors and stress signals from the intracellular operating systems where it can stop the cell cycle progression until normalization of cellular conditions²⁵¹. Several HCMV proteins are known to interact with p53 and pRb proteins as discussed previously. In addition HCMV IE-72 mediates transactivation of E2F-responsive promoters by

alleviating the p107 transcriptional repression and thus induce the cellular proliferation in quiescent cells ²⁵².

2.6 Angiogenesis

Angiogenesis is the process by which new blood vessels are formed. It is an essential component for the development of solid tumor which requires blood vessels for its progression and maintenance. The initiation of such process is controlled by a relative balance between the anti-angiogenic factors (e.g. thrombospondin-1 (TSP-1)) and the pro-angiogenic ones (e.g. basic fibroblast growth factor (bFGF) and VEGF) with an imbalance in favor of pro-angiogenic factors which will result in an enhanced formation of blood vessels followed by a huge effect on the proliferation of tumor and the formation of metastasis (as reviewed in ²⁵¹).

Currently, several pro-angiogenic and anti-angiogenic factors are known to be present in different tumor types ²⁵³. Angiogenesis can also be regulated indirectly either through the activation of oncogenes or by the loss of tumor suppressor genes which induce VEGF expression ^{254,255}. P53 loss of function, which can be seen in most human tumors can cause the downregulation of thrombospondin-1 (TSP-1) ²⁵⁶ and thus favoring the balance toward the pro-angiogenic factors. HCMV is able to modulate several ways to promote angiogenesis and favor a pro-angiogenic environment ²⁵⁷. HCMV IE can induce angiogenesis either by inducing the production of IL-8^{258,259} or through suppressing the expression of TSP-1 ²⁶⁰. Moreover, HCMV-mediated COX-2 activation can enhance angiogenesis. This effect is mediated by HCMV pUS28. COX-2 further upregulates iNOS, PDGF, bFGF, TGF- α , and VEGF in tumor cell and thus favors angiogenesis and cell migration ²⁶¹. In addition, pUS28 was shown in infected glioblastoma cells to favor an angiogenic phenotype by inducing VEGF production ²²⁵. HCMV gB proteins had also been proposed to bind to PDGFR- α leading to intracellular phosphorylation of the receptor and thus enhance the migratory and angiogenic capacities of GBM cells ²⁶².

The potential link between HCMV and breast cancer

Worldwide, breast cancer is the most frequently diagnosed cancer among women²⁶³. According to WHO 2015, breast cancer was the leading cause of 571,000 deaths worldwide, with a progress in the number of cases annually (WHO). Depending on the transcriptomic profile, breast cancer had been shown to involve several categories including: the luminal epithelial cell type A and B; the normal breast epithelial-like group, and the basal-like type⁹². According to Lehman et al. 2011, the basal-like breast cancer is a subtype of the triple negative breast cancer which constitute 10%–20% of all breast cancers²⁶⁴. The treatment of patients with basal-like cancer is challenging because of the absence of any well-defined molecular target. They lack the expression of estrogen receptors (ER) and progesterone receptors (PR) and also the human epidermal growth factor receptor 2 (HER2)^{265–267}. In general, TNBC tumors are of higher grade, larger in size, have an involvement of lymph nodes at diagnosis, and are more aggressive. In addition, patients with TNBC have poorer prognosis than patients with other breast cancer subtypes²⁶⁸. Several internal and external factors are implicated in the development of breast cancer including obesity, alcohol consumption, family history of breast cancer, genetic predisposition, radiation exposure, dense breast tissue, lifestyle, ethnicity, hormonal replacement therapy and hormonal contraception (WHO,²⁶⁹).

Furthermore, infectious agents mainly viruses had been known to be involved in several cancers in humans²⁰². We are interested in studying the oncogenic potential of human cytomegalovirus and breast cancer. Actually, breast milk is considered as an important transmission route for HCMV in humans. Moreover, the blood monocytes and tissue macrophages are important cellular reservoirs for HCMV. They are responsible for the dissemination of the virus and may act as a site for latency^{270–272}. Noteworthy, HCMV is able to induce the polarization of distinct inflammatory (M1) and immunosuppressive (M2) macrophages²⁷³ which is associated with the secretion of cytokines that could have an important role in the replication and fitness of the virus and thus favoring the promotion of breast cancer^{274–276}. Furthermore, an HCMV strain with macrophage tropism could have a pivotal role in the development of tumor-associated macrophages (TAM), which is a marker of poor prognosis in human breast cancer and in glioblastoma^{277–279}. As we mentioned previously, HCMV has a wide cellular tropism,

and both monocyte/macrophages and human mammary epithelial cells (HMECs) are shown to be of its targets ²⁸⁰.

1- HCMV infection of HMECs and macrophages polarization

It had been well known that HCMV exhibits broader tropism. It can infect most organs of the body including breast, brain, colon, kidney, eye, blood, lungs, and liver. Reports showed that HCMV can replicate in different cell types ranging from monocytes to neural stem cells ^{270,281–283}. Two potential scenarios could be implicated in the HCMV infection of HMECs and the development of breast tumor. The first scenario is through the transmission of HCMV through breast milk. The mammary epithelial cells, that line the duct responsible for the conversion of most precursors into milk constituents and then their transportation into the mammary lumen, might be infected directly by HCMV present in milk. Subsequently, the macrophages present in the breast tissue can also be infected with HCMV. This will favor a protumoral microenvironment. The second scenario is the transmission of HCMV through the blood (viremia). HCMV present in blood could infect the circulating monocytes which will differentiate into macrophages upon their migration into breast tissue. These HCMV-infected macrophages might transmit the virus to the mammary epithelial cells ²⁶⁹. Blood monocytes and tissue macrophages are widely recognized as important reservoirs of HCMV. They are also responsible for the viral dissemination throughout the body ^{272,273}. Moreover, HCMV infection of the monocytes has a potential to reprogram these monocytes leading to their polarization towards M1 inflammatory macrophages that also exhibits properties of the immunosuppressive M2 macrophages. Upon HCMV infection, the induction of NF-KB and PI3K is the responsible of this polarization ²⁷³, where the prolonged secretion of inflammatory factors from the polarized macrophages is often linked with cancer development (reviewed in ²⁷⁴).

In response to viral infection, cellular immunity includes the activation of M1 macrophages which are induced by T helper (Th1)-type through the induction of interferon-gamma. M1 macrophage induction through interferon gamma displays a proinflammatory profile with high levels of proinflammatory cytokines such as interleukin (IL)-6, IL-1beta, and tumor necrosis factor alpha (TNF-alpha) ²⁸⁴. Furthermore, chemokines secretions are classically increased including macrophage

inflammatory protein (MIP)-1alpha (CCL3), MIP-1beta (CCL4) and RANTES (CCL5)^{284,285}. Antiviral immunity is increased to achieve the immune activation, with Th1 activity enhancement and an increase in the levels of IL-2, IL-12, IL-18 and IFN gamma, especially in the lymph nodes of the subjects infected with the virus²⁸⁶. Thus, the role played by M1 macrophages in cellular immunity is pivotal. M1 macrophages are involved in responses towards tissue damage in immunodeficiency syndromes and delayed-type hypersensitivity reactions²⁸⁷. Moreover, they have an important anti-cancer role. They are involved in the stimulation of Th1-cytotoxic T cells (CTLs) and also other effector cells which will fight the viral infection and ultimately improve the patient's survival. Thus, they could be an important target for the development of a successful immunotherapy in cancer^{288,289}.

In addition, the activation of M2 differentiation pathway through the production of specific cytokines (e.g. IL-4 and IL-13) in the presence of Th-2 type response is also possible. These M2 macrophages display tissue repair and anti-inflammatory properties^{287,290}. They can promote tumor growth due to their role in matrix remodeling and their immunosuppressive function. Alternatively, IL-4 and IL-13 may activate macrophages through a common receptor chain (IL-4Ra)²⁸⁷, where IL-4 is produced by Th-2 cells and by mast cells and basophils²⁹¹. IL-13 is secreted by the activated T cells. The impact of IL-4 and IL-13 on macrophages causes the upregulation of MHC class II and mannose receptor expression. This will stimulate endocytosis and antigen presentation. In addition, the expression of macrophage-derived chemokine (CCL22) is upregulated. Moreover, IL-13 causes an upregulation in the expression of IL-1 receptor alpha-chain and IL-1 decoy receptor, which counteract the pro-inflammatory effects of IL-1²⁸⁷.

In the tumorous environment, a differentiation of macrophages towards a specific phenotype occurs, these macrophages are known as the tumor associated macrophages (TAMs)²⁹². Most of TAM exhibit a phenotype close to that of M2 macrophages. Macrophages may acquire the M2 state after entering the tumorous tissue near the breast cancer cells. These M2 macrophages secrete a little amount of pro-inflammatory cytokines and large amounts of the immunosuppressive cytokines, e.g., TGF-beta and IL-10 (reviewed in²⁹³). Thus, being immunosuppressives in nature M2 macrophages could favor the development of breast cancer²⁹³. Generally the number of TAMs is correlated with poor prognosis and in solid tumors they constitute 5 to 40 % of the tumor mass²⁹².

2- Cytomegalovirus targeting macrophages

HCMV infection is characterized by the persistence of viral genome at defined anatomical sites of the normal host for months or even years in a nonproductive form¹¹⁶. There are several involved strategies that allow the virus to avoid its immune system elimination. Such strategies include exploiting immunologically privileged tissues for replication (*i.e.*, epithelial cells of the salivary glands which don't express sufficient amount of MHC Class I molecules to trigger clearance by the CD8⁺ cell), expressing genes that are able to interfere with the immune response, and inducing a state of latency²⁸. Blood monocytes and tissue macrophages role is central in case of HCMV infection. Being a target in infected organs, they can serve as disseminators of the virus throughout the host but also as sites of viral latency²⁹⁴. In HCMV-infected organs, macrophage is the most prevalent infiltrating cell type^{32,295}. Experimentally in the laboratory setup, the cell type that is most widely used to grow HCMV and which produces high titers of infectious virus is the fibroblast. In addition, HCMV can also grow on macrophages but with lower viral production rate than in fibroblasts^{31,270}. By comparison with fibroblasts, infecting macrophages either with the laboratory strains (AD169) or with the clinical isolates (HCMV-DB) had shown low-level sustained growth, indicating that HCMV can infect macrophages cultures with the production of much lower viral titers in the supernatants as compared to fibroblasts²⁷⁰.

The viral growth kinetics difference between fibroblasts (high growth) and macrophages (low growth) is neither due to different viral attachment and adsorption²⁹⁶ nor due to a difference in the entry of the virus²⁷⁰, though the early phases of the viral cycle could contribute in the cellular tropism of the virus. It is believed that the restriction of HCMV replication in some cell types depends mainly on a block that occurs after viral entry and prevents the viral gene expression³⁵. Recently, reports show that following the HCMV infection of cancer cells, several blocks occur in the viral cycle²⁹⁷. For an optimal viral replication, the virus must be able to inhibit the macrophage differentiation. This could be a factor of getting the low rate of viral growth in macrophages cultures^{298,299}. Moreover, several studies mentioned that the presence of an intact ULb' sequence in the genome of HCMV clinical isolates is required for an efficient viral growth in endothelial cells, epithelial cells, and macrophages^{26,40,282,300}. This ULb' region is lost in the HCMV laboratory strains, which results in an inefficient replication in epithelial cells, endothelial

cells and macrophages²⁶. This reflects the importance of focusing on the clinical strains rather than the laboratory ones while studying the HCMV replication and pathogenesis.

Though the susceptibility of HCMV infection is higher in M2 macrophages, it can also infect the classically activated macrophages. HCMV is able to establish a productive and persistent infection in either macrophages, which acquire similar features of classical activation later and secrete the pro-inflammatory cytokines and chemokines in high levels^{301–303}. Furthermore, during the acute HCMV infection, HCMV IE1 protein is involved in the production of TNF alpha³⁰⁴. Also, there are several significant cellular signal transduction events that occur in macrophages infected with HCMV. During entry, the gB glycoprotein of HCMV binds to cellular epidermal growth factor receptor (EGFR) and directly initiates the activation of apoptosis suppressor Akt. Such activation of Akt will promote the Akt-dependent prosurvival state after monocytes infection³⁰⁵. Additionally, other signals are also targeted by HCMV in order to let the infected monocytes pass the 48-hours cell fate decision checkpoint required to start maturation into macrophages. EGFR activation rapidly induces the expression of HSP27 and Mcl-1, which work together to precisely control the activity of caspase-3. During the viral dissemination, the control of caspase-3 activity is a key process for allowing viral persistence³⁰⁶. Infection of monocytes differentiating into macrophages will activate the caspase-dependent or independent cell death programs. These cell death programs are activated by HCMV pUL36¹⁸⁷. Moreover, the anti apoptotic protein, Bcl-XL, provides a protection against the CMV-induced apoptosis³⁰⁷. In addition, reports indicate that the binding activity of NF-kB is increased in the nuclear extracts of macrophages and fibroblasts as a response to HCMV infection²⁷⁰. Studies also showed that NF-kB is activated in response to HCMV infection in monocytes³⁰⁸ and fibroblasts³⁰⁹ through the ligands of the HCMV UL55 (gB)³⁰⁸. In addition to the conventional activation of p50/p65 NF-KB, the alternative p52/Bcl-3 complexes are also activated after macrophage infection and can target the HCMV major immediate early promoter (MIEP)²⁷⁰. Through the NF-kB sites, the activated complexes of p52/Bcl-3 will regulate the HCMV MIEP. In addition Bcl-3 activates a number of human genes among others Bcl-2^{310–312}, cyclin D1^{313–316}, inducible NO synthase³¹⁷, P-selectin³¹⁸, and EGFR^{319,320}. Most of the activated cellular genes favor the cellular survival and lead to an optimal infection by HCMV. Histone deacetylases (HDAC) can also have a role in the latency of HCMV in macrophages, although their supposed recruitment by p52³¹⁵ and Bcl-3^{321–323} had not

been fully characterized yet. In addition to HDACs, reports showed that the activity of MIEP is inhibited by histone methyltransferases which could explain the negative autoregulation of MIEP by the HCMV IE2^{65,324}. In case of concomitant bacterial infection, HCMV enhances the bacterial induction of macrophage inflammatory responses that are mediated through NF- κ B pathway, thus promoting the organ inflammation in tissues infected with HCMV³²⁵. By comparing the HCMV-infected macrophages to the mock-infected macrophages, studies reported that the infection with HCMV maintains the surface expression of TLR5, TLR4, and CD14, in which they decline over time in the mock-infected macrophages. The phosphorylation of NF- κ B and I κ B α is also enhanced, along with the expression of the adaptor protein MyD88³²⁵. HCMV replication in monocytes is poor; however it is enhanced in monocytes derived macrophages^{306,326}. After the HCMV binding to monocytes, an intracellular increase in Ca²⁺ levels is induced and increased Ca²⁺ levels result in the blockage of monocytes ability to differentiate into macrophages. Observations suggest that HCMV has an efficient strategy that allows its interference with the cellular differentiation pathways, and this can also explain the development of generalized immunosuppression in patients infected with HCMV^{298,327}. HCMV infection of CD14⁺ monocytes results in the generation of latency-specific transcripts, the conservation of the viral genomes, and the ability of the virus to enter the lytic cycle again³²⁸.

3- HCMV, macrophages and mammary epithelial cell transformation: a new paradigm?

There are two distinct phenomena that could be observed after macrophages infection by HCMV. First, NF- κ B switch regulates the expression of viral genes, and results in a viral persistence via a sustained viral replication at low levels. In addition, HCMV-infected macrophages might have a role in the disease progression by spreading the infection to other cells that are present in their vicinity. Such cells could be more permissive than macrophages, as epithelial cells and/or fibroblasts^{62,272}. Second, HCMV infection of macrophages could cause changes in the expression of cellular genes giving rise to an adjusted cellular phenotype, for instance the M2 phenotype that favors a protumoral microenvironment^{270,273}. Several attempts were made to find the link between HCMV infection and the development of breast cancer. El-Shinawi et al. observed that

patients with inflammatory breast cancer (IBC) had a higher prevalence of anti-HCMV IgG than patients with non-IBC invasive ductal carcinoma (IDC). They also observed that NF- κ B is more activated and the levels of HCMV DNA were higher in tumorous tissues that were isolated from IBC as compared to IDC patients. The cause of an enhanced NF- κ B activation can either be the HCMV infection of breast cells or the cytokine production in the tumorous microenvironment^{329,330}. Richardson et al. hypothesized that the incidence of breast cancer could be elevated by late HCMV exposure³³¹. This hypothesis was based on the correlation between HCMV seroprevalence and the incidence of breast cancer. Cox and colleagues had enrolled 399 invasive breast cancer patients and 399 control patients in their study to investigate the correlation between the development of breast cancer and the levels of HCMV IgG. Their results showed that there was a significant correlation between the elevated levels of HCMV IgG and breast cancer development in women³³². Moreover, testing milk samples of HCMV seropositive women for the presence of HCMV, revealed that HCMV was detected in more than 90% of the studied samples^{79,333}. HCMV DNA was also found in the normal breast tissue³³⁴.

To show that the breast epithelium constitutes an important HCMV reservoir in humans, the detection of HCMV antigens in breast biopsies could provide a positive argument. The prevalence of HCMV antigens in the neoplastic epithelium of breast cancer patients is higher than in the non-tumoral breast tissue of breast cancer patients and in non cancer patients²⁸¹. Recent reports showed that HCMV DNA and proteins are present either in the breast cancer tissue or in the sentinel lymph node metastases tissue^{278,281}.

A model where HCMV infects macrophages and mammary epithelial cells and initiates epithelial cells transformation in a favorable protumoral microenvironment might explain both, the presence of TAMs and the detection of HCMV DNA and/or antigens in breast tumors (Annex 2, Article 1 TOVJ). In vitro, HCMV had been shown to be implicated in promoting the human mammary epithelial cells (HMECs) transformation with activating several signaling pathways such as PI3K/AKT, Myc and Ras²⁸⁰. Moreover, HCMV primes the macrophages polarization towards an M2 phenotype with enhanced Bcl3 activity²⁷⁰. Priming HMECs toward the epithelial transformation and macrophages toward the M2 phenotype could develop a favorable tumor microenvironment for the formation of breast tumor.

HMECs also secrete CSF-1 in high levels, which could be involved in promoting the proliferation of breast cancer cells ³³⁵. CCL2 secretions can also be induced in myeloid cells and fibroblasts by the replication of HCMV ^{336–338}. TAMs can secrete EGF that will bind to the EGFR of breast cancer cells. In human breast cancer cells, the development of a transformed phenotype is enhanced by the benign mammary epithelial cells ³³⁹. Finally, breast cancer cells invasion is stimulated in culture in the presence of the human mammary fibroblasts, which increase the development of stroma in mouse xenograft experiments ³⁴⁰. This will then accelerate the development and the dissemination of breast cancer. Once the definitive transformation of HMECs occurred, the persistence of HCMV that initiated the transformation is no more required, and a potential paradigm is that the virus could be cleared from breast cancer cells. Actually, HCMV is unable to efficiently replicate in the transformed cells such as the hepatocellular carcinoma HepG2 cells and fibroblasts which express SV40 T antigen and oncogenic H-Ras ^{297,341}. In addition, in M2 (TAM) macrophages, HCMV replicates in low levels because of the Bcl3-p52 NF-kB complex binding on the MIEP which results in a reduced viral transcription ²⁷⁰ (Annex 2, Article 1 TOVJ).

Reports demonstrated that macrophages, being a part of the tumor stroma, will promote the migration of breast cancer cells and the activity of stem cell ³⁴². A breast cancer cell lines (BCCLs) co-culture with THP-1 cell line revealed that there is an increase in the migration of ER-positive breast cancer cell lines with all types of macrophages. Also, in this monocytes-macrophages differentiation model, it had been shown an increase in the formation of mammospheres with M2-macrophages as compared to M1-macrophages. Actually, HCMV is able to favor stemness of the transformed cells with tumorspheres formation in cellular models of colon cancer, glioblastoma, and hepatocarcinoma in vitro ^{341–346}. Such results indicate that HCMV might favor the development of a more aggressive phenotype with a higher risk of metastasis and a poorer prognosis. Interactions between the basal-like breast cancer (BBC) cells and the stromal cells are important determinants of the tumor biology. Furthermore, inflammatory cells that are associated with the stroma have a key role in cancer progression. A coculture of BBC cells with monocytes-like THP-1 cells had led to alterations in the expression of genes with an upregulation of both M1 and M2 markers. Monocytes to macrophages differentiation increased in BBCs, in relative to luminal

breast cancers, with enhanced migration of macrophages. Observations revealed the secretions of distinct cytokines patterns in co-cultures of macrophages-BBCs. These include the upregulation of MIG, NAP-2, osteoprotegerin, MCP-1, MCP-3, and interleukin (IL)-1 β ³⁴⁷.

The epithelial-mesenchymal transition (EMT) of cancer cells which are at the invasive front of tumors, close to TAMs, suggest that there could be a mutual interaction between these two types of cells ^{292,348}. The activation of macrophages to TAM-like phenotype occurs via the effect of mesenchymal-like breast cancer cells through GM-CSF secretion. CCL18 secreted by TAMs induces EMT of cancer cells and enhances metastasis. In the breast cancer biopsies, GM-CSF which is expressed at high levels is associated with an increase in CCL18 (+) macrophages, EMT, and metastasis parallel to a reduction in patient survival. These findings suggest that an important positive feedback loop between GM-CSF and CCL18 exists and participate to breast cancer metastasis. ³⁴⁹(Annex 2, Article 1 TOVJ).

In human breast carcinomas, overexpressed macrophage colony-stimulating factor (CSF-1) and its receptor (CSF1-R) have been correlated to poor prognosis. At the tumor site, the expression of CSF1-R is restricted to macrophages and allows the regulation of TAMs infiltration and function by CSF-1 ³⁵⁰. The epidermal growth factor (EGF) is first expressed by macrophages and increases the formation of elongated protrusions and cell invasion by carcinoma cells. CSF-1, which is produced by carcinoma cells, promotes the expression of EGF by macrophages. Furthermore, EGF also enhances the expression of CSF-1 by carcinoma cells, thus generating a positive feedback loop ³⁵¹(Annex 2, Article 1 TOVJ). A large cohort study was done on the infiltration and activation of macrophages using immunohistochemistry from human primary breast tumors. The study showed that in tumors with negative estrogen receptor and of histological ductal type, the poor differentiation and fast proliferation was associated with the presence of high M2 macrophages numbers ³⁵².

Finally, though macrophages infected with HCMV might favor a protumoral microenvironment, it might also result in the containment of the viral dissemination. A murine model of infection indicates that the peripheral murine cytomegalovirus (MCMV) infection spreads through the lymph nodes where MCMV infects the infiltrating

macrophages but with a highly restricted viral replication in this latter cell type. By depleting these macrophages, MCMV infects quickly the surrounding fibroblasts and spreads faster ³⁵³. Moreover, depleting the splenic macrophages enhances the replication of MCMV in the spleen, rather than controlling it ³⁵³. Thus tissue macrophages can protect from CMV spread to other cell types present in their vicinity which are usually highly permissive for the virus. This might have implications for the pathogenesis of breast cancer and its dissemination ²⁷⁹.

Objective of the study:

Several research groups studied the role played by HCMV in the development of inflammatory diseases and cancer^{60,341,354}. Either antigens or DNA of HCMV have been detected in various tumor tissues including colon, liver, prostate, brain (glioblastoma, medulloblastoma), and breast cancer^{277,278,281,355–357}. The implication of HCMV infection in various cancers had led to the development of the “oncomodulation” paradigm. It means that HCMV can infect the tumor cells causing an increase in their malignancy²⁷⁹. For example in cells with a tumorigenic phenotype, the HCMV pUS 28 protein promotes the progression of the cell cycle and the expression of cyclin D1²²⁶. However, in nontumorigenic cells, pUS28 protein induces apoptosis²²⁵.

Blood monocytes and tissue macrophages are recognized as an important cellular reservoir responsible for HCMV dissemination and can also be sites for the establishment of latency^{271,272,328,358}. Therefore macrophage-tropic HCMV strains such as the HCMV-DB clinical isolate³⁵⁸ might be involved in the appearance and development of tumor-associated macrophages (TAM), which are known to be a marker of poor prognosis both in breast carcinoma and glioblastoma^{269,274,346,359}. Thus, searching for new HCMV isolates that target the monocytes/macrophages and understanding their role not only in the infection of monocytes/macrophages, but also of other cellular targets such as epithelial cells could be critical to assess the role of HCMV in oncomodulation but also oncogenesis, especially in adenocarcinoma.

HCMV-DB (KT959235), a clinical isolate from a cervical swab specimen of a 30-year-old pregnant woman that was previously isolated in our lab at the University of Franche-Comté³⁵⁸. It is a highly macrophage-tropic strain which triggers an M2 phenotype and causes an upregulation of the Bcl-3 proto-oncogene³⁵⁸. It is close from other primary clinical isolates such as PH and TR strains which also infect macrophages²⁶.

The clinical isolate HCMV-DB infects productively the human mammary epithelial cells (HMECs) in vitro²⁸⁰. HMECs infected with HCMV-DB resulted in a pro-oncogenic cellular environment with Rb hyperphosphorylation and a decreased functional activity of p53, upregulation of c-Myc, overexpression of mRNA with enhanced telomerase activity, activation of Akt and STAT3, and upregulation of cyclin D1 causing an enhanced

cellular proliferation²⁸⁰. Furthermore, HCMV-DB had shown its potential to transform the primary HMECs by colony formation assay on soft agar²⁸⁰.

HCMV-DB showed a complete life cycle in HMECs as confirmed by detecting both, the early and late viral transcripts and proteins²⁸⁰. In addition to the productive infection in HMECs, HCMV-DB also shaped the cellular environment favoring its transformation. P53 was upregulated in HMECs infected with HCMV-DB similar to the results obtained in other cell types^{280,360}. HCMV IE2 protein was shown to bind to p53 in HMECs infected with HCMV-DB as proven previously in human fibroblasts^{280,361}. A decreased binding of p53 to its target gene promoters (p21 and MDM2) was also observed in HMECs infected with HCMV-DB. This could reveal the failure of p53 response to have an efficient protection in HCMV-infected cells against cell cycle promotion and cellular proliferation²⁸⁰. HMECs infected with HCMV-DB showed upregulation of the telomerase activity²⁸⁰ that usually results in cellular immortalization³⁶². The level of phosphoRb was also elevated in HMECs infected with HCMV-DB²⁸⁰. The HCMV UL-97 is involved in the phosphorylation and inactivation of Rb family proteins and thus could promote the cell cycle progression³⁶³. Moreover, reports showed that the retinoblastoma family proteins are downregulated by pp71 (pUL82)³⁶⁴

Furthermore, HMECs infected with HCMV-DB revealed an elevated expression level of phospho-STAT3, phospho-AKT, and cyclin-D1 in addition to the upregulation of c-Myc. The expression of Ki67 antigen was also elevated upon infection²⁸⁰. HCMV-DB was also able to induce colony formation in soft agar seeded with infected HMEC. The soft agar assay is widely known to be the most reliable assay for the determination of malignant transformation²⁸⁰. Moreover, HMEC cultures infected with HCMV-DB for a prolonged time resulted in the formation of several clusters of spheroid cells called CMV-transformed HMECs or CTH cells around day 20 post infection in some of the cultures²⁸⁰. These formed clusters of spheroid CTH cells were gently detached and cultivated in HMEC Ready medium for several passages (currently up to >160 passages) and suggested to have undergone immortalization. The injection of these CTH cells in the mammary fat pad of NOD SCID Gamma (NSG) mice had led to tumor development two weeks later²⁸⁰.

The three main objectives of our study are as follows:

- Objective 1. To characterize the genomic profile of the HCMV-DB strain.
- Objective 2. To study the transcriptomic profile of HMECs infected with HCMV-DB.
- Objective 3. To screen the CTH cell genome for the presence of HCMV-DB DNA.

Results

1. Molecular analysis of the HCMV-DB strain.

1.1. Genomic profile of the HCMV-DB strain.

We compared the genomic sequence of HCMV-DB to that of ten HCMV strains, including eight clinical isolates with low passages in culture (Toledo, PH, TR, Davis, Merlin, JP, TB40/E, VR1814) and two laboratory adapted strains (AD169, towne)^{26,365}. The total length of the complete HCMV-DB genomic sequence is 235,512 bp (Fig 8, table 1). By comparison, Merlin strain sequence is approximately of the same size (235,645 bp). The shortest sequence corresponds to the laboratory adapted strain AD169 (229,354 bp), and the longest sequence is for TB40/E clinical strain (237,683 bp). The total length of clinical isolates with low passages in culture ranges from 229,700 bp for PH to 237,683 bp for TB40/E (Fig 8, table 1).

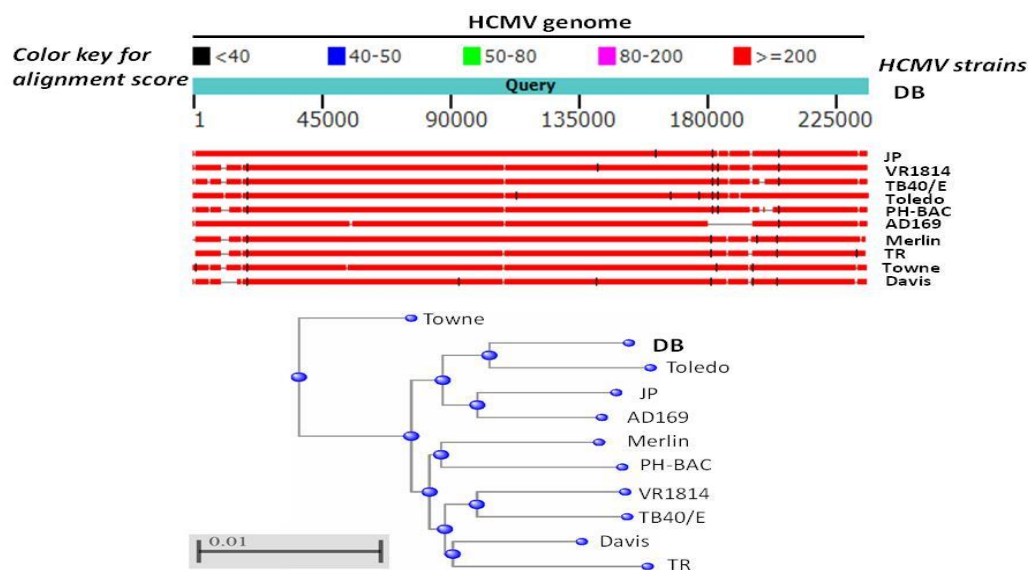


Figure 8. Comparison of HCMV-DB genomic sequence with other genomic sequences of clinical and laboratory adapted HCMV strains. The upper panel shows the genomic sequence of HCMV-DB as compared to eight low passages clinical isolates (Merlin, Toledo, TR, PH, VR1814, Davis, JP, and TB40/E) and two laboratory adapted strains (AD169 and Towne). The lower panel shows the alignment tree corresponding to the whole viral genome with a comparison between HCMV-DB genome and the genome of ten other HCMV strains.

Depending on the sequence similarities among HCMV strains, the highest total blasting scores were measured for Toledo and JP strains as compared to DB (Table 1). JP and Toledo share 99% and 98% identity with DB respectively, by linear full genome alignment (Table 1).

Table 1: Genomic comparison of HCMV-DB with other HCMV strains.

HCMV strain	Clinical	Geographical	DNA	Passages	Nucleotide length	Genes Mutated	Max. score (e+05)	Total score (e+05)	Query cover	Identity	Accession
DB	Pregnant women	France	Clinical material on macrophage cultures	low passage on fibroblasts	235512	RL13, UL9			100	100	KT959235
Towne	Urine from a congenitally infected infant	USA	Fibroblast culture cells	many	235147	many: RL13, UL1, UL40, UL130, US1	1.316	3.929	95	99	FJ616285
AD169	Adenoid tissue	USA	Fibroblast culture	many	229354		1.238	4.036	92	98	X17403.1
DAVIS	Liver biopsy from a congenitally infected infant	USA	Fibroblast culture	many	229768	RL5A, RL12, RL13, UL1, UL2, UL4, UL5, UL6, UL99, UL130	1.29	3.936	95	98	JX512198.1
JP	Post mortem prostate tissue from an AIDS patient	UK	Clinical material	low passage on fibroblasts	236375		2.838	4.129	98	99	GQ221975.1
Merlin	Urine from a congenitally infected infant	UK	Clinical material	low passage on fibroblasts	235646	RL13	2.841	4.034	97	98	AY446894
PH	Bone marrow transplant recipient	USA	Clinical material	low passage on fibroblasts	229700		1.545	3.856	95	98	AC146904
TB40/E	Throat wash of a bone marrow transplant recipient	Germany	Fibroblast culture	Few	237683	RL13, UL128, IRS1, US1, US2	1.555	3.962	96	98	KF297339
Toledo	Urine from a congenitally infected infant	USA	Fibroblast culture	several	235404	RL13, UL9, UL128	1.548	4.171	99	98	GU937742
TR	Vitreous humor from eye of HIV-positive male	USA	Fibroblasts	several	235681		1.539	3.955	96	98	KF021605.1
VR1814 (FIX)	Cervical secretions of a pregnant woman with a primary HCMV infection	Italy	Clinical material	low passage on fibroblasts	235233		1.564	3.995	97	98	GU179289

All the analyzed clinical isolates share 98% identity with DB except for JP that shares 99% identity (Table 1). RL13 and UL9 genes of HCMV-DB strain are mutated, however the ULb' region is not (Table 1). Altogether, our analyses reveal that the genomic sequence of HCMV-DB is highly similar to that of the clinical strains Toledo and JP.

1.2. Phylogenetic classification of the HCMV-DB strain based on genes involved in virus entry.

Since the HCMV-DB strain is highly macrophage-tropic³⁵⁸, we did a phylogenetic analysis to compare genes that code for the viral envelop and are involved in the tropism (Fig 9)^{366–368}. The minimal complex gB:gH/gL is required for the entry of HCMV into fibroblasts^{367,369}. However, the pentameric complex gH/gL/pUL128/pUL130/pUL131 in addition to gB, is required for the entry of HCMV into macrophages, monocytes, endothelial cells, and epithelial cells^{38,282,370–372}. We noticed that the sequences of gB:gH/gL from DB are close to that of VR1814, JP and Merlin, respectively (Fig 9A).

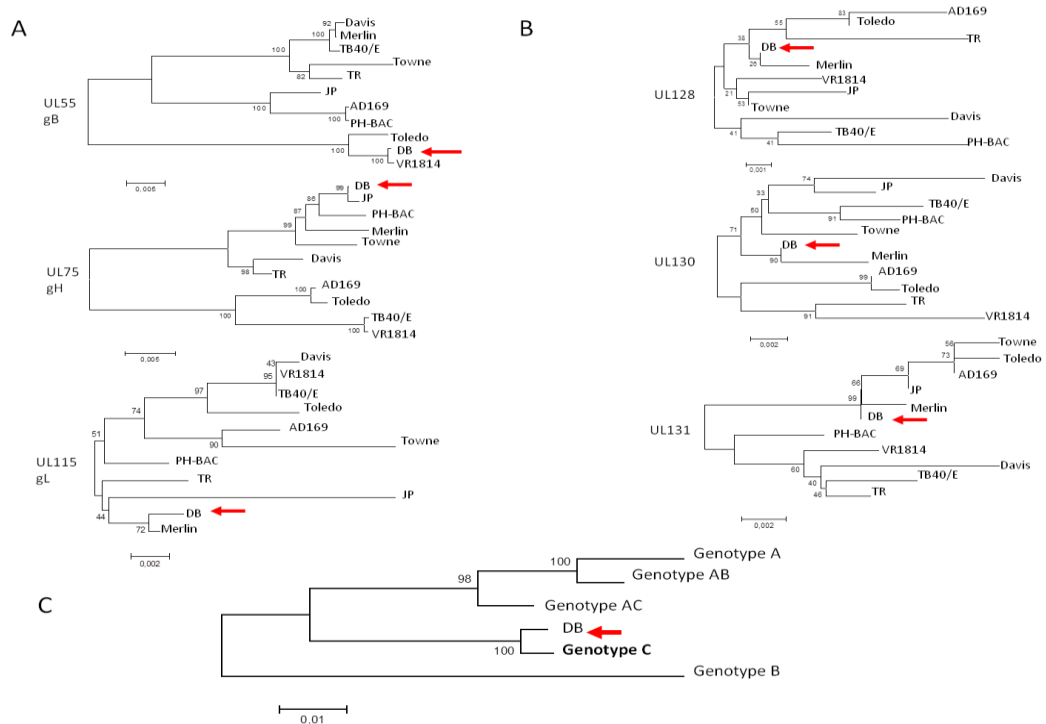


Figure 9. Phylogenetic analyses comparing several HCMV strains for viral genes coding for proteins involved in cellular tropism and the genotype classification of HCMV-DB according to the UL-144 gene analysis. (A) Phylogenetic analysis of genes coding for the viral envelop glycoproteins that are required for the entry into cells: UL55 (gB), UL75 (gH), and UL115 (gL). (B) Phylogenetic analysis of genes coding for pUL128, pUL130, and pUL131 required in addition to the envelop glycoproteins for the viral entry into monocytes, macrophages, epithelial cells, and endothelial cells. (C) HCMV-DB strain belongs to the genotype C based on the phylogenetic analysis of the UL-144 gene.

The pentameric complex of HCMV-DB was close to that of VR1814 for gB, to that of JP for gH. For the rest of the pentameric complex: gL, pUL128, pUL130 and pUL131, HCMV-DB is close to Merlin (Fig 9B). By considering sequences of genes that are involved in the virus entry, we can conclude that the HCMV-DB strain is close to the Merlin strain.

The UL144 gene is situated in the ULb' region of the viral genome. It includes amino-acid sequence similar to that of the members of the tumor necrosis factor receptor superfamily and sequence similarities with the herpes simplex virus entry mediator (HveA or HVEM). We observed that HCMV-DB belongs to genotype C of UL144 (Fig 9C).

2. Analysis of the transcriptome of HMECs infected with HCMV-DB.

2.1. The transcriptome of HMECs infected with HCMV-DB displays a triple negative basal-like phenotype.

HMECs infected with HCMV-DB either at low MOI=1 or at high MOI=10 at day 1 post infection and uninfected HMECs were used for the screening with human breast cancer and oncogenes/tumor suppressor genes RT² profiler PCR assays (PAHS-131ZA and PAHS-502Z, respectively). MDA-MB231 and MCF-7 breast cancer cell lines were used as positive controls with basal-like and luminal phenotypes, respectively. Each of the RT² Profiler PCR arrays profiles the expression of 84 key genes -which overlap sometimes in the two assays- commonly involved in tumor classification, signal transduction, and other commonly affected pathways such as angiogenesis, adhesion, proteolysis, cell cycle, and apoptosis.

Regarding the expression of ER/PGR/HER2 transcripts, HMECs infected with HCMV-DB and MDA-MB231 cells showed a similar transcriptome that was clearly distinct from the transcriptome of MCF-7 cells (Fig.10). Although the triple negative ER-/PGR-/HER2- phenotype was observed in both the infected and the uninfected HMECs, the levels of estrogen receptor (ESR1), progesterone receptor (PGR) and HER2 (ERBB2) transcripts were higher in HMECs infected with HCMV-DB as compared to uninfected HMECs (Fig 10A). The gene expression of luminal markers (KRT18, GATA3, KRT19, and TFF1) and estrogen marker (ESR1) was low in HCMV-DB infected HMECs similar

to the phenotype of MDA-MB231 cells (Fig. 10B, Annex 2, Article 3, Sci Rep sup tables 2 and 3). In contrast, MCF-7 cells expressed high levels of luminal markers transcripts (Fig 10B).

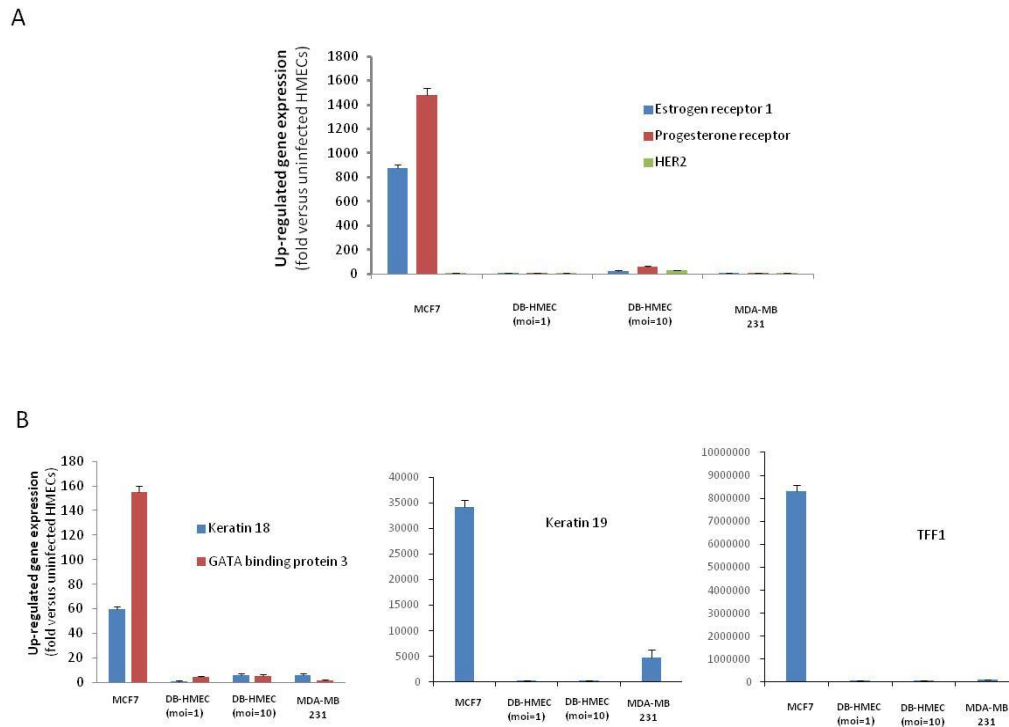


Figure 10. Transcriptome analysis of HMECs infected with HCMV-DB displays a triple negative basal-like phenotype. (A) HMECs infected with HCMV-DB and MDA-MB-231 cells show a similar pattern of ER-/PGR-/HER2- transcripts, in contrast to the ER+/PGR+/HER2- transcripts detected in MCF-7 cells. (B) Low levels of gene expression of luminal markers (KRT18, KRT19, GATA3, TFF1) in both HCMV-DB infected HMECs and MDA-MB-231 cells as compared to the luminal MCF-7 cells.

2.2 The transcriptome of HMECs infected with HCMV-DB presents oncogenic traits with enhanced cellular proliferation

HMECs infected with HCMV-DB showed an upregulation in the gene expression of several oncogenes as compared to the uninfected HMECs, including: Myc (MYC), Fos (FOS), HRas (HRAS), KRas (KRAS), NRas (NRAS), and Jun (JUN) (Fig 11A). Moreover, the transcripts of numerous other oncogenes are also upregulated (RUNX1, RUNX3, ETS1, ABL1, RET, ROS1, RARA, PRKCA, RAF1, PIK3CA, PML, NFKBIA, MET, MYB, MCL1, KITLG) (Annex 2 Article 3 Sci Rep, sup tables 2 and 3). We observed also the upregulation in the gene expression of the tumor suppressor genes that

code for p53 protein (TP53) and the retinoblastoma protein (RB1), as reported previously for the basal-like triple negative cell lines as MDA-MB231 cells (Fig 11B and Annex 2, Article 3, Sci Rep, supp table 3) ^{373,374}.

Furthermore, there was also upregulation of several other tumor suppressor genes in HMECs infected with HCMV-DB especially at low moi (moi=1) compared to uninfected HMECs. This includes: TP73, TGFB1, SMAD4, TSC1, VHL, FHIT, and STK11 (Fig 11B).

Reports mentioned the activation of the IL-6/JAK-STAT3/Cyclin D1 axis in biopsies of breast cancer patients ³⁷⁵. We observed an upregulation in the gene expression of IL-6, IL-6 receptor and JAK2 in HMECs infected with HCMV in comparison to the uninfected HMECs especially at high moi (moi=10), with a stable expression of STAT3 gene (Fig 11C). Also we observed in HCMV-DB infected HMECs (moi=10) an upregulation of the gene expression of cyclin dependent kinase 4 (CDK4) and other cyclins (CCNA1, CCNA2, CCND1, CCNE1) especially cyclin D1 which plays a role as regulator of CDKs (fig 11D, left panel). The gene expression of CDK inhibitors (CDKN) 1A (p21), 2A (p16), 2B (p15), and 3 (CDKN1A, CDKN2A, CDKN2B, CDKN3) is up-regulated by 5 folds in HMECs infected with HCMV-DB as compared to uninfected cells (Fig 11D, right panel).

In agreement with activation of the IL6/JAK/cyclin D1 pathway in HMECs infected with HCMV-DB, we observed an upregulation in the expression of proliferation marker genes such as the Ki67 antigen gene (MKI67) and the topoisomerase 2 gene (TOPO2A) in HMECs infected with HCMV-DB in comparison to uninfected HMECs (Fig. 11E). The expression of genes involved in cell survival (AKT1, PIK3C2A, NFKB1, REL, BCL-2) is also upregulated in HMECs infected with HCMV-DB as compared to uninfected HMECs, which indicates a prosurvival signal in infected cells (Fig 11F.). In agreement with the induction of several prosurvival genes in infected cells as compared to uninfected controls, we observed a down-regulation of caspase 8 transcript (CASP8) in HMECs infected with HCMV-DB at moi 1 compared to uninfected cells (Fig 11F).

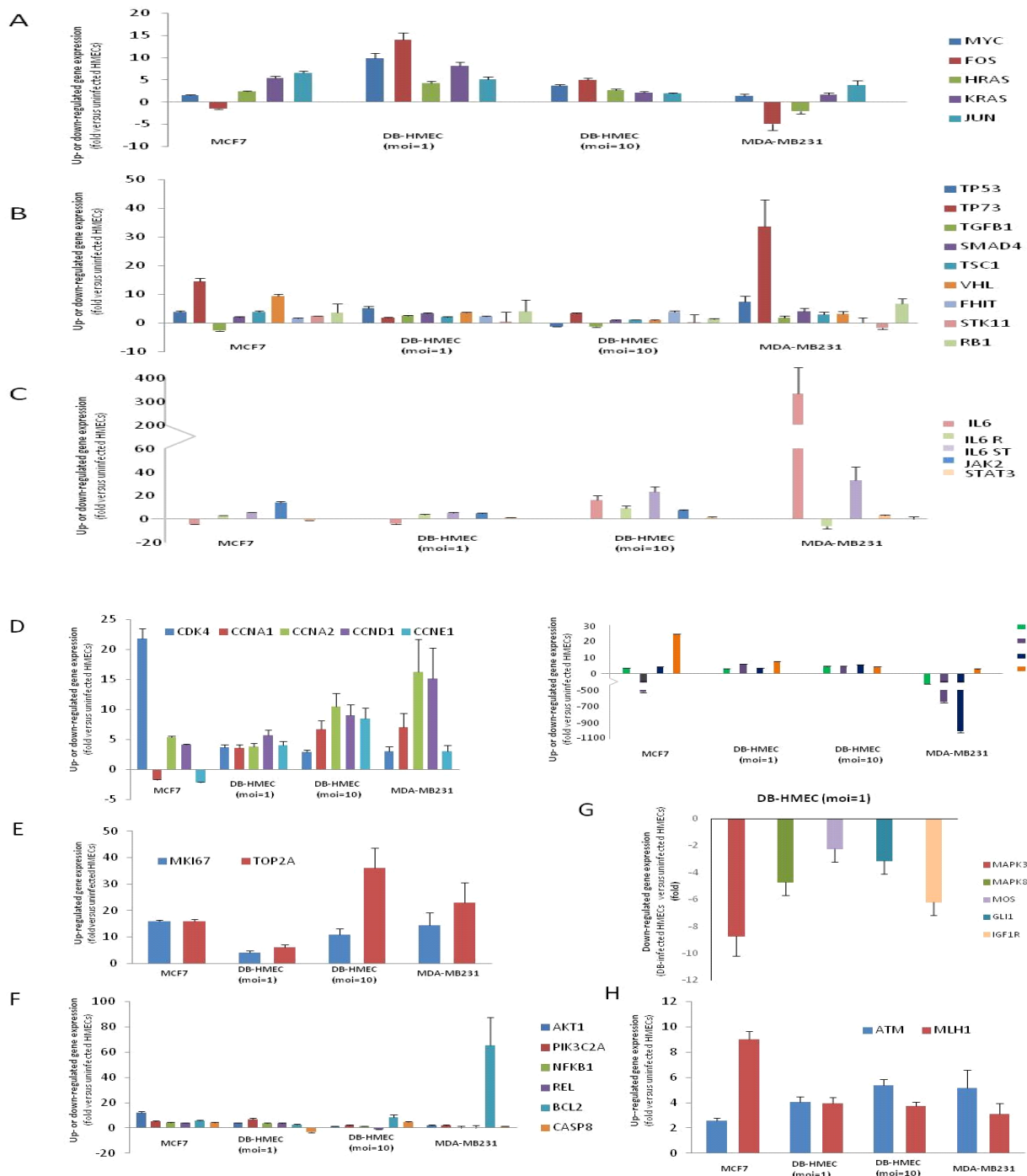


Figure 11. The transcriptome of HMECs infected with HCMV-DB displays oncogenic traits. (A) Upregulation of the gene expression of oncogenes: Myc (MYC), Fos (FOS), Jun (JUN), KRas (KRAS), HRas (HRAS) and NRas (NRAS), (B) Upregulation of the gene expression of tumor suppressor genes: TP53, TP73, FHIT, VHL, SMAD4, TGFβ1, STK11, TSC1, RB1. (C) Expression of genes from the IL-6/JAK-STAT3 axis. (D) Left panel, Upregulation of genes coding for the cyclins: CCNA1, CCNA2, CCND1, CCNE1 and the cyclin dependent kinase 4 CDK4. Right panel, Upregulation of genes coding for the cyclin dependent kinase inhibitors: CDKN 1A, CDKN 2A, CDKN 2B and CDKN 3. (E) Upregulation of the expression of genes coding for proliferation markers, the Ki67 antigen (MKI67) and the topoisomerase 2 (TOPO2A). (F) Upregulation of the expression of genes involved in cell survival (NFKB1, REL, AKT1, PIK3C2A, BCL-2) and downregulation of the expression of caspase 8 (CASP8). (G) Downregulation of gene expression of the members of the MAPK cascade (MAPK3, MAPK8, MOS, GLI1, IGF1R). (H) Upregulation of the expression of genes involved in DNA repair: the ataxia telangiectasia mutated (ATM) and human MutL homolog (MLH1). The up- and down-regulation were measured in HMECs infected with HCMV-DB (moi, 1 and 10) as compared to uninfected HMECs.

2.3 The transcriptome of HMECs infected with HCMV-DB displays modifications in cell signaling, angiogenesis and proteolysis.

The expression of genes corresponding to the MAPK cascade members (MAPK3, MAPK8, MOS, GLI1, IGF1R) is downregulated in HCMV-DB infected HMECs (Fig 11G). However, the gene expression of the transcription factors, tumor suppressor gene hypermethylated in cancer 1 (HIC1) and forkhead box D3 (FOXD3) is up-regulated in HCMV-DB infected HMECs at high moi (Annex 2 Article 3 Sci Rep, supp fig 1). We also observed an upregulation in the expression of ataxia telangiectasia mutated (ATM) and human MutL homolog (MLH1) genes, that are both involved in DNA reparation in HMECs infected with HCMV-DB compared to uninfected HMECs (Fig 11H).

Moreover, in HMECs infected with HCMV-DB at high moi (moi=10) we observed upregulation in the genes expression of E-cadherin (CDH1), claudin 7 (CLDN7), beta-catenin (CTNNB1) and alpha 6 integrin (ITGA6) (Fig 12A). Also there was both up- and downregulation of genes involved in angiogenesis (upregulation: IL-6, SERPINE1, THBS1, S100A4, EGF; downregulation: ID1, SLIT2) and proteolysis (upregulation: MMP9; downregulation: CST6, CTSD) (Fig 12B, Annex 2 Article 3 Sci Rep supp table 3).

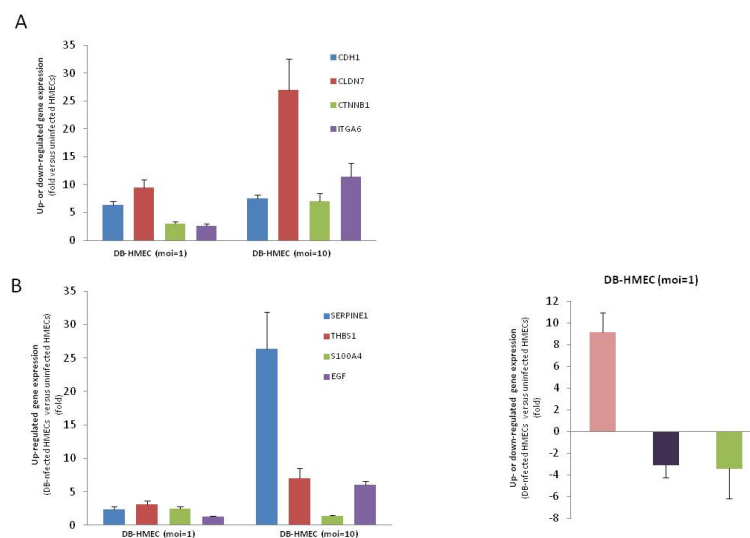


Figure 12. Modification of the transcriptome of genes involved in cell adhesion, angiogenesis and proteolysis in HMECs infected with HCMV-DB. (A) Upregulated gene expression of E-cadherin (CDH1), claudin 7 (CLDN7), beta-catenin (CTNNB1), alpha 6 integrin (ITGA6); (B) Upregulated expression of genes involved in angiogenesis (SERPINE1, THBS1, S100A4, and EGF) and proteolysis (MMP9), and downregulated expression of genes involved in proteolysis: CST6 and CTSD. The up- and down-regulation were measured in HMECs infected with HCMV-DB (moi, 1 and 10) as compared to uninfected HMECs.

2.4 The transcriptome of HMECs infected with HCMV-DB reveals a global hypomethylation state.

Since epigenetic regulation of chromatin has been associated with breast cancer classification and prognosis, we performed a Human Epigenetic Chromatin Modification Enzymes RT² Profiler PCR Array (PAHS-085A) which profiles the expression of 84 key genes encoding enzymes known or predicted to modify genomic DNA and histones to regulate chromatin accessibility and therefore gene expression (Annex 2, Article 3 Sci Rep, Suppl. Table 4). Considering a standard two-fold increase or decrease of RNA expression between HMECs infected with HCMV-DB and uninfected HMECs to be biologically significant, performed analysis indicated the down-regulation of most (n=50) of the genes including genes coding for SET domain proteins with a histone methyltransferase activity (ASH1L, MLL3 (KMT2C), MLL5 (KMT2E), NSD1, SETD1A, SETDB1, SETDB2, SETD2, SETD3, SETD5, SETD6, SETD7, SETD8, SUV420H1, WHSC1), DNA methyltransferase genes (DNMT3A, DNMT3B), histone methyltransferase genes (PRMT1, PRMT2, PRMT8, SMYD3, SUV39H1), histone acetyltransferase genes (ATF2, CIITA, CSRP2BP, KAT2A, KAT6B, NCOA1), DNA/histone demethylase genes (KDM1A, KDM5C, KDM4C, KDM6B), histone deacetylase genes (HDAC2, HDAC4, HDAC5, HDAC6, HDAC9, HDAC10), genes involved in histone ubiquitination (MYSM1, RNF2, RNF20, UBE2A, UBE2B, USP21, USP22), and histone kinase genes (AURKA, AURKB, AURKC, NEK6, RPS6KA3) (Fig. 13A, Annex 2 Article 3 Sci Rep Suppl. Table 4), and the upregulation of few genes (n=5) including histone methyltransferase genes (PRMT3, PRMT5, PRMT6), a gene coding for SET domain proteins with a histone methyltransferase activity (SETD4) and the histone deacetylase HDAC3 (Fig. 13B, Annex 2 Article 3 Sci Rep Suppl. Table 4). Altogether our results indicate an overall down-regulation of transcripts involved in methylation of DNA and histones in HMECs infected with HCMV-DB. In addition, transcripts of histone acetyltransferase and deacetylase genes are mostly downregulated in HMECs infected with HCMV-DB compared to uninfected controls.

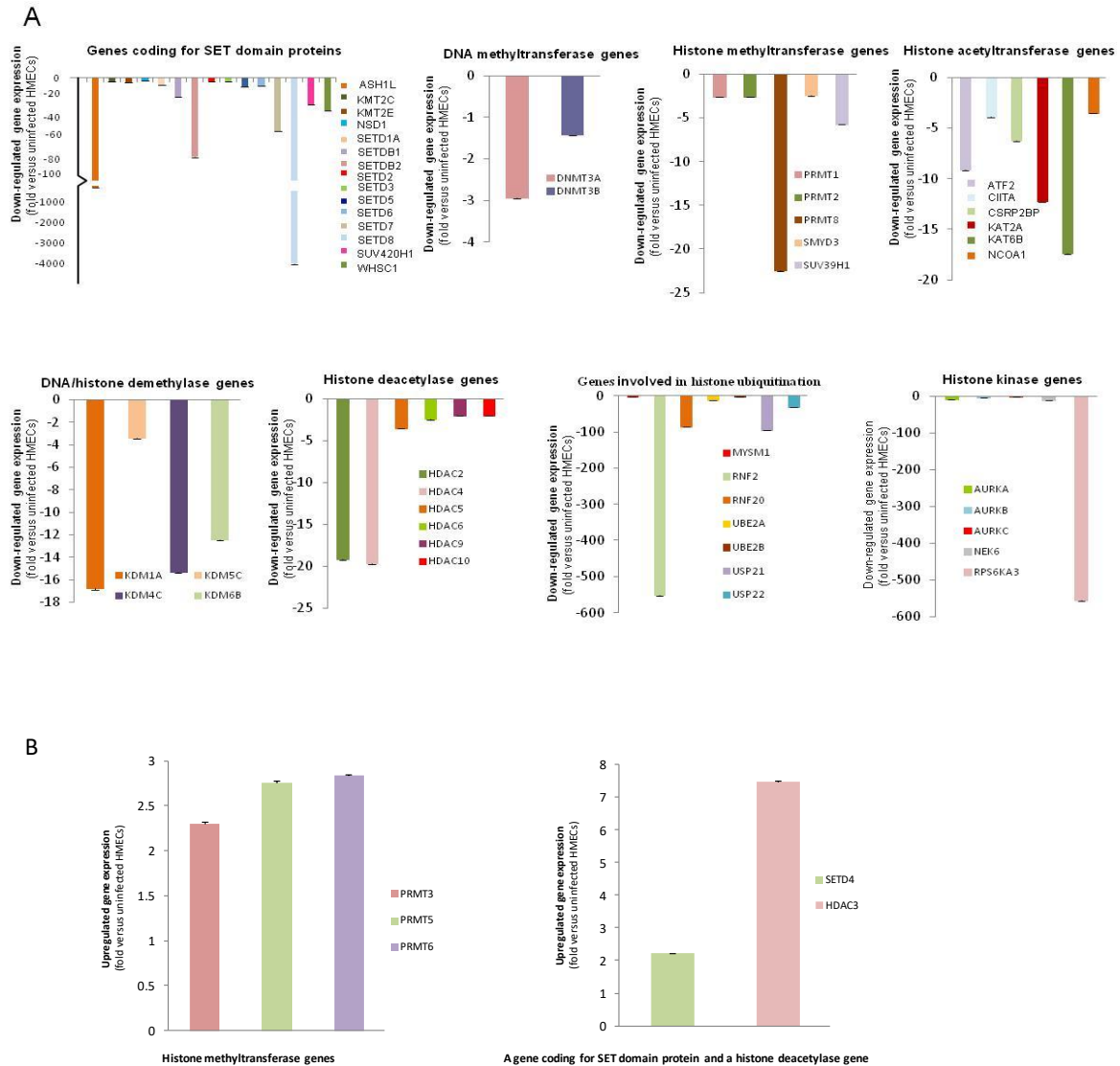


Figure 13. A general hypomethylation state was observed in HMECs infected with HCMV-DB. (A) Downregulation of the gene expression of DNA methyltransferase genes (DNMT3A), histone methyltransferase genes (PRMT1, PRMT2, PRMT8, SMYD3, SUV39H1), genes coding for SET domain proteins with a histone methyltransferase activity (ASH1L, MLL3 (KMT2C), MLL5 (KMT2E), NSD1, SETD1A, SETDB1, SETDB2, SETD2, SETD3, SETD5, SETD6, SETD7, SETD8, SUV420H1, WHSC1), histone acetyltransferase genes (ATF2, CIITA, CSRP2BP, KAT2A, KAT6B, NCOA1), histone kinase genes (AURKA, AURKB, AURKC, NEK6, RPS6KA3), genes involved in histone ubiquitination (MYSM1, RNF2, RNF20, UBE2A, UBE2B, USP21, USP22), DNA/histone demethylase genes (KDM1A, KDM5C, KDM4C, KDM6B) and histone deacetylase genes (HDAC2, HDAC4, HDAC5, HDAC6, HDAC9, HDAC10). (B) Upregulation of the gene expression of histone methyltransferase genes (PRMT3, PRMT5, PRMT6), genes coding for SET domain proteins with a histone methyltransferase activity (SETD4) and HDAC3. The up- and down-regulation were measured in HMECs infected with HCMV-DB (moi, 1 and 10) as compared to uninfected HMECs.

2. Screening CTH cells for the presence of HCMV-DB DNA.

Since the CMV-transformed HMECs (CTH cells) resulted from the transformation of HMECs following HCMV-DB infection *in vitro*, we screened CTH cells for the presence of HCMV-DB sequence(s) that would definitively confirm a direct link between HCMV-DB and the appearance of CTH cells in culture. Two main hypotheses could explain the transformation of a cell by a virus: the “hit and run” hypothesis and the direct presence of viral gene(s) in the transformed cells. The “hit and run” mechanism was proposed by Shen and his colleagues after the transformation of primary baby rat kidney (BRK) resulting from the transient expression of IE1 and IE2. In this case the presence of viral DNA namely IE1/IE2 DNA was not observed in the clonal cell lines that are derived from transformed BRK foci ²³⁷. The second mechanism of viral transformation allows the detection of viral genes especially viral oncogenes in the transformed cells. This is the case for the cellular transformation observed with DNA oncogenic viruses such as HPV, HBV, HHV-8, and EBV. To exclude a “hit and run” mechanism to explain the appearance of CTH cells in the culture, it is critical to demonstrate that HCMV-DB DNA can be detected in CTH cells. Therefore, we prepared primers sets that cover almost all the HCMV-DB genome (Figure 21), and we screened CTH cells of different passages for the presence of HCMV-DB sequence(s) using the conventional PCR.

3.1 Screening approach.

For each PCR amplification, DNA extracted from uninfected HMECs and MRC5 cells was used as negative controls in addition to the no template control (NTC). As positive controls, DNA extracted from HCMV-DB viral stock and HCMV-DB infected HMECs 1 day post infection was used.

Primers were designed for genes of different stages of viral expression. Three genes coding for the immediate early HCMV proteins were targeted ³⁷⁶: UL122 (IE2), UL123 (IE1), and TRS1, in addition to the major immediate early promoter (MIEP). Eight genes coding for early HCMV proteins were also screened ³⁷⁶: UL33, UL54, UL55, UL56, UL57, UL77, UL78, and UL112. Five genes coding for early-late HCMV proteins were tested ³⁷⁶: UL72, UL84, UL89, UL96, UL97. Finally four genes corresponding to late HCMV proteins were analyzed ³⁷⁶: UL83, UL93, UL94. Moreover, we screened also the

four long non coding (lnc) RNA genes present in the HCMV genome: RNA 2.7, RNA 1.2, RNA 4.9, and RNA 5.0 in addition to the lytic origin of DNA replication (Orilyt), RL1, UL76, and US1.

3.2. Most of the HCMV genes screened were not detected in CTH cells.

3.2.1. Immediate early HCMV genes.

PCR amplifications for genes coding for the HCMV-DB immediate early proteins did not show any positive result in DNA extract from CTH cells. The amplicon size and position of each amplicon in the HCMV-DB genome are summarized in Table 2. The positive controls were fine with amplicons of the correct size in the HMECs infected with HCMV-DB and HCMV-DB viral stocks. No specific amplification was obtained neither in the CTH cells nor in the negative controls (Fig 14, table 2).

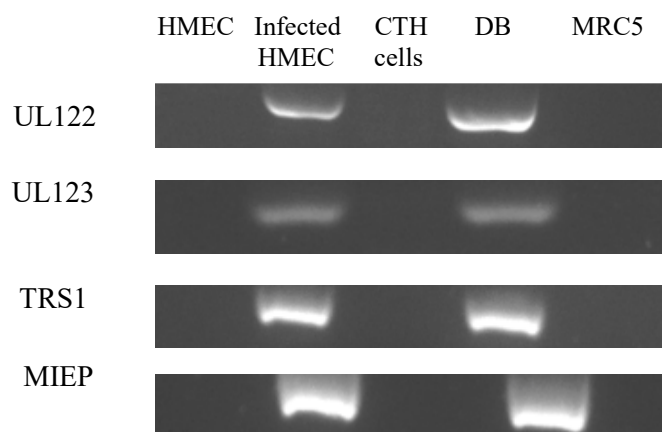


Figure 14. Agarose gel electrophoresis for PCR analysis of HCMV-DB DNA of immediate early proteins in CTH cells. PCR conditions: Initial denaturation T=95°C, 10 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T(59°C for UL-122, 57°C for UL-123, 59°C for TRS-1, and 57 °C for MIEP), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 10 min. Volume: 25µL. 5 µL of PCR product loaded to a 2.5% agarose gel; running at 67 volts for 2h . Bands with the correct size for each gene were detected in the infected HMEC and DB positive control. No bands were detected neither in CTH cells nor in the negative control.

3.2.2. Early HCMV genes.

We did not detect any of the early HCMV genes in CTH cell extracts although the positive and negative controls worked fine (Fig 15, table 2).

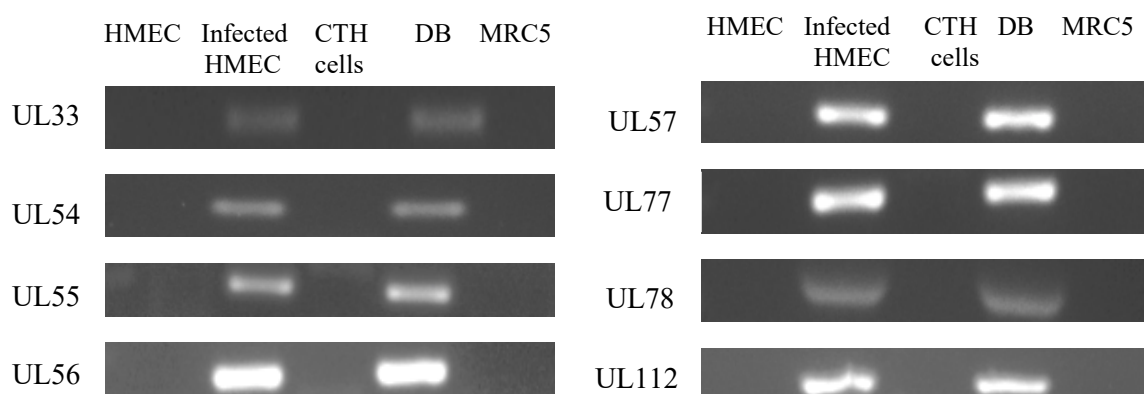


Figure 15. Agarose gel electrophoresis for PCR analysis of HCMV-DB DNA of early proteins in CTH cells. Left panel: PCR conditions: Initial denaturation T=95°C, 5 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T(48°C for UL-33 and UL-54, and 52°C for UL-55 and UL-56), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 10 min. Right panel: PCR conditions: Initial denaturation T=95°C, 5 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T(50°C for UL-77, 52°C for UL-57 and UL-78, and 59°C UL-112), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 10 min. Volume: 25µL. 5 µL of PCR product loaded to a 2.5% agarose gel; running at 67 volts for 2h . Bands with the correct size for each gene were detected in the infected HMEC and DB positive control. No bands were detected neither in CTH cells nor in the negative control.

Table 2: Results summary of PCR amplifications of immediate early and early proteins in CTH cells.

	Gene	Amplicon size (bp)	Amplicon position (bp)	PCR result in CTH cells
Immediate early	UL-122	431	172221-172652	Negative
	UL-123	393	172717-173109	Negative
	TRS1	128	232343- 232470	Negative
	MIEP	311	174837-175147	Negative
Early	UL33	109	44415-44523	Negative
	UL54	138	80280-80417	Negative
	UL55	197	83490-83686	Negative
	UL56	175	86317-86491	Negative
	UL57	203	90544-90746	Negative
	UL77	135	112358- 112492	Negative
	UL78	191	114452- 114642	Negative
	UL112	158	161737- 161894	Negative

3.2.3. Early-late HCMV genes.

We did not find specific amplification in CTH cell extracts for UL72, UL84, UL89, UL96, and UL97 genes (Fig 16, table 3). The positive and negative controls did work fine.

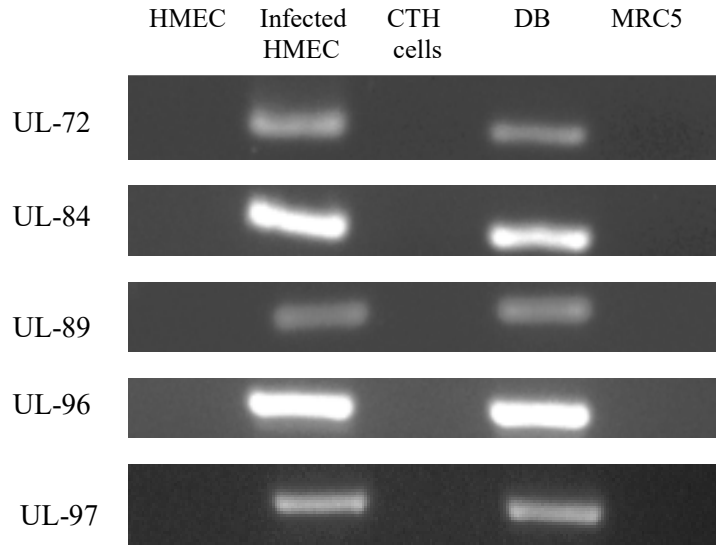


Figure 16. Agarose gel electrophoresis for PCR analysis of HCMV-DB DNA of early-late proteins in CTH cells. PCR conditions: Initial denaturation T=95°C, 5 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T(52°C for UL-72, UL-84 and UL-89, 55°C for UL-96, and 50.3 °C for UL-97), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 10 min. Volume: 25µL. 5 µL of PCR product loaded to a 2.5% agarose gel; running at 67 volts for 2h. Bands with the correct size for each gene were detected in the infected HMEC and DB positive control. No bands were detected neither in CTH cells nor in the negative control.

Table 3: Results summary of PCR amplifications of early-late proteins in CTH cells.

	Gene	Amplicon size (bp)	Amplicon position (bp)	PCR result in CTH cells
Early-Late	UL72	192	106506- 106699	Negative
	UL84	213	122981- 123193	Negative
	UL89	166	134430-134595	Negative
	UL96	288	141135-141422	Negative
	UL97	325	141983- 142307	Negative

3.2.4. Late HCMV genes.

None of the screened genes coding for late HCMV proteins such as UL83, UL93, and UL94 were detected in CTH cell lysates (fig 17, table 4).

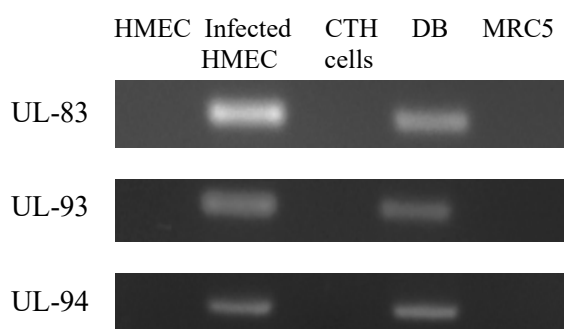


Figure 17. Agarose gel electrophoresis for PCR analysis of HCMV-DB DNA of late proteins in CTH cells. PCR conditions: Initial denaturation T=95°C, 5 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T(57°C for UL-83, and UL-93, and 54°C for UL-94), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 10 min. Volume: 25µL. 5 µL of PCR product loaded to a 2.5% agarose gel ; running at 67 volts for 2h. Bands with the correct size for each gene were detected in the infected HMEC and DB positive control. No bands were detected neither in CTH cells nor in the negative control.

Table 4: Results summary of PCR amplifications of late proteins in CTH cells.

	Gene	Amplicon size (bp)	Amplicon position (bp)	PCR result in CTH cells
Late	UL83	448	120649-121096	Negative
	UL93	158	136170- 136327	Negative
	UL94	122	137534- 137655	Negative

3.2.5. Other HCMV-DB genes.

Finally, no specific amplicons corresponding to the lytic origin of DNA replication (orilyt), RL1 , UL76, and US1 were detected in CTH cell extracts (Fig 18, table 5).

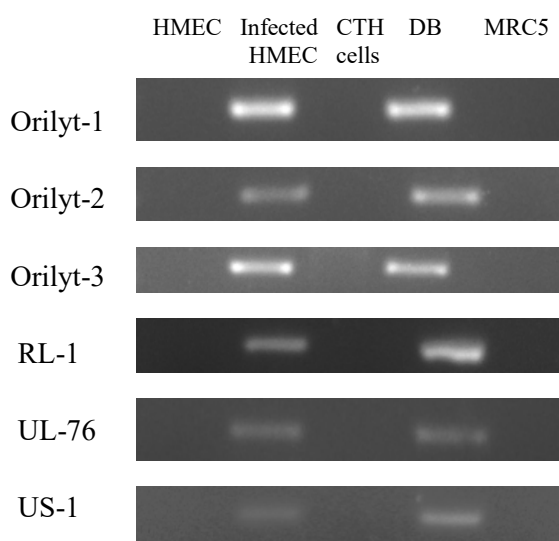


Figure 18. Agarose gel electrophoresis for PCR analysis of HCMV-DB DNA in CTH cells.PCR conditions: Initial denaturation T=95°C, 10 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T(52°C for Orilyt-1 and UL-76, 55°C for Orilyt-2 and Orilyt-3, 57°C for RL-1 and 58°C for US-1), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 10 min.Volume: 25µL. 5 µL of PCR product loaded to a 2.5% agarose gel; running at 67 volts for 2h . Bands with the correct size for each gene were detected in the infected HMEC and DB positive control. No bands were detected neither in CTH cells nor in the negative control.

Table 5: Results summary of PCR amplifications in CTH cells.

Gene	Amplicon size (bp)	Amplicon position (bp)	PCR result in CTH cells
Orilyt set 1	188	91907- 92094	Negative
Orilyt set 2	202	93617- 93818	Negative
Orilyt set 3	224	93115-93338	Negative
RL1	139	1797- 1935	Negative
UL76	231	111755- 111985	Negative
US1	141	198323- 198463	Negative

3.3. Detection of long non-coding RNA 4.9 (lncRNA4.9) HCMV sequences in CTH cells.

During the viral infection, host cells generate several lncRNAs that have a role in counteracting the infection. Similarly, viruses also express several lncRNAs to resist the cellular antiviral activity³⁷⁷. We screened CTH cells for the presence of the four HCMV-DB lncRNAs: lncRRNA2.7, lncRNA1.2, lncRNA5.0 and lncRNA4.9. We did not find any specific amplification in CTH cell extracts for RNA 2.7, RNA1.2 , and RNA5.0 (Fig 19, table 6).

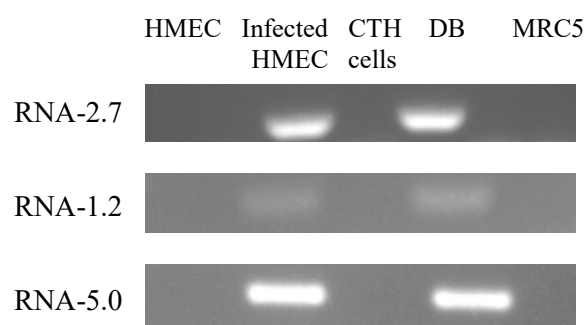


Figure 19. Agarose gel electrophoresis for PCR analysis of HCMV-DB DNA of lncRNA in CTH cells.PCR conditions: Initial denaturation T=95°C, 5 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T(53°C for RNA-2.7, 50°C for RNA-1.2, and 48°c for RNA-5.0), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 10 min.Volume: 25µL. 5 µL of PCR product loaded to a 2.8% agarose gel ; running at 67 volts for 2h . Bands with the correct size for each gene were detected in the infected HMEC and DB positive control. No bands were detected neither in CTH cells nor in the negative control.

However, we detected two positive amplicons of the right size for the HCMV-DB lncRNA4.9 gene in CTH cell extracts (Fig 20, table 6) (Annex 2, Article 2 EBioMed). A first amplicon of 126 bp which corresponds to the nucleotides 95592-95717 of lncRNA4.9 was detected in CTH cell extracts. A second amplicon of 90 bp which corresponds to the nucleotides 99014-99104 of lncRNA4.9 was also detected in CTH cell extracts. Thus, we detected two sequences of HCMV-DB genome in DNA extract of CTH cells.

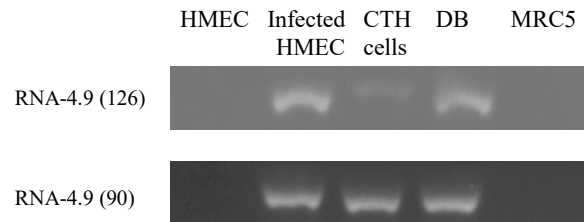


Figure 20. Agarose gel electrophoresis for PCR analysis of HCMV-DB DNA of lncRNA4.9 in CTH cells.

PCR conditions: Initial denaturation T=95°C, 5 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T(54°C for RNA-4.9 (126), and 58°C for RNA-4.9 (90), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 10 min. Volume: 25µL. 5 µL of PCR product loaded to a 2.8% agarose gel ; running at 67 volts for 2h . Bands with the correct size for each gene were detected in CTH cells in addition to the infected HMEC and DB positive control. No bands were detected in the negative control.

Table 6: Results summary of PCR amplifications of lncRNA in CTH cells

	Gene	Amplicon size (bp)	Amplicon position (bp)	PCR result in CTH cells
LnRNA	RNA2.7	122	2584-2705	Negative
	RNA1.2	93	6821-6913	Negative
	RNA5.0	181	160159-160337	Negative
	RNA4.9(126)	126	95592-95717	Positive
	RNA4.9(90)	90	99014-99104	Positive

The following map summarizes all the genes of HCMV-DB that were screened by PCR amplifications with their positions.

3.4. Sequencing of lncRNA4.9 HCMV amplicons present in CTH cells.

The 126 bp and 90 bp amplicons obtained following amplification with lncRNA4.9 primer sets were sent for sequencing at two independent companies (Genoscreen, Lille, France and GATC, Cologne, Germany) for confirmation by Sanger's sequencing. Table 7 includes the list of primers used for sequencing and the passage number of CTH cells used in each PCR amplification.

After receipt of the sequencing results, we compared the amplicon sequence obtained with the reference sequence of the HCMV-DB genome (Genbank number: KT959235) using a Blast software. We confirmed that the two 126bp and 90 bp amplicons matched exactly two sequences present in the HCMV-DB strain which was used to infect HMEC culture and that resulted in the appearance of clusters of CTH cells. PCR amplifications and Sanger's sequencing were repeated twice and gave similar results independent on the screening company used.

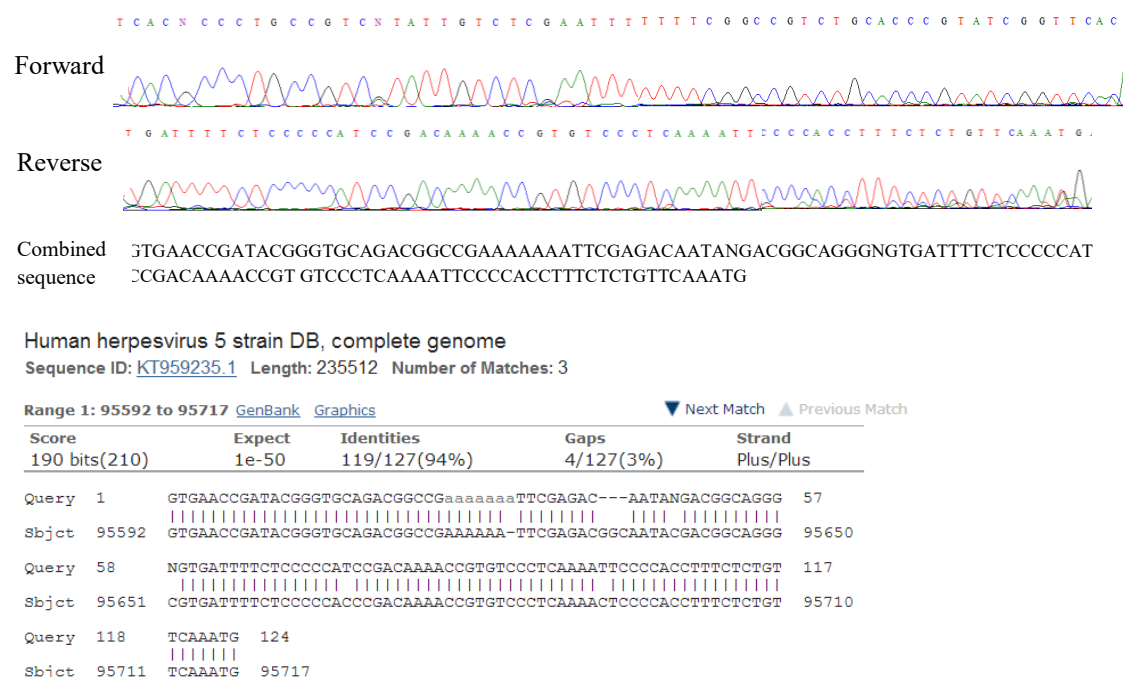


Figure 22: Sequencing results of lncRNA4.9 (126bp) and its blast with HCMV-DB (KT959235)

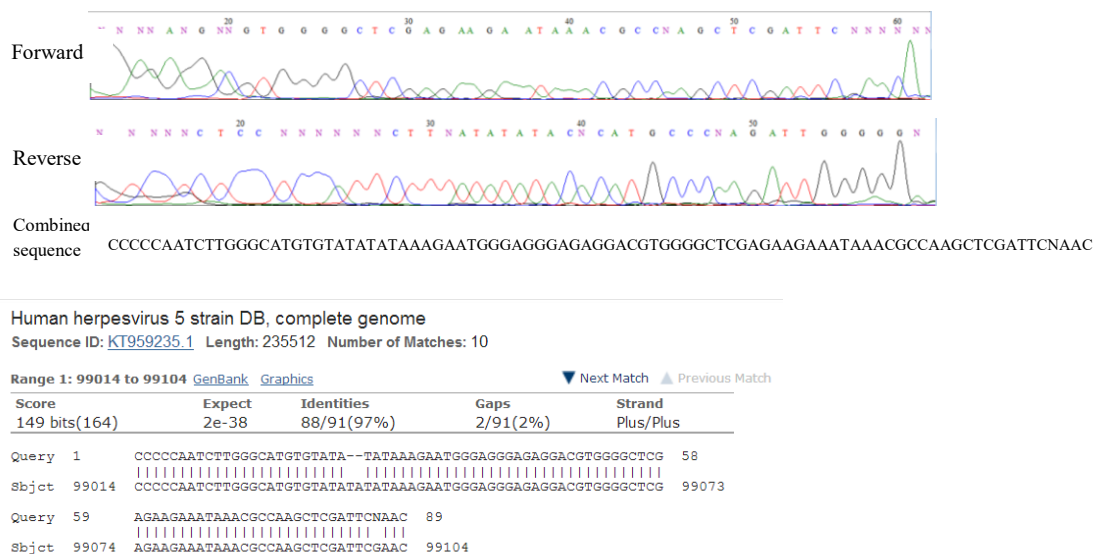


Figure 23 : Sequencing results of LncRNA4.9 (90bp) and its blast with HCMV-DB (KT959235)

Table 7: List of primers used in sequencing

Sequenced genes	Primers used for sequencing		Passage number of CTH cells	Purified for sequencing
RNA 4.9	Set (126 bp)	5' GTGAACCGATACGGGTGGAG 3'	135	No
		5' CATTTGAACAGAGAAAGGTGG 3'		
	Set (90bp)	5' CCCCCAATCTTGGGCATGTG 3'	135	No
		5' GTTCGAATCGAGCTTGGCGTA 3'		

The DNA of CTH cells used in PCR amplification experiments was extracted from CTH cells with various passage numbers in culture. To confirm that the passage number will not affect the obtained results, we chose three sets of primers for which we obtained negative results in CTH cells: MIEP, UL-83, and UL123; and the RNA 4.9 (90bp and 126bp) sets for which we obtained a positive signal in CTH cells. We then did PCR amplifications with all these sets using CTH cell DNA from the same vial (same passage number in culture, passage 35 or P35). The obtained results confirmed the previous results where there was no amplification in CTH cells with MIEP, UL-83, and UL-123 primer sets. Nevertheless, MIEP, UL83 and UL123 amplicons of the correct sizes (311 bp, 448 bp, and 393 bp respectively) were obtained in positive controls namely in HCMV-DB infected HMEC and in HCMV-DB viral stock (Fig 24; left panel).

PCR amplifications for lncRNA4.9 gave amplicons of the expected sizes (90bp, and 126 bp) in C TH cells in agreement with the positive controls namely HCMV-DB infected HMEC and HCMV-DB viral stock (Fig 24; right panel). These results show that using DNA extracted from CTH cells with different passage number will not affect the obtained results.

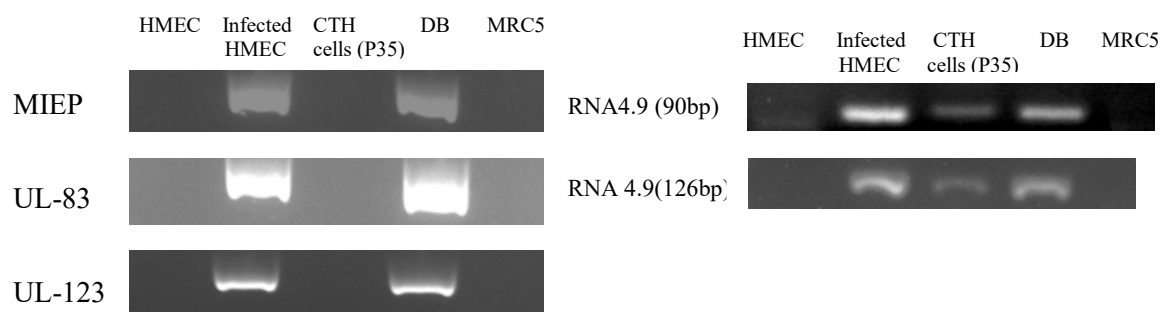


Figure 24. Agarose gel electrophoresis for PCR analysis of HCMV-DB DNA in CTH cells from the same passage number in culture (P35). Left panel: PCR conditions: Initial denaturation T=95°C, 5 min. 40 Cycles : Denaturation T=95°C, 30sec; Annealing T=57°C, 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 5 min. No bands were detected neither in CTH cells nor in the negative control. Right panel: PCR conditions: Initial denaturation T=95°C, 5 min. 40 Cycles: Denaturation T=95°C, 30sec; Annealing T 58°C for RNA 4.9 (90bp) and 54°C for RNA 4.9 (126bp), 30 sec ; Elongation T=72°C, 30 sec. Final elongation T=72°C, 5 min. Bands with the correct size for each gene were detected in CTH cells in addition to the HCMV-DB infected HMEC and HCMV-DB viral stock. No bands were detected in the negative controls (uninfected HMEC and MRC5 cells).

Materials and Methods

Cell cultures. Human primary mammary epithelial cells (HMECs) were obtained from Life Technologies (Carlsbad, CA, USA). They were cultivated in HMEC medium (Life Technologies, Carlsbad, CA, USA) supplemented with HMEC supplement and bovine pituitary extract (Life Technologies, USA). MDA-MB231 and MCF-7 cells were provided by Institut Hiscia (Arlesheim, Switzerland) and cultivated in DMEM medium with 10% FBS. MRC5 human fibroblasts were obtained from BioMe'rieux and cultivated in DMEM medium with 10% FBS. Cell viability assay was performed by trypan blue exclusion. Cultures were free of mycoplasma. Cell culture flasks and cell scrapers were purchased from Nunc.

HCMV infection of HMECs. The clinical isolate HCMV-DB was isolated in our laboratory from a cervical swab specimen from a 30-year-old pregnant woman [khan 2009]. Cell-free virus stocks were prepared by propagating HCMV-DB in macrophages. The MDM cultures were infected at different multiplicities of infection (MOI) at days 7–10 post isolation, incubated for 12 h at 37°C, washed thoroughly, and covered with fresh medium. Cells were infected with a viral isolate when the monolayer was confluent. Supernatants were clarified by centrifugation and stored at -80°C until use (khan 2009). Virus titers were determined by plaque forming assay in MRC5 human fibroblasts according to standard procedures. MRC5 human fibroblasts were cultured in MEM with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 µg/ml). HMECs (1×10^6) were infected at a multiplicity of infection (MOI) of 1 or 10 for 2 h at 37°C, washed thoroughly (three times with 1X PBS), and covered with fresh medium. Supernatants were clarified by centrifugation and stored at -80°C until use.

The purity of our HCMV stocks was confirmed by the absence of detection of other viruses (HSV-1, HSV-2, varicella-zoster virus, Epstein-Barr virus, adenovirus, BK virus) using PCR screening (data not shown). Following HCMV infection of HMECs, viral replication was assessed by the appearance of a cytopathic effect (CPE) in the cultures and by detection of IE1 antigen (clone E13, Argene-Biosoft, Varihes, France) using immunofluorescence microscopy (Nikon Eclipse E400, Kanagawa, Japan). Quantification of viral titer in cell culture supernatants was performed by qPCR as previously described (khan 2009).

RT² Profiler™ PCR Arrays. Total RNA was extracted from uninfected HMECs and HMECs infected with HCMV-DB at MOI of either 1 or 10 at day 1 post infection, MCF-7 and MDA-MB 231 using TRIzol reagent (Life Technologies, Grand Island, NY), and the first strand cDNA synthesis was achieved using the RT² First Strand Kit (SABiosciences, Valencia, CA) following the manufacturer's instructions. Three RT² Profiler™ PCR Arrays for human breast cancer (PAHS-131ZA), oncogenes/tumor suppressor genes (PAHS-502Z) and human epigenetic chromatin modification enzymes (PAHS-085A) (all three from SABiosciences) were applied on the Strategene Mx3005P real-time PCR system (Agilent Technology, Santa Clara, CA). Each 96-well RT² Profiler™ PCR Array contains 84 genes related to human cancers, wells for different housekeeping genes, a genomic DNA contamination control, three replicate reverse transcription controls, and three replicate positive PCR controls. Data analyses were performed using the web based analysis software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>)

Genomic analysis of HCMV-DB. The analysis of HCMV-DB genome and its comparison to other HCMV strains: AD-169 and Towne (laboratory adapted strains) and Merlin, Toledo, TR, PH, VR1814, Davis, JP, and TB40/E (clinical strains with low passages in culture) was done using the NCBI nucleotide blast tool (<https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi>)

Phylogenetic analysis. Phylogenetic analysis was determined among various HCMV strains (described in Table 1) for UL55, UL75, UL115, UL128, UL130, and UL131 genes. For UL144 gene the phylogenetic analysis was performed as previously reported³⁷⁸. Multi-sequence alignments (MSA) were performed using MEGA7 by CLUSTAL W with the following parameters: a gap opening penalty of 15 and gap extension penalty of 6.66. Phylogenetic tree was constructed using the neighbor-joining method. Each clustering was confirmed by the bootstrap method with 1,000 replicates. The analysis was conducted using MEGA7 (<http://www.megasoftware.net/>).

PCR amplifications. Classical PCR amplifications were done for several parts of the HCMV genome. Primers were either designed in house or (when specified) taken from references. Primers were ordered from Eurogentec. Dream Taq polymerase was used. It was ordered from Thermofisher (EP0702, Lithuania).

Primers design: Primers used in HCMV-DB genome screening were designed using the NCBI primers designing tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The obtained primers were chosen after checking for the hairpins and primer dimers formation using the oligoanalyzer tool.

Gel electrophoresis: Amplified products were electrophoresed in agarose gel (the concentration of the gel was determined depending on the amplicon size) and stained with Sybr green I nucleic acid stain.

Gene sequencing. Positive PCR results were sent either to Genoscreen (Lille, France) or to GATC (Cologne, Germany) for sequencing, to confirm their positivity. The obtained sequences were prepared using the BIOEDIT software. Sequences were blasted with DB strain (KT959235) using NCBI nucleotide blast tool (<https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi>).

Statistical analysis. The reported values are the means and SD of independent experiments. Statistical analysis was performed using Mann Whitney U test, and differences were considered significant at a value of $P < 0.05$. Microsoft Excel was used to construct the plots.

Discussion

Our results indicate that the genomic profile of HCMV-DB strain is close to other clinical strains mainly Toledo, Merlin, and JP clinical strains. We observed that the HCMV-DB infects HMECs and the transcriptome of the HCMV-DB infected HMEC cells shows pro-oncogenic characteristics with an upregulation in the expression of several oncogenes, activation of pro-survival genes, and upregulation of cell proliferation markers, stemcellness and EMT. Also our results showed that part of the HCMV-DB genome especially a lncRNA4.9 signature is detected in HCMV-DB-transformed HMECs, namely CTH cells indicating a potential role for HCMV in oncogenicity and cellular transformation.

Although the clinical isolates of HCMV display a broad cellular tropism infecting among others monocytes, macrophages, endothelial cells, epithelial cells, stromal cells, fibroblasts, smooth muscle cells, neural stem/progenitor cells, and hepatocytes, the growth of HCMV laboratory strains is only restricted to fibroblasts²⁸². Some clinical isolates of HCMV can infect the blood monocytes and tissue macrophages with inducing a distinct (M1) inflammatory and (M2) immunosuppressive macrophage polarization and establish latency in the infected monocytes/macrophages^{271–273,358}. HCMV strains tropism could have an important role in activating the M1/M2 macrophages, enhancing viral fitness and potentially favoring the promotion of breast cancer^{274–276}. The studied strain HCMV-DB (KT959235) was isolated previously from a cervical swab specimen from a pregnant women of 30- years old³⁵⁸. HCMV-DB is a highly macrophage tropic strain which triggers an M2 phenotype and upregulates the proto-oncogene Bcl-3³⁵⁸. HCMV-DB strain is close from other primary clinical isolates such as the JP strain which was isolated from the prostate of an HIV-infected patient after his death^{26,365}. Both JP

and DB strains infect the prostate and the mammary glands respectively in which they share several similarities concerning their physiology, appearance and pathology³⁷⁹. The overall analysis of DB strain genome sequence revealed that it is highly close to that of JP strain (99% identity) and with a lesser extent to the Toledo strain (98% identity). Furthermore, by taking in consideration the viral genes that are involved in cell tropism, the phylogenetic analysis reveals that the DB strain is close to the Merlin strain²⁷⁴.

HCMV-DB infected HMECs showed a triple negative ER-/PR-/HER2- phenotype with a low level in the expression of the luminal markers (KRT19, KRT18, GATA3, and TFF1). The oncogenic conversion of HMECs is requiring a limited set of growth-deregulating changes³⁸⁰. These changes include the inactivation of the pathways of P53 and retinoblastoma (Rb) proteins³⁶⁴, telomere maintenance^{241,362}, and the activation of Akt pathway and of mitogenic signal which is provided by Myc and Ras oncogenes^{380–382}. We observed that in HCMV-DB infected HMECs the transcripts of Rb and P53 are upregulated. It has been reported in fibroblasts infected with several strains of HCMV that p53 transcript and its protein expression are increased³⁶⁰. In order to explain the functional inactivation of p53 that is required to explain the development of cellular transformation previous reports mentioned that there is a potential inhibitory effect of HCMV-IE2-86 protein on p53 transcriptional activity³⁶¹. In accordance with p53 transcript upregulation, we previously reported the enhanced p53 protein expression in HMECs infected with HCMV-DB, its binding to HCMV-IE2 and the functional inactivation of p53²⁸⁰.

HMECs infected with HCMV-DB showed 7-fold higher expression of retinoblastoma protein (Rb) as compared to the uninfected HMECs at day 1 post infection. Rb transcript was also upregulated in MDA-MB231 cells in agreement with

previous reports that showed the upregulation of pRb protein in MDA-MB 231 cells ³⁷³. Although there was an upregulated expression of Rb gene in HCMV-DB infected HMECs, reports showed that there is a post translational phosphorylation of pRb which has a pivotal impact on its inactivation ³⁸². The UL97 protein (pUL97) of HCMV has activities similar to that of the cellular cyclin dependant kinase (CDK) complexes. Thus UL97 will phosphorylate the pRb protein causing its inactivation. The HCMV pUL97 protein is lacking the amino acid residues that are conserved in CDKs and which are responsible for allowing the attenuation of kinase activity, and also it is not inhibited by the CDK inhibitor p21. Thus its activity is immune from the normal CDK control mechanisms ^{229,363}. Moreover, HCMV UL82 encodes for pp71 which play a role in degrading the retinoblastoma proteins family ²²⁷. In accordance with this hypothesis, we had shown previously that UL82 transcript and its corresponding protein expression (pp71) were upregulated at day 1 and 3 post infection of HMECs with HCMV-DB ²⁸⁰. Thus, both proteins, p53 and pRb, could be inactivated in HMECs infected with HCMV-DB and therefore potentially block the cellular senescence and thus enhance the unchecked cell division ³⁸⁰.

In addition, we observed that the IL-6/STAT3 axis is activated in the infected cells with an increased expression of pUS28 (Annex 2, Article 3, Sci Rep, figure 3), which is in agreement with other studies confirming the HCMV pUS28 role in mediating cellular proliferation through the activation of this axis ^{248,383}.

The proto-oncogenes c-Myc and Ras provide the acquisition of a mitogenic signal that could further permit the complete in vitro transformation of HMECs infected with HCMV-DB. In agreement with the enhanced Myc and Ras transcripts expression in HCMV-DB infected HMECs, we had shown previously the enhanced Myc and Ras

proteins expression starting at day 1 post infection ²⁸⁰. The overexpression of other oncogenes transcripts such as Jun and Fos could also be involved in the HCMV-DB induced transformation of HMECs ^{384,385}. Myc is also overexpressed in cervical carcinoma, and its region is located on chromosome 8q24.21 which is a known site for the frequent integration of the human papillomavirus (HPV) ^{381,386,387}.

The activation of Fos and Myc is induced in HMECs infected with HCMV-DB by the IE1 and IE2 proteins ³⁸⁸. Additionally, MYB gene is also induced in the HCMV-DB infected HMECs similar to the enhanced MYB gene expression in carcinoma induced by HPV ²⁹³.

Moreover, the activation of PI3K/Akt pathway in breast cancer is critical for the anchorage-independent growth and transformation of HMECs ^{389,390}. We observed the enhanced expression of the PI3K/Akt pathway genes, which is in accordance with the threonine-308 and serine-473 phosphorylation of Akt in HCMV-DB infected HMECs ²⁸⁰. We also observed the upregulation of pro-survival gene transcripts such as Akt, Bcl-2, and NF- κ B in parallel to the decreased caspase-8 transcript levels. These results show that the transcriptome of HCMV-DB infected HMECs displays a pro-survival phenotype.

HCMV-DB infected HMECs has also revealed an upregulation in the gene expression of markers of proliferation as topoisomerase 2, Ki67 antigen and the transcription factor E2F1 as compared to the uninfected HMECs. These obtained results come in agreement with the observed increased proliferation in HCMV-DB infected HMECs cultures ³⁵⁸.

HCMV-DB infected HMECs had also revealed a dysregulation of the cell cycle, with an enhanced expression of cyclins genes (cyclin A1, D1 D2, E1). Cell cycling

inhibitors (CDKNs) were also expressed in a lesser extent. The induction of CDKN2A gene in HCMV-DB infected HMECs is in agreement with the highly induced CDKN2A gene with HPV infection in which is widely used as a surrogate marker in HPV carcinoma ^{293,391}.

Beside the enhanced HCMV-DB infected HMECs proliferation, a dysregulation in cell cycling which could lead to the inhibition in the progression of G1 cell cycle was also noticed. This will cause a blockage in G1/S transition, as other groups had previously reported in fibroblasts infected with different HCMV strains ³⁹². The presence of an exquisite balance between cell cycling enhancement (cyclin gene expression) and cell cycling inhibition (CDKN gene expression), might favor the enhanced cell proliferation as detected by the increased TOP2A and Ki67Ag transcripts and enhanced Ki67 antigen detection in HCMV-DB infected HMECs²⁸⁰.

The decreased activation of MAPK had been reported as pejorative in triple negative breast cancers ³⁹³. HCMV-DB infected HMECs which exhibit a ER-/PR-/HER2-phenotype display a decreased expression in the genes of the MAPK cascade (MAPK3, MAPK8).

Previous reports had mentioned that several herpesviruses lead to genomic instability with activation of DNA repair pathway with increased ATM gene expression ³⁹⁴⁻³⁹⁶. Our results showed that the ATM gene expression is upregulated in HCMV-DB infected HMECs as was previously reported in fibroblasts infected with other strains of HCMV ³⁹⁵. Though the enhanced expression of ATM could favor the DNA damages repair (such as chromosomal breaks) as already reported in HCMV infected fibroblasts ^{232,236}, it could also cause a genomic instability and thus favoring the cellular transformation ³⁹⁷.

Several studies have mentioned that triple negative breast cancers are characterized by the most extensive hypomethylation, opposite to the cancers with positive luminal estrogen receptors that show the highest degree of hypermethylation ^{398,399}. HCMV-DB infected HMECs showed that the expression of several histone methyltransferase genes and of genes coding for SET domain proteins with histone methyltransferase activity is downregulated. DNA methyltransferase 3A (DNMT3A) gene expression is also downregulated in the infected HMECs.

Suzuki et al study on the progression of mammary tumor reported that there is a marked reduction of histone acetylation in ductal carcinoma in situ (DCIS) as compared to the normal mammary epithelium whereas most cases showed the presence of similar acetylation levels in DCIS in comparison to the invasive ductal carcinoma ⁴⁰⁰. The authors also described a decrease in HDAC1, HDAC2, and HDAC6 protein levels during the progression of tumor ⁹⁰. We observed that HCMV-DB infected HMECs display a decreased expression of genes that are involved in histone acetylation (histone acetyl transferases) as compared to uninfected HMECs. Also we observed that the gene expression of several histone deacetylases (HDAC 2, 4,5,6,9, and 10) was also decreased. Altogether, our results show that HCMV-DB infected HMECs display alterations of histone acetylation starting at day 1 post infection in which they have been evoked as an early breast tumor progression event.

The transcriptomic analysis of HCMV-DB infected HMECs revealed that there is a modulation in genes that are involved in angiogenesis and proteolysis, and thus that are potentially involved in tumor microenvironment remodeling ^{401,402}. The expression of IL-6-JAK gene was also upregulated in HCMV-DB infected HMECs as compared to the uninfected HMECs which has been reported to play a role in tumorspheres appearance in HCMV infected hepatocytes cultures ³⁴¹.

Furthermore, HCMV-DB infected HMECs showed an upregulation in the gene expression of the marker of stemcellness, alpha 6 integrin (ITGA6). Also the proto-oncogene tyrosine-protein kinase Src which is a gene involved in EMT is upregulated in the infected cells. Altogether, these results show that there is a potential trend towards the development of cancer stem cells (CSCs) in HCMV-DB infected HMECs. These CSCs are required for the sustained tumor growth, invasiveness, and the formation of metastasis ⁴⁰³. CSCs expansion could be measured by tumorsphere formation. In accordance with IL-6-JAK pathway activation, alpha 6 integrin upregulation, and src gene expression, we noticed the formation of tumorspheres in HCMV-DB infected HMECs cultures (Annex 2, Article 3, Sci Rep, figure 3).

Since CTH cells appeared in some of the cultures of HMECs infected with HCMV-DB, it was critical to show that a HCMV-DB genomic signature can be found in CTH cells. Therefore, we screened CTH cells with primer sets specific for HCMV-DB. Our PCR screening for HCMV genome in CTH cells indicates that none of the screened genes coding for the immediate early proteins (IE1, IE2, and TRS1) was expressed. Shen et al. had studied the role of IE1/IE2 in cellular transformation of primary BRK. In accordance to our results, they were unable to detect the expression of these two immediate early proteins after transformation ²³⁷. They suggested that IE1 and IE2 products can induce mutations accumulation, where they may be involved in increasing the frequency of error in the cellular DNA polymerases or could interfere with the cellular DNA repair process ²³⁷. Their finding that mutated p53 alleles are present in many of the transformed BRK cell lines suggests that one of the mechanisms involved in the contribution of IE1 and IE2 in transformation is the p53 mutation ²³⁷. They proposed the “hit-and-run” mechanism for HCMV contribution to oncogenesis, where it induces cellular genes mutations ²³⁷.

The MIEP is the major immediate early promoter for IE1 and IE2 genes. In agreement with the absence of detection of IE1 and IE2 genes in CTH cells, we did not detect the MIEP sequence by PCR. However, our PCR screening showed the presence of signatures of lnc RNA 4.9 in two different positions of the gene. lncRNA 4.9 is a long non coding RNA. lncRNA4.9 gene is located between the oriLyt and the UL-69 gene ⁴⁰⁴. lncRNAs have emerged as important regulators of the expression of genes in human cells ⁴⁰⁵. Interaction with chromatin modifying complexes is a well-defined mechanism ⁴⁰⁵. lncRNA can mediate epigenetic changes in histones by acting as a molecular scaffolds, which results either in the activation or the suppression of gene expression ^{406–409}.

Rosseto et al study on CD14 (+) monocytes with latent HCMV infection suggested that lncRNA4.9 might be a regulatory RNA with a potential to control both the cellular and the viral gene expression. It was the first report of an HCMV encoded lncRNA that can interact with polycomb repressive complex (PRC). PRC2 is primarily consisted of enhancer of zeste homolog2 (EZH2), SUZ12, RbAp48, and EED. It catalyzes histone H3 lysine 27 (H3K27) methylation to generate the trimethyl-H3K27 repressive mark H3K27me3 ⁴¹⁰. The presence of this H3K27me3 mark was also assessed at the MIEP ⁴⁰⁵. Recent studies showed that H3K27me3 is associated with the latency of herpes virus genomes and the repression of their lytic gene expression ^{411–414}. This could explain the role of the lncRNA4.9 in mediating gene silencing ^{415,416}. The physical interaction of lncRNA4.9 with the latent genome of HCMV at the MIEP region together with its interaction with PRC2 proteins that bound to the same locus, could also explain the repression of IE2 expression during latency ⁴⁰⁵. There is growing evidence demonstrating that EZH2, the catalytic subunit of PRC2, is essential for the initiation of cancer, its development and progression, metastasis, and resistance of drugs ⁴¹⁷. Altogether the

interaction of lncRNA4.9 with EZH2 could be one of the mechanisms by which HCMV develops oncogenicity.

Moreover, our results showed that except for lncRNA4.9, none of the screened lncRNAs (lncRNA1.2, lncRNA 2.7, and lncRNA5.0) was detected in CTH cells. These lncRNAs in addition to the lncRNA4.9 constitute the major viral RNA production during the lytic HCMV infection ⁴⁰⁴.

Our screening for genes coding for either the early or the late proteins showed no expression for any of them. Rossetto et al study on the expression of HCMV genes in the latent infection showed that there are several early and late genes that are detected ⁴⁰⁵. The fact that we were unable to detect any of these genes is because we are studying transformed cells where we suggest that after several passages of cells in culture, most of the viral genome will be eliminated except for the genomic part that could be the responsible for cellular transformation, as in HPV where its integration into the host genome showed a disruption in the E1 and E2 open reading frames while keeping the E6 and E7 ORFs and LCR intact ^{418–420}.

However even these genomes could be eliminated too with retaining the oncogenic phenotype as in adenovirus where the cells retain their oncogenic phenotype even after total loss of all the viral DNA ⁴²¹.

In conclusion, we described the genomic characteristics of the HCMV-DB strain, we observed that the transcriptome of HMECs infected with HCMV-DB clearly indicates a pro-oncogenic trend. Moreover, our finding of a signature of HCMV-DB in the transformed cells constitutes the presence of a link between HCMV-DB infection of HMEC and the developed cellular transformation and gives rise for a suggestion that this LncRNA4.9 might be responsible for the development of such transformation. Further work is to be done to elucidate the exact role of this lncRNA4.9 in the cellular transformation and to clarify the mechanism by which cells were transformed.

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Annex 1:

Table : Primers used for HCMV screening in PCR amplifications.

Primer	Sequence	Amplicon size	Reference	Position
RL1-F	5'GTTAGAACGTCCATCCGCG3'	139 bp	Sureselect	1797- 1935
RL1-R	5' AGTCGTAGCATGTGGGTGC 3'			
RNA 2.7 F	5' GCGAATCTCAAAGTTCTCACGA 3'	122 bp	Sureselect	2584-2705
RNA 2.7 R	5' TGACTTCATCGCAACGATCCA 3'			

RNA 1.2 F	5' TGGTTCATAAGGTAACGATGCT 3'	93 bp	Sureselect	6821-6913
RNA 1.2 R	5' TCGGAACCTTTTAGGAACCAGC 3'			
UL-33 F	5'GAGATCGCAGCAGAATCAGC 3'	109 bp	Sureselect	44415-44523
UL-33 R	5' CTGATTGCAGTTTTTGCTATTG 3'			
UL-44 F	5'TACGCATGTTTTTCACGCCG 3'	215 bp	In house design	57015-57229
UL-44 R	5'TCTAGATTTTCGGCGTGGTGG 3'			
UL-54 F	5' GCCATAGTGTGAGCTTATAG 3'	138 bp	In house design	80280-80417
UL-54 R	5' CTTTCCCTCGGCTTCTCACAA 3'			
UL-55 F	5' GGGGTCGGACATGTTCACTT 3'	197 bp	In house design	83490-83686
UL-55 R	5' TGGCTTTTCTTGAACGTGCG 3'			
UL-56 F	5' CAGGTTGTACTCCCTCAGCG 3'	175 bp	In house design	86317-86491
UL-56 R	5' GTCAAGCCAGTCCGATGTGA 3'			
UL-56-1 F	5' ACCGTTATTCCTCTGACTGTGA 3'	80 bp	In house design	85789-85868
UL-56-1 R	5' CGATGTCGGGGACGGGTA 3'			
UL-57 F	5' CTGAAGGCCAACTCGCAAAC 3'	203 bp	In house design	90544-90746
UL-57 R	5' ACGTCATGGTGTCAATCGCT 3'			
UL-57-1 F	5' TCGCAAGTTCTCCAGCGAAA 3'	107 bp	In house design	88026- 88132
UL-57-1 R	5' TGCTCTTCTTCGTGGATGGC 3'			
Orilyt F	5' CGATCCCCGAAAACACCGTA 3'	188 bp	In house design	91907- 92094
Orilyt R	5' CGGCGATTGAGGTTACGAGA 3'			
Orilyt-1 F	5' TCTACGAAAACGGACTGGCG 3'	202 bp	In house design	93617- 93818
Orilyt-1 R	5' CCACATCCCATAATCCGCGT 3'			
Orilyt-2 F	5' GTTAGGGTTCCACCGTCCTC 3'	224 bp	In house design	93115-93338
Orilyt-2 R	5' GTCAGGGGTACGTGAGAAG 3'			
RNA 4.9 F	5' GTGAACCGATACGGGTGGAG 3'	126 bp	In house design	95592-95717
RNA 4.9 R	5' CATTTGAACAGAGAAAGGTGG 3'			
RNA 4.9 F	5' CCCCCAATCTTGGGCATGTG 3'	90 bp	Sureselect	99014-99104
RNA 4.9 R	5' GTTCGAATCGAGCTTGCGTA 3'			

Table : Primers used for HCMV screening in PCR amplifications.

Primer	Sequence	Amplicon size	Reference	Position
UL-71 F	5' GGAGTGCAAGAAATCGCAGC 3'	203 bp	In house design	104883- 105085
UL-71 R	5' CGAGCATGTACGCGCTTTTC 3'			
UL-72 F	5' GCTCGCGTGATAGCCAAATG 3'	192 bp	In house design	106506- 106699
UL-72 R	5' CATTTACGCTGCCCCAATCG 3'			
UL-73 F	5' TCGACGACTAGTGCCAAACC 3'	228 bp	In house design	107091- 107318
UL-73 R	5' TTTGGTGGTGGTCGCAGTAA 3'			

UL-74 F	5' ACGAATCAGTACGTGCGTGT 3'	247 bp	In house design	107602- 107848
UL-74 R	5' TACCGCTTTCAACGTCACCA 3'			
UL-75 F	5' GAGGTAGTGAGTTCGCGGAG 3'	250 bp	In house design	109235- 109484
UL-75 R	5' CCTCATCATCACCCAGACGG 3'			
UL-76 F	5' TGTTGGTGTTTTTACCGCCG 3'	231 bp	In house design	111755- 111985
UL-76 R	5' CCAGACTGGGCACTACGTTC 3'			
UL-77 F	5' CTGTTGAACCAGCAGTGCG 3'	135 bp	Banerjee	112358- 112492
UL-77 R	5' ACTATAAAGACCGTGTGGGACG 3'			
UL-78 F	5' TTCGACGGCGTTGTTTTTCC 3'	191 bp	In house design	14452- 114642
UL-78R	5' GCCAACGGTAGTCCAGTGAA 3'			
UL-82 F	5' ACGCTTGATCTTGTCGGTT 3'	218 bp	In house design	119182- 119399
UL-82 R	5' CCAATCAGACTCACGACACCA 3'			
UL-83 F	5' TCGTACTGACGCAGTTCCAC 3'	128 bp	In house design	121174- 121301
UL-83 R	5' CATTTTGGGCTGCTGTGTCC 3'			
UL-84 F	5' AGCGTGGCGGTACAAAGTAA 3'	213 bp	In house design	122981- 123193
UL-84 R	5' GACGATTGGAGCTAGACCCG 3'			
UL-89 F	5' AAACATGTTGGCCGTCTTGC 3'	166 bp	In house design	134430-134595
UL-89 R	5' CAACAATGCGCCCTTTGACA 3'			

Table: Primers used for HCMV screening in PCR amplifications.

Primer	Sequence	Amplicon size	Reference	Position
UL-93 F	5' CCCACTGGAGTTGCTCGATT 3'	158 bp	In house design	136170- 136327
UL-93 R	5' CCAGTCTCAGGTGTAGCGTC 3'			
UL-94 F	5' CTTTGCGAGACCGATTCCAGA 3'	122 bp	In house design	137534- 137655
UL-94 R	5' AAGATCGTCGAACGGCAAGC 3'			

UL-96 F	5' ACAGCTCTTAAAGGACGTGATGCG 3'	288 bp	Ranganathan et al	141135-141422
UL-96 R	5' ACCGTGTCCCTTCAGCTCGGTAAA 3'			
UL-97 F	5' CTTCGGACAAGGAGAACCAG 3'	325 bp	Ranganathan et al	141983- 142307
UL-97 R	5' AGCTCCACGTCGTTTTCTTC 3'			
UL-98 F	5' TTTCCACTGCGACCACATCC 3'	97 bp	Banerjee	145398- 145494
UL-98 R	5' ACGGTCTAGCACGGTAGAGA 3'			
UL-98-1 F	5' AGTCGCGCAACTCGCTGTGG 3'	320 bp	Ranganathan et al	144302- 144621
UL-98-1 R	5' GAGCACGCCGAAGCACAGGT3'			
UL-98-2 F	5' GAGGTAGCGCGCTTTTCACT3'	200 bp	In house design	145174- 145373
UL-98-2 R	5' CAGCCCAGTTCGCTTTCCTC 3'			
UL-100 F	5' GTGTTGAAACCAAGCGCTAC 3'	125 bp	In house design	146850- 146974
UL-100 R	5' ACGCAGTACCGCAGCTACA 3'			
UL-104 F	5' CCAGGTCTGGTTGAGCGTG 3'	107 bp	Banerjee	151863- 151968
UL-104 R	5' GGACATCCGCGACCTGTTTA 3'			
RNA 5.0 F	5' CTGAAATGAATCTATGAAGGTGC 3'	181 bp	In house design	160159- 160337
RNA 5.0 R	5' ATTACGAGTAGTAGTGGTATGTA 3'			
UL-112 F	5' ATGGATCTGCCTACTACCGT 3'	158 bp	In house design	161737- 161894
UL-112 R	5' TGCAGCACCTTGCCATCGC 3'			
UL-122 F	5'AGCACCATCCTCCTCCTTCTCTGG3'	431 bp	Ranganathan et al.	172221-172652
UL-122 R	5' AAGCGGCCTCTGATAACCAAGCC 3'			
IE1-F	5' TCCTCGGTCACTTGTTCAAA 3'	117 bp	Ranganathan et al.	173583- 173700
IE-1 R	5' CGACGTTCTGCAGACTATG 3'			
US1-F	5' GAAGCCCGTTCTTCCGAGTC 3'	141 bp	In house design	198323- 198463
US1-R	5' AAGCTCCTCGTCATCCTCCG 3'			
TRS-1 F	5' GTTGGCATGGGACGATCAATC 3'	128 bp	In house design	232343- 232470
TRS-1 R	5' GACTCCTGGTACGACTTGGA 3'			

Annex 2: Publications

Publication 1. Open Virol J. 2017 Mar 31;11:15-27.



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REVIEW ARTICLE

Cytomegalovirus, Macrophages and Breast Cancer

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Abstract: The human cytomegalovirus (HCMV) is a betaherpesvirus that is highly host specific, infects among others epithelial cells and macrophages, and has been recently mentioned as having oncomodulatory properties. HCMV is detected in the breast tumor tissue where macrophages, especially tumor associated macrophages, are associated with a poor prognosis. In this review, we will discuss the potential implication of HCMV in breast cancer with emphasis on the role played by macrophages.

Keywords: HCMV, Tumor, Macrophages, Breast cancer.

MACROPHAGES, HETEROGENEOUS CELL POPULATIONS

Against pathogens, macrophages (Ms) constitute the first defensive line of the organism where they become differentially activated in response to the microenvironment. Cellular immunity response to viral infection includes the well-established, classically activated macrophages (M1) that are induced by T helper 1 (Th1)-type responses through the interferon-gamma induction. This interferon (INF)-gamma induction of type 1 macrophages displays a proinflammatory profile with high levels of interleukin (IL)-1 beta, IL-6, tumor necrosis factor alpha (TNF alpha), and other proinflammatory cytokines (Fig. 1A), in both of the plasma and lymph nodes, especially during systemic viral infections [1]. Classically, chemokines secretions are increased in patients with a M1 phenotype after viral infection. These chemokines include macrophage inflammatory protein (MIP)-1alpha, MIP-1beta and RANTES (CCL3, CCL4 and CCL5 respectively) (Fig. 1A) [1, 2]. Immune activation is achieved by the increase of antiviral immunity with the enhancement of Th1 activity and the increase in IFN gamma, IL-12, IL-2 and IL-18 levels, especially in the lymph nodes of virally-infected subjects [3]. Therefore, M1 macrophages have a pivotal role in cellular immunity. They are involved in immunodeficiency syndromes, and in responses towards tissue damage and delayed-type hypersensitivity reactions [4]. Furthermore, M1 macrophages are important anti-cancer players. They stimulate the Th1-cytotoxic T cells (CTLs) and other effectors cells, thus improving patient survival. This makes them an important target that might be used to make a successful immunotherapy of cancers [5, 6].

Another activated macrophage (M2) differentiation pathway is possible in the presence of Th-2 type response and through the production of specific cytokines, such as IL-4 and IL-13 (Fig. 1B). These M2-macrophages are part of the defense against parasitic infections, and display anti-inflammatory and tissue repair properties [4, 7]. Due to their participation in matrix-remodeling and their immunosuppressive activity, they can favor tumor growth. Macrophages may be activated alternatively by the action of IL-4 and IL-13 through a common receptor chain (IL-4Ra) (Fig. 1B) [4, 8, 9]. IL-4, a pleiotropic cytokine, is produced by Th-2 cells, which constitute a specific subset of CD4⁺ T cells, and by basophils and mast cells [10]. IL-13, secreted by activated T cells, has been shown to induce major changes in human monocytes phenotype. Their antigen presenting capabilities are increased by upregulating the expression of multiple

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cell surface molecules. The effect of IL-4 and IL-13 on macrophages leads to the upregulation of the mannose receptor and MHC class II molecules expression which stimulate endocytosis and antigen presentation. This alternative activation pathway also upregulates macrophage-derived chemokine (CCL22) expression [8]. In addition, IL-4 and IL-13 counteract the pro-inflammatory actions of IL-1 through the increased expression of the IL-1 receptor alpha-chain and the IL-1 decoy receptor, *in vitro* and *in vivo* (Fig. 1B) [4].

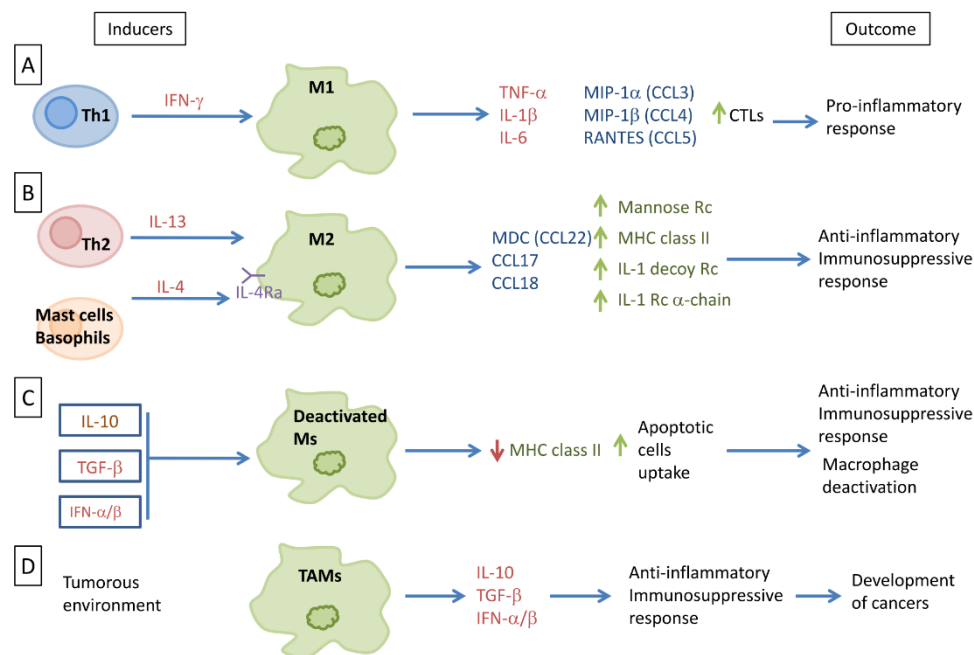


Fig. (1). Macrophages, heterogeneous cell populations. (A) Th1 induction of M1 through interferon-gamma signaling. M1 releases specific cytokines and chemokines as part of the proinflammatory response. (B) M2 differentiation *via* IL-13 and IL-4 released from Th2, mast cells, and basophils. Differentiated M2 then releases factors that favor the anti-inflammatory and immunosuppressive responses. (C) Ms deactivation through the IL-10, TGF-beta, and IFN-alpha/beta signaling. Deactivation downregulates the expression of MHC class II and increases the uptake of apoptotic cells, resulting in an anti-inflammatory and immunosuppressive response. (D) Macrophages differentiation towards a specific phenotype present in the tumorous environment (TAMs), where they release large amount of immunosuppressive cytokines and a little amount of pro-inflammatory cytokines indirectly promoting the development of cancer.

CTLs: Th1-cytotoxic T cells ; IFN: Interferon ; IL: Interleukine ; MHC: Major Histocompatibility Complex ; MIP: Macrophage Inflammatory Protein ; TNF: Tumor Necrosis Factor ; TAMs: Tumor Associated Macrophages ; Th: T helper ; Rc: Receptor ; CCL : C-C Motif Chemokine Ligand ; M : Macrophage ; MDC : Macrophage-Derived Chemokine ; RANTES : Regulated on Activation, Normal T cell Expressed and Secreted ; TGF : Transforming Growth Factor.

The deactivation of macrophages is mostly induced by the production of another cytokine: IL-10. IL-10 acts on a plasma membrane receptor that is distinct from those for IL-4 and IL-13 [7, 11]. Similar to IL-10, TGF-beta and IFNalpha/beta are also involved in macrophage deactivation with strong anti-inflammatory properties and downregulation of MHC class II molecules on the plasma membrane [7]. Macrophages deactivation leads to immune suppression, by creating an anti-inflammatory response, through the increase of apoptotic cells intake and the reduction of MHC class II molecules surface expression (Fig. 1C) [12 - 14].

In the tumorous environment, macrophages will differentiate towards a specific phenotype, known as tumor-associated macrophages (TAMs) [15]. Most TAMs exhibit a phenotype close to M2 macrophages. After entering tumorous tissue near breast cancer cells, macrophages may acquire an M2 state. Immunosuppressive cytokines, *e.g.*,

IL-10 and TGF-beta, are secreted in large amounts by these M2 macrophages, along with a little amount of pro-inflammatory cytokines (Fig. 1D) (reviewed in [16]). These immunosuppressive M2 macrophages indirectly promote the development of cancers. In general, there is a correlation between the number of TAMs and poor prognosis, depending on the tumor type. In solid tumors, TAMs make up 5 to 40% of the tumor mass [15].

MACROPHAGES, AN EXQUISITE TARGET FOR CYTOMEGALOVIRUS INFECTION

Human cytomegalovirus (HCMV) is a virus causing asymptomatic infection in healthy people and infecting a huge part of the population worldwide. It is an opportunistic, species-specific betaherpesvirus that can cause, in the absence of an effective immune response, severe disease, especially in patients with AIDS, in immunocompromised solid-organ and bone marrow allograft recipients, and in cancer patients. Immunohistochemical studies showed that HCMV infected cells can be present in virtually all body organs. A variety of cell types can be the *in vivo* host to the virus, including fibroblasts, macrophages, epithelial cells, stromal cells, smooth muscle cells, hepatocytes, endothelial cells and neuronal cells [17, 18].

A characteristic feature of the infection by HCMV is the persistence of the viral genome, as nonproductive form for months or even years, at specific anatomical sites in the normal host [19]. Several strategies enable the virus to avoid its elimination by the immune system, including: exploiting tissues that are immunologically privileged for replication (*i.e.*, epithelial cells of the salivary glands that express an insufficient amount of MHC Class I molecules to trigger the clearance by CD8⁺ cell), inducing a latent state of infection, and expressing genes interfering with the immune response [20]. HCMV also has the ability to exploit mechanisms interfering with chemokine-driven inflammation which enables the evasion from the immune response [21]. Four immunomodulatory glycoproteins, namely gpUS2, gpUS3, gpUS6 and gpUS11 are encoded by the human CMV. They decrease the expression of the major histocompatibility complex (MHC) class I proteins on the cell surface [22]. Proteins encoded by these four functional genes are dispensable for the replication of the virus but should impede the immune surveillance of cytotoxic CD8⁺ T lymphocyte (CTL). This occurs by reducing the MHC class I proteins levels on the infected cells surfaces [22, 23]. Furthermore, gpUS2 impedes the translocation of MHC class II proteins and could give CMV the ability to evade CD4⁺ T cell effector functions [24]. NK cells are also shown to be involved in the control of CMV infection [25]. In humans, normal cells are protected from NK cells by the expression of MHC Class I HLA-E molecules which bind to NK cells CD94 inhibitory receptors. HCMV UL 40 gene encodes for a protein, homologous to MHC Class I molecules, resulting in the up-regulation of Class I HLA-E molecules expression. This allows HCMV target cells to escape from NK cell attack [26].

The role of blood monocytes and tissue macrophages is central in case of infection. These cells serve as targets in the infected organs where they function either as viral disseminators throughout the host or as sites of HCMV latency [27]. The most prevalent infiltrating cell type found in HCMV-infected organs is the macrophage [28, 29]. Experimentally, fibroblast is the most widely used cell type to grow HCMV. It produces high titers of infectious virus after *in vitro* infection. Macrophages are also permissive for HCMV replication but in a lower manner than fibroblasts and the rate of viral production in these cells is considerably less [29, 30]. Macrophages infection either with clinical isolates (HCMV-DB) or with laboratory strains (AD169) had resulted in low-level sustained growth in comparison to fibroblasts. This indicates that HCMV can infect macrophages cultures, producing much lower viral titers in the culture supernatants as compared to fibroblasts [30]. In fact, macrophages produce only low levels of HCMV and murine CMV (MCMV) [19, 25, 26].

The difference in viral growth kinetics between macrophages (low growth) and fibroblasts (high growth) is neither due to differential attachment and adsorption of the virus [31] nor a difference in viral entry [30], although the early phases of viral cycle might be a contributing factor in HCMV cell tropism. The restriction of HCMV replication in some cell types is believed to be mainly dependent on a post entry block to viral gene expression [32]. Recently, it has been reported that several blocks occur in the viral cycle following HCMV infection of cancer cells [33]. The virus ability to inhibit macrophage differentiation is required for optimal viral replication, and might be a factor of the low rate of viral growth in macrophage cultures [34, 35]. In addition, studies showed that the presence of an intact ULb' sequence in the genome of clinical HCMV isolates had lead to their efficient growth in macrophages, epithelial cells and endothelial cells [36 - 39]. On the other hand, this ULb' region is lost in the HCMV laboratory strains which leads to the inefficient replication in macrophages and epithelial cells [37]. This reflects the importance of testing clinical strains rather than laboratory ones to study HCMV replication and pathogenesis. Finally, although HCMV infection susceptibility is higher in M2 macrophages, HCMV also infects classically activated macrophages. HCMV establishes a productive and persistent infection in both types of macrophages, which then acquire similar features of classical

activation and secrete high levels of pro-inflammatory cytokines and chemokines [40 - 42]. Moreover, during acute HCMV infection, the IE1 CMV protein is involved in the production of the TNF alpha cytokine [43].

Significant cellular signal transduction events occur in the HCMV-infected macrophages. During entry, HCMV glycoprotein gB binds to the cellular epidermal growth factor receptor (EGFR) initiating directly the activation of the apoptosis suppressor Akt (Fig. 2). This activation promotes an Akt-dependent prosurvival state after monocytes infection [44]. In addition, HCMV also targets other signals in order to allow infected monocytes to pass the 48-hours cell fate decision checkpoint, which is required to begin maturation into macrophages. EGFR activation rapidly induces HSP27 and Mcl-1 expression (Fig. 2), which function together to control caspase-3 activity precisely. Caspase-3 activity is a key process in the viral dissemination and it is responsible for permitting virus persistence [45]. The infection of monocytes differentiating into macrophages activates caspase-dependent or independent cell death programs. Both activated death programs are controlled by the HCMV UL36 gene [46]. In addition, the antiapoptotic Bcl-XL protein provides protection against CMV-induced apoptosis [47]. Furthermore, it is observed that in response to HCMV infection, NF-kB binding activity is increased in nuclear extracts of macrophages and fibroblasts [30]. Previous studies also reported that NF-kB is activated in response to HCMV infection in fibroblasts [48] and monocytes [49] through the HCMV UL55 (gB) ligands [49].

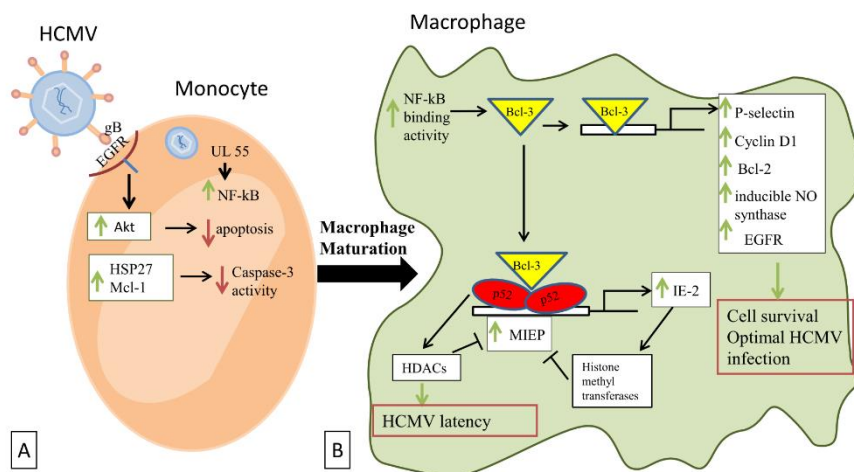


Fig. (2). Cellular transduction events occurring after macrophages infection by HCMV. (A) HCMV binds to the cellular EGFR through its glycoprotein gB and directly activates Akt, thus promoting an Akt-dependent prosurvival state. EGFR activation rapidly induces the expression of HSP27 and Mcl-1 that act together to downregulate caspase-3 activity and permit the virus persistence. **(B)** In the differentiated macrophages, NF-kB binding activity is increased allowing Bcl-3 to activate a number of human genes that favor cellular survival and optimal infection by HCMV. Activated p52/Bcl-3 complexes also act through the NF-kB sites to regulate the MIEP of HCMV and thus affecting the virus latency *via* HDACs. MIEP is also under by negative autoregulation through IE2 expression which in turn activates histone methyltransferases.

EGFR: Epidermal Growth Factor Receptor ; HDACs: Histone Deacetylases ; HCMV: Human Cytomegalovirus ; MIEP: Major Immediate Early Promoter ; Bcl: B-cell lymphoma encoded protein ; gB: Glycoprotein B ; HSP: Heat Shock Protein ; IE: Immediate Early ; Mcl-1: Induced myeloid leukemia cell differentiation protein ; NF-kB: Nuclear Factor-Kappa B.

In addition to the NF-kB activation, p52/Bcl-3 complexes and the major immediate early promoter (MIEP) of HCMV are also shown to be activated after macrophage infection [30]. Through the NF-kB sites, activated p52/Bcl-3 complexes regulate the MIEP of HCMV, while Bcl-3 activates a number of human genes as reported by studies including P-selectin [50], cyclin D1 [51 - 54], Bcl-2 [55 - 57], inducible NO synthase [58], and EGFR [59, 60] (Fig. 2). Most of the activated cellular genes favor cell survival to allow optimal infection by HCMV.

Histone deacetylases (HDAC) may play a role in HCMV latency in macrophages, although their possible recruitment by p52 [53] and Bcl-3 [61 - 63] is not fully characterized yet. In addition to HDACs, inhibition of MIEP

activity by histone methyltransferases has been reported and could explain its negative autoregulation by IE2 (Fig. 2) [64, 65].

In case of concomitant bacterial infection, HCMV enhances bacterial induction of macrophage inflammatory responses that are mediated through NF- κ B pathway and thus promoting organ inflammation in HCMV-infected tissues [66, 67]. The comparison between HCMV-infected and mock-infected macrophages had lead to the observation that HCMV infection maintains CD14, TLR4 and TLR5 surface expression, which declines over time in mock-infected macrophages. I κ B α and NF- κ B phosphorylation is also enhanced, along with the expression of MyD88, an adaptor protein [67].

HCMV replication is poor in monocytes while it is enhanced in monocytes derived macrophages [45, 68]. When HCMV binds to monocytes, it induces an intracellular increase in Ca²⁺ levels. One of the results of the Ca²⁺ rise is the block of the monocytes ability to differentiate into macrophages. Observations suggest that the virus has an efficient strategy permitting it to interfere with cellular differentiation pathways, and this may also elucidate the generalized immunosuppression that is often observed in HCMV-infected patients [34, 69]. HCMV infection of CD14⁺ monocytes gives rise to the generation of latency-specific transcripts, conservation of viral genomes, and the capacity of the virus to reenter the lytic cycle. In addition, latent virus has an effect on the level of STAT1 phosphorylation leading to disruption in type I and II signaling [70].

MACROPHAGES, CYTOMEGALOVIRUS AND CELLULAR TRANSFORMATION IN BREAST CANCER

Two distinct phenomena could be observed after HCMV infection of macrophages. First, the viral gene expression is regulated by the NF- κ B switch, resulting in viral persistence through sustained low levels of viral replication. Additionally, HCMV-infected macrophages could fuel the progression of the disease by permitting the infection to spread to cells in the vicinity of infected macrophages, which could be more permissive cells, like fibroblasts and/or epithelial cells. During viremia, the virus present in blood could infect circulating monocytes [71, 72]. Upon migration of infected monocytes into breast tissue, they could, after their differentiation into macrophages, transmit the virus to the surrounding mammary epithelial cells [73]. Most of the breast cancers are carcinomas that have their origin at cells lining the milk-forming ducts of the mammary gland, especially transformed epithelial mammary cells. Moreover, these mammary epithelial cells lining the duct could be infected directly by HCMV present in the milk [74]. The infection of mammary epithelial cells by HCMV primary clinical isolates could favor their transformation (GH and AK unpublished data). Second, HCMV infection of macrophage could alter the expression of cellular genes in HCMV-infected cells resulting in an adjusted cellular phenotype, *e.g.*, an M2 phenotype that will favor a protumoral microenvironment [30, 75].

Monocytes and macrophages constitute important HCMV reservoirs and their responsibility in the viral dissemination is well known [68, 72, 73]. Furthermore, HCMV infection of monocytes has the potential to reprogram them, giving rise to their polarization toward the inflammatory macrophages (M1), that also displays properties of immunosuppressive macrophages (M2) [75]. HCMV-infected monocyte transcriptome exhibits an unique M1/M2 polarization signature skewed towards the classical M1 activation phenotype [76]. This is mediated by induction of NF- κ B and PI3K activities in these monocytes upon HCMV infection [30, 75]. M1 macrophages exhibit inflammatory cytokines expression, where their prolonged secretion is often connected with the development of cancer (reviewed in [77]). El-Shinawi and co-workers study has shown that the prevalence of HCMV IgG in patients with inflammatory breast cancer (IBC) is higher than that in non-IBC invasive ductal carcinoma (IDC) patients. They also observed that HCMV DNA levels are higher and NF- κ B is more activated in cancerous tissues isolated from IBC in comparison with IDC patients. NF- κ B enhanced activation can either be a result of HCMV infection of breast cells or of cytokine production in the tumor microenvironment [78, 79]. In another instance, we have observed in a patient oriented study that there is a positive correlation among the seroprevalence of HCMV IgG, elevated levels of IL-6, and the incidence of liver cancer [80].

Several attempts have been made to find a link between HCMV and the development of breast cancer. The large protein repertoire of the HCMV has the potential ability either to initiate or to promote neoplastic changes in cells. Richardson hypothesized that late exposure to HCMV could increase the incidence of breast cancer [81]. The basis for this hypothesis is the correlation between breast cancer incidence and HCMV seroprevalence. The correlation between levels of HCMV IgG and breast cancer development was investigated in a study conducted by Cox and colleagues, where they enrolled 399 invasive breast cancer patients and 399 control patients. Results showed a statistically significant correlation between HCMV IgG levels elevation and the development of breast cancer in women [82].

Furthermore, searching for HCMV in milk samples from HCMV seropositive women revealed that HCMV is present in more than 90% of the samples [83, 84], while the presence of HCMV DNA was also reported in normal breast tissue [85]. More direct evidence that the breast epithelium form an important reservoir for HCMV in humans was provided by the detection of HCMV antigens in breast biopsies. HCMV antigens prevalence was higher in the neoplastic epithelium of breast cancer patients as compared to normal breast tissue of breast cancer patients and non-cancer patients [86]. The presence of HCMV DNA and proteins both in breast cancer tissue and in sentinel lymph node metastasis tissue has also been recently reported [86, 87]. A model in which the HCMV infection of both mammary epithelial cells and macrophages initiates the transformation of epithelial cells in a favorable protumoral microenvironment could explain both the detection of HCMV DNA and/or antigens in breast tumors parallel to the presence of TAMs associated with poor prognosis.

In vitro, HCMV promotes oncogenic transformation in human mammary epithelial cells (HMECs) with activation of several signaling pathways such as PI3K/AKT, Myc and Ras (AK and GH, unpublished data). In addition, HCMV favors the activation of macrophages toward a M2 phenotype with increased Bcl3 activity (Fig. 3) [30]. The priming of HMECs toward epithelial transformation and of macrophages toward M2 phenotype could create a favorable tumor microenvironment for breast tumor formation. Furthermore, HMECs secrete CSF-1 in high levels that could promote the proliferation of breast cancer cells [88]. The replication of HCMV can induce CCL2 secretions in fibroblasts and myeloid cells [89 - 91]. In turn TAMs can secrete EGF that bind to EGFR on the breast cancer cells. The development of a transformed phenotype in human breast cancer cells is enhanced by the benign mammary epithelial cells [92]. Finally, the invasion of breast cancer cells is stimulated, in culture, in the presence of human mammary fibroblasts, which increase, in mouse xenograft experiments, the development of stroma [93]. This will further promote the transformation of breast cancer cells and ultimately could lead to the development of breast adenocarcinoma.

Once HMECs had been definitively transformed, there will be no more requirements for the presence of HCMV that initiated the transformation, so it will be cleared from the breast cancer cells. In fact, we and others have reported that HCMV cannot replicate efficiently in transformed cells such as hepatocellular carcinoma HepG2 cells and in fibroblasts expressing SV40 T antigen and oncogenic H-Ras [18, 33]. HCMV replicates in a low level in M2 (TAM) macrophages because of the binding of a Bcl3-p52 NF- κ B complex on the MIEP promoter that result in a reduced viral transcription (Fig. 2) [30]. Altogether, this model of mammary epithelial cell transformation could rely on a « hit and run » theory in which HCMV (alone or with other prooncogenic factors) could promote the appearance of breast adenocarcinoma through a dual control of HMEC transformation and M2/TAM shift that will feed the initiation of the tumor which will then no more depend on HCMV existence and take its future in its own hands. Targeting TAMs has been proposed as a new therapeutic approach to fight breast cancer [94, 95].

It has been demonstrated that macrophages, as part of the tumor stroma, promote breast cancer cell migration and stem cell activity [96]. The co-culture of breast cancer cell lines (BCCLs) with THP-1 cell lines showed that migration of ER-positive breast cancer cell lines increased with all types of macrophages. And in this model of monocyte-macrophage differentiation, an increase in mammosphere formation occurs with M2-macrophages in comparison with M1-macrophages. In fact, HCMV favors the stemness of transformed cells with formation of tumorspheres in cellular models of glioblastoma, hepatocarcinoma and colon cancer *in vitro* [18, 96 - 100]. These results indicate that HCMV could favor the appearance of a more aggressive phenotype with increased risk of metastasis and poorer prognosis. Basal-like breast cancer (BBC) does not have any biologically targeted therapy yet, while it is considered an aggressive subtype of breast cancer. Important determinants of the tumor biology are the interactions of the stromal cells with the BBC cells. Inflammatory cells associated with the stroma play a key role in the progression of cancer. BBC cells co-culture with monocytes-like THP-1 cells have resulted in gene expression alteration with upregulation of both M1 and M2 markers. In relative to luminal breast cancers, the differentiation of monocytes to macrophages increased in BBCs with enhanced macrophage migration. It is observed that a distinct pattern of cytokines is secreted in macrophage-BBC co-cultures, including upregulation of NAP-2, MIG, MCP-1, MCP-3, osteoprotegerin, and interleukin (IL)-1 β [101].

The epithelial-mesenchymal transition (EMT) of cancer cells that are present at the invasive front of tumors, in a close vicinity with TAMs, suggest that a mutual interaction might be present between these two cell types [15, 102]. Macrophages are activated to TAM-like phenotype by the effect of mesenchymal-like breast cancer cells *via* GM-CSF. CCL18 from TAMs induces cancer cell EMT and increases cancer metastasis. In breast cancer samples, the expression of GM-CSF in high levels is associated with the increase in CCL18(+) macrophages and the realization of EMT by cancer cells, which both translate into an increase of metastasis and a reduction of patient survival. This suggests the importance of a positive feedback loop between GM-CSF and CCL18 in breast cancer metastasis (Fig. 3) [103].

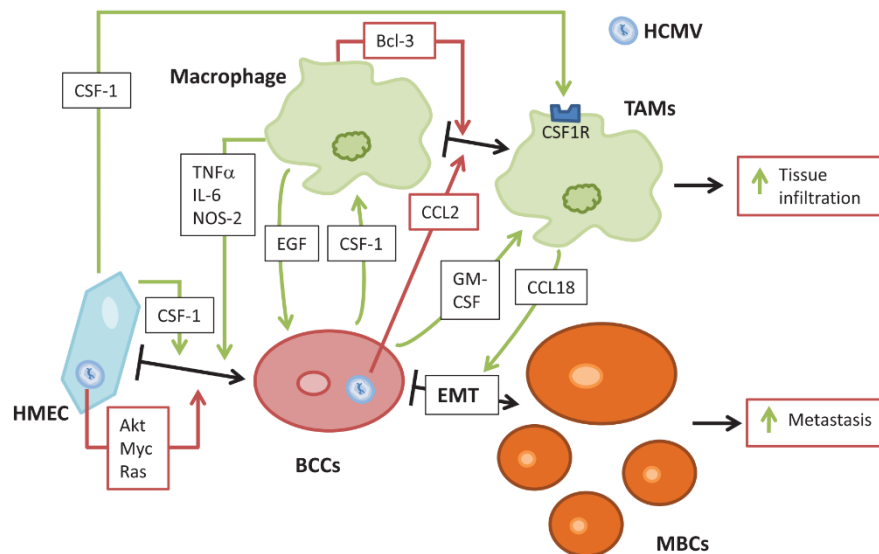


Fig. (3). Potential interplay between macrophages, mammary epithelial cells and HCMV in breast cancer. HCMV infection of HMEC induces their priming towards epithelial transformation, by activation of several signaling pathways (Akt, Myc, Ras). HCMV also favors macrophages differentiation towards M2 phenotype. HMEC produces CSF-1, that will enhance cell transformation. This creates a favorable microenvironment, in which infected HMEC produce CCL-2 that promotes TAMs differentiation. In turn, TAMs secrete EGF, that promote breast cancer cells proliferation. Once HMEC had been transformed, they produce GM-CSF, which activate macrophages. In turn, TAMs secrete CCL18, that promotes the epithelial-mesenchymal transition (EMT) of breast cancer cells. This interplay favors cells activations and transformations, leading toward increased metastasis of breast cancer cells and increased tissue infiltration by TAMs.

BCCs: Breast Cancer Cells ; CSF: Colony Stimulating Factor ; EMT: epithelial-mesenchymal transition ; HMEC: Human Mammary Epithelial Cells ; MBCs: Metastatic Breast Cancer cells ; TAMs: Tumor Associated Macrophages ; Bcl: B-cell lymphoma encoded protein ; CCL : C-C Motif Chemokine Ligand ; EGF: Epidermal Growth Factor ; GM: Granulocyte Macrophage ; HCMV : Human Cytomegalovirus ; IL: Interleukin ; NOS: NO Synthase ; TNF: Tumor Necrosis Factor.

The overexpression of macrophage colony-stimulating factor (CSF-1) and its receptor (CSF1-R) has been correlated to poor prognosis in human breast carcinomas. CSF-1R expression is restricted, at the tumor site, to macrophages, allowing the regulation of infiltration and function of TAMs by CSF-1. This action of CSF-1 could enhance the metastatic potential of tumor [104]. For their comigration and invasion into collagen I, which involves a paracrine loop, it is necessary and sufficient for macrophages and tumor cells to be present. Epidermal growth factor (EGF) is first expressed by macrophages and increases the formation of elongated protrusions and cell invasion by carcinoma cells. CSF-1 produced by carcinoma cells enhances the expression of EGF by macrophages. Moreover, EGF promotes CSF-1 expression by carcinoma cells thereby generating a positive feedback loop (Fig. 3) [105]. Infiltration and activation of macrophages have been studied by immunohistochemistry in human primary breast tumors, from a large cohort. A poor differentiation and a fast proliferation was associated with high numbers of M2-macrophages in tumors showing estrogen receptor negativity and histological ductal type [106].

Finally, although HCMV infection of macrophages might favor a protumoral microenvironment, it could also result in the containment of the viral dissemination. In a murine model, peripheral MCMV infection spreads *via* lymph nodes and MCMV infects filtering macrophages, which support virus replication poorly. When these macrophages are depleted, MCMV infects susceptible fibroblasts and spread faster [107]. Also, depletion of splenic macrophages significantly enhances, rather than deters, replication of MCMV in the spleen [107]. Thus, tissue macrophages may protect other highly permissive cell types present in their vicinity from CMV infection parallel to the enhancement of a stemless protumoral environment, which could be critical in breast cancer and dissemination. Finally, once the CMV has played its role as an initiator and/or enhancer of tumorigenesis, viral expression within transformed cells not only will be limited ([33], GH and AK unpublished data), but also could limit cellular transformation. In fact, recent reports

indicate that HCMV and MCMV infection could favor the clearance of tumoral cells [108, 109].

CONCLUSION

A primary feature of breast cancer is a deleterious inflammation. Accumulating evidence demonstrates that macrophages have a critical role at each stage of cancer progression, as they are, in mammary tumors, the most abundant leukocyte population. These tumor-associated macrophages (TAMs) facilitate neoplastic transformation, are responsible for the tumor escape from immune responses and could lead to the subsequent metastatic cascade. HCMV infects macrophages and favors the appearance of a M2 phenotype, close to the TAM phenotype, parallel to the activation of oncogenic pathways in mammary epithelial cells. Breast tumors could induce and exploit, with the help of HCMV, trophic macrophages in order to subvert immune responses, avoiding the destruction of malignant cells. Therefore, macrophage- and/or HCMV-targeted intervention strategies have to be evaluated in order to curtail breast cancer morbidity and mortality.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Research Paper

The Human Cytomegalovirus Strain DB Activates Oncogenic Pathways in Mammary Epithelial Cells



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ABSTRACT

Background: Human cytomegalovirus (HCMV) establishes a persistent life-long infection and increasing evidence indicates HCMV infection can modulate signaling pathways associated with oncogenesis. Breast milk is an important route of HCMV transmission in humans and we hypothesized that mammary epithelial cells could be one of the main cellular targets of HCMV infection.

Methods: The infectivity of primary human mammary epithelial cells (HMECs) was assessed following infection with the HCMV-DB strain, a clinical isolate with a marked macrophage-tropism. The impact of HCMV-DB infection on expression of p53 and retinoblastoma proteins, telomerase activity and oncogenic pathways (c-Myc, Akt, Ras, STAT3) was studied. Finally the transformation of HCMV-DB infected HMECs was evaluated using soft agar assay. CTH cells (CMV Transformed HMECs) were detected in prolonged cultures of infected HMECs. Tumor formation was observed in NOD/SCID Gamma (NSG) mice injected with CTH cells. Detection of long non coding RNA4.9 (lncRNA4.9) gene was assessed in CTH cells, tumors isolated from xenografted NSG mice and biopsies of patients with breast cancer using qualitative and quantitative PCR.

Results: We found that HCMV, especially a clinical strain named HCMV-DB, infects HMECs *in vitro*. The clinical strain HCMV-DB replicates productively in HMECs as evidenced by detection of early and late viral transcripts and proteins. Following infection of HMECs with HCMV-DB, we observed the inactivation of retinoblastoma and p53 proteins, the activation of telomerase activity, the activation of the proto-oncogenes c-Myc and Ras, the activation of Akt and STAT3, and the upregulation of cyclin D1 and Ki67 antigen. Colony formation was observed in soft agar seeded with HCMV-DB-infected HMECs. Prolonged culture of infected HMECs resulted in the development of clusters of spheroid cells that we called CTH cells (CMV Transformed HMECs). CTH cells when injected in NOD/SCID Gamma (NSG) mice resulted in the development of tumors. We detected in CTH cells the presence of a HCMV signature corresponding to a sequence of the long noncoding RNA4.9 (lncRNA4.9) gene. We also found the presence of the HCMV lncRNA4.9 sequence in tumors isolated from xenografted NSG mice injected with CTH cells and in biopsies of patients with breast cancer using qualitative and quantitative PCR.

Conclusions: Our data indicate that key molecular pathways involved in oncogenesis are activated in HCMV-DB-infected HMECs that ultimately results in the transformation of HMECs *in vitro* with the appearance of CMV-transformed HMECs (CTH cells) in culture. CTH cells display a HCMV signature corresponding to a lncRNA4.9

Abbreviations: ChIP, chromatin immunoprecipitation; HMECs, human mammary epithelial cells; HCMV, human cytomegalovirus; MOI, multiplicity of infection; UV, ultraviolet rays; HI, heat inactivated; Rb, retinoblastoma; hTERT, human telomerase reverse transcriptase; IE, immediate early; LA, late antigen.

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genomic sequence and give rise to fast growing triple-negative tumors in NSG mice. A similar lncRNA4.9 genomic sequence was detected in tumor biopsies of patients with breast cancer.

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1. Introduction

Worldwide breast cancer is the most common cancer diagnosed among women (Warner, 2011). Notably, majority of the breast cancers classified as carcinomas have been found to be originated from the mammary epithelial cells lining the duct responsible for converting most precursors into milk constituents and transporting them to the mammary lumen (Dimri et al., 2005). Breast cancer exhibits heterogeneous molecular characteristics and utilizing gene expression patterns several types of breast cancer have been identified including a normal breast epithelial-like group, a luminal epithelial cell type A, a luminal epithelial cell type B, an ER2-overexpressing group, a basal-like group and a claudin low group (Lehmann et al., 2011). Etiological factors involved in breast cancer include genetic and environmental risk factors (Hüsing et al., 2012), and among these latter viruses could be involved with close to one-fifth of all cancers in the world caused by infectious agents (Zur Hausen, 2009).

The human cytomegalovirus (HCMV), a highly host specific pathogen, is a member of *Betaherpesviridae* family. HCMV generally causes asymptomatic to mild infection in immunocompetent host. However, its infection in immunocompromised host may result in serious complications (Coquette et al., 2004). HCMV infects a broad range of cells including monocytes, macrophages, fibroblasts, endothelial cells, epithelial cells, stromal cells, hepatocytes, smooth muscle cells, and neural stem/progenitor cells (Belzile et al., 2014; Khan et al., 2009; Lepiller et al., 2013; Wang and Shenk, 2005). Although HCMV clinical isolates display a broad cellular tropism infecting among others fibroblasts and epithelial cells, the growth of laboratory HCMV strains is restricted to fibroblasts (Wang and Shenk, 2005). In infected patients, the blood monocytes and tissue macrophages are regarded as an important HCMV cellular reservoir responsible for the dissemination of virus and may also act as a site for the establishment of latency (Hargett and Shenk, 2010; Khan et al., 2009; Smith et al., 2004). Noteworthy, HCMV has the ability to induce a distinct inflammatory (M1) and immunosuppressive (M2) macrophages polarization (Chan et al., 2009). In addition, macrophage polarization into M1/M2 phenotype is associated with the secretion of cytokines that could play a pivotal role in viral replication and fitness, and favor breast cancer promotion (Grivnikov et al., 2010; McKinney et al., 2014; Teng et al., 2012).

Role of HCMV in inflammatory diseases and cancer has been well speculated (Cobbs et al., 2002; Lepiller et al., 2011; Söderberg-Nauclér, 2006). Earlier studies demonstrated that HCMV was able to induce the *in vitro* transformation of human embryo lung fibroblasts (Clanton et al., 1983; Geder et al., 1976). More recently, HCMV DNA or antigen has been found in tumor tissues from brain (glioblastoma, medulloblastoma), colon, prostate, liver and breast cancer (Banerjee et al., 2015; Baryawno et al., 2011; Bhattacharjee et al., 2012; Harkins et al., 2010; Samanta et al., 2003; Taher et al., 2013). Besides a direct role of HCMV in cellular transformation, HCMV could infect the tumor tissue and acts as a cofactor in amplifying mechanisms of oncogenesis, a paradigm called as oncomodulation (Michaelis et al., 2009).

Previously, we isolated a clinical HCMV strain from a 30-year-old pregnant woman named as HCMV-DB (KT959235), which is highly macrophage-tropic, triggers a M2 phenotype and upregulates the proto-oncogene Bcl-3 (Khan et al., 2009). HCMV-DB strain is close from other primary clinical isolates which also infect macrophages such as PH and TR strains (Suppl. Fig. S1) (Michaelis et al., 2009). There is scarcity of direct evidence suggesting the involvement of HCMV in transformation of human mammary epithelial cells (HMECs) (Herbein and Kumar, 2014). Here, we assessed the potential direct

oncogenic role of HCMV in primary human mammary epithelial cells (HMECs) *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Reagents

Anti-p53, anti-Rb, anti-Ras, anti-pAktThr308, anti-pAktSer473, anti-Akt and anti-cyclinD1 antibodies were purchased from Cell signaling (Danvers, MA, USA). Anti-pSTAT3, anti-STAT3 and anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-pp65, anti-pp85 (pUL25), anti-pp71 (pUL82), anti-IE1 and anti-IE2 antibodies were purchased from Santa Cruz Biotechnology. Recombinant Raf1-GST was purchased from Millipore (Molsheim, France).

2.2. Cell Cultures

Human primary mammary epithelial cells (HMECs) were obtained from Life Technologies (Carlsbad, CA, USA). MDA-MB-231 and MCF-7 cells were provided by Institut Hiscia (Arlesheim, Switzerland). HMECs were cultivated in HMEC medium (Life Technologies, Carlsbad, CA, USA) supplemented with HMEC supplement and bovine pituitary extract (Life Technologies, USA). Cell viability assay was performed as previously described (Khan et al., 2009). Cultures were free of mycoplasma.

2.3. HCMV Infection of HMECs

AD169 is a highly passaged laboratory strain of HCMV originally isolated from the adenoids of a child (Murphy et al., 2003). The clinical isolate HCMV-DB was isolated in our laboratory from a cervical swab specimen from a 30-year-old pregnant woman (Khan et al., 2009). Cell-free virus stocks were prepared by propagating AD169 in MRC5 human fibroblasts, meanwhile HCMV-DB were grown in macrophages, as described previously (Khan et al., 2009). The TB40/E strain was isolated from a throat wash of a bone marrow transplant recipient (Tomasec et al., 2005). MRC5 human fibroblasts were cultured as described previously (Coquette et al., 2004; Khan et al., 2009). HMECs (1×10^6) and MRC5 cells (1×10^6) were infected at a multiplicity of infection (MOI) of 1 or 10 for 2 h at 37 °C, washed thoroughly (three times with $1 \times$ PBS), and covered with fresh medium. Where specified, HCMV heat-inactivated at 95 °C for 10 min or treated with UV ($1200 \mu\text{J}/\text{cm}^2$ for 15 min) were used as controls. Supernatants were clarified by centrifugation and stored at -80°C until use. Virus titers were determined by plaque-forming assay in MRC5 human fibroblasts as described previously (Khan et al., 2009). The purity of our HCMV stocks was confirmed by the absence of detection of other viruses (HSV-1, HSV-2, varicella-zoster virus, Epstein-Barr virus, adenovirus, BK virus) using PCR screening (data not shown). Following HCMV infection of HMECs, viral replication was assessed by the appearance of a cytopathic effect (CPE) in the cultures and by detection of IE1, IE2, pp65 and pp85 by western blotting and IE1 antigen (clone E13, Argene-Biosoft, Varis, France) using immunofluorescence microscopy (Nikon Eclipse E400, Kanagawa, Japan). For the detection of HCMV mRNA using RT-PCR assay, total RNA was extracted from uninfected, UV-treated and HCMV-DB infected HMECs with RNeasy mini kit (Qiagen). Total 2 μg of RNA was reverse transcribed into cDNA with Superscript III RT (Life Technologies) using oligo (dT) primers. The 5 μl of reverse transcription reaction product was amplified using primers against IE1, US28 and UL82 primers as

described previously (Lepiller et al., 2013). The beta-globin gene was amplified as an internal control (sense, 5'-TCCCTCTACCTACTTCTTA-3'; antisense, 5'-TGCCTGGACTAATCTGCAAGAG-3'). The PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide. Quantification of viral titer in cell culture supernatants was performed by qPCR as previously described (Khan et al., 2009). The UL128, UL131, UL133 and UL138 genes were amplified using the following primers: UL128 (sense, 5'-GATTCGCGGGATCGTCACCA-3'; antisense, 5'-TCACTGGAGCATATAGCCCA-3'); UL131 (sense, 5'-ATGTGTATGATGTCTCATAATAAAGC-3'; antisense, 5'-TCAACGTGACGTCCACGAGC-3'); UL133 (sense, 5'-AGACTCCGTAATCGACCTCC-3'; antisense, 5'-GATGACCGTATTGGACCATGTC-3') and UL138 (sense, 5'-ACGATCTGCCGCTGAATGTC-3'; antisense, 5'-ACAGCTCGCAACAGCGGATC-3'). Quantification of c-Myc (MYC) and cyclin-D1 (CCND1) transcripts in HMECs infected with HCMV-DB (moi = 1, day 1 postinfection) and uninfected HMECs was performed using the human breast cancer RT² profiler PCR assay (PAHS-131ZA) (Qiagen). HCMV entry assays were performed as previously described (Khan et al., 2009).

2.4. Western Blotting

Cellular extracts of HMECs either uninfected or infected with HCMV or UV-treated HCMV strains were used to examine the expression of p53, Rb, pRb, c-Myc, Ras, pAkt(Thr308), pAkt(Ser473), Akt, pSTAT3, STAT3, cyclin D1, IE1, IE2, pp65, pp85 and β -actin protein by western blotting as described previously (Khan et al., 2009). Where specified, protein levels were quantified by densitometry using ImageJ 1.40 software (National Institutes of Health, Bethesda, MA, USA).

2.5. Pull-Down Assay

For GST-pull down assay, 20 μ g of GST-Raf (Millipore) proteins were immobilized on glutathione agarose beads (Thermo Fisher Scientific, Rochester, NY), washed five times with 1 \times PBS, and were incubated overnight at 4 °C with 500 μ g of lysates. The suspension was then washed three times with 1 \times PBS, denatured and the expression of active-Ras was analyzed by SDS-PAGE and autoradiography.

2.6. Immunoprecipitation

HMECs were mock-treated or were infected with HCMV strains (MOI = 1). At day 3 post infection, the cell lysates were precleared by adding 50 μ l of protein A magnetic beads (Millipore) for 1 h at 4 °C. The clear supernatants were removed, combined with anti-p53 antibody (Cell Signaling) or isotype control antibody (Millipore) and incubated overnight at 4 °C. The lysates were further incubated with 50 μ l protein A magnetic beads (Millipore) at 4 °C for 2 h. Immune complex were washed in the presence of protease inhibitors (Roche, Meylan, France) and bound protein was eluted with sample buffer and run on 10% SDS-PAGE gels. Expression of IE2 was determined by western blotting.

2.7. Chromatin Immunoprecipitations Assay

p53 and H3K9me3 chromatin immunoprecipitations (ChIPs) were performed with EZ-Magna ChIP™ A - Chromatin Immunoprecipitation Kit (Millipore). Briefly, 10⁷ HMECs were infected with HCMV-DB and at day 1 post infection cells were fixed and sonicated as per manufacturer's instructions. Sonication conditions were as follows: 9 pulses, 40% amplitude, pulse duration 9 s and gap between each cycle 30 s. p53 and H3K9me3 ChIP assays were performed as described previously (Soria et al., 2010). DNA was purified and analyzed using PCR followed by agarose gel electrophoresis.

2.8. Flow Cytometry Analysis

For proliferation assays, HMECs were left uninfected or were infected with HCMV. Proliferation was measured using the quantification of Ki67 Ag expression by intracellular flow cytometry as described previously (Lepiller et al., 2013). To discriminate between infected and uninfected HMECs, the pp71 HCMV expression was detected by flow cytometry using an anti-pp71 antibody.

2.9. Assessment of Telomerase Activity

Telomerase activity was assayed by using TRAPEZE Telomerase detection kit as recommended by the manufacturer (Chemicon, Temecula, CA). Control and HCMV-infected HMECs were suspended in 3-[(3-cholamidopropyl) dimethyl ammonio] propanesulfonic acid (CHAPS) lysis buffer (10 mM Tris - HCl [pH 7.5], 1 mM MgCl₂, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM P3-mercaptoethanol, 0.5% CHAPS, 10% glycerol) for 20 min on ice and centrifuged at 20,000g for 20 min at 4 °C. The amount of protein in the supernatants was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). For each assay of telomerase activity, 1 μ g of protein was used, and 30 PCR cycles were performed after the elongation reaction using telomerase primers. The PCR products were resolved by polyacrylamide gel electrophoresis and visualized by staining with SYBR Green I (Roche). For the detection of hTERT transcripts, total cellular RNA was isolated from uninfected or HCMV-infected HMECs with an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized with OligodT20 primers using a SuperScript III First-Strand Synthesis System (RT-PCR; Invitrogen Life Technologies, Carlsbad, CA). hTERT mRNA expression was analyzed by RT-PCR with primers specific for hTERT mRNA (accession no. AF015950): 5'-CGGAAGAGTGCTCGGA GCA-3' and 5'-GGATGAAGCGGAGTCTGGA-3'.

2.10. Soft Agar Colony Formation Assay

Colony formation in soft agar seeded with uninfected HMECs, HMECs infected with wild-type or heat-inactivated HCMV, MCF-7 cells and MDA-MB-231 cells was assayed using Cell Biolabs Cytosolic Cell Transformation Assay kit (Colorimetric assay, CB135; Cell Biolabs Inc., San Diego, CA) as per the manufacturer's protocol. Starting 1 day post infection, cells were incubated for 14 days (15 days post-infection) in the semisolid agar medium before solubilization and detection using the provided MTT solution for quantification of the formation of colonies in soft agar (Cayman Chemical, Ann Arbor, MI) and a microplate reader (A570nm). Colonies were observed under an Olympus microscope (Center Valley, PA). Following DNA extraction from soft agar colonies, the HCMV major immediate early promoter (MIEP) and beta-globin sequences were amplified using the following primers: MIEP (sense, 5'-TGGGACTTTCCTACTTGG-3'; antisense, 5'-CCAGGCGACTGACGGTT-3') and beta-globin (sense, 5'-TCCCTCTCTACCTCTACTTTCTA-3'; antisense, 5'-TGCTGCTGACTAATCTGCAAGAG-3').

2.11. Isolation and Growth of CTH Cells

HMECs cultures were infected with HCMV-DB at a MOI of 1. Several clusters of spheroid-cells were observed in HMECs infected with HCMV-DB around day 20 post infection in some of the cultures. These spheroid-cell clusters detected in HMEC cultures infected with HCMV-DB were gently detached and the floating detached cells named CTH cells were cultured in HMEC Ready medium (Cat#12752010, Gibco, Grand Island, NY) for numerous passages, currently >150 passages.

2.12. Animals

Six-week-old female NOD/SCID Gamma (NSG) mice purchased from Charles River Laboratories (L'Arbresle, France) were kept under strict

pathogen-free conditions at the Central Animal facilities of University of Franche-Comté (notification d'autorisation n° 05085.02). Cell viability was determined by trypan blue staining and cells were counted using haemocytometer. After harvesting and during injection, cells were maintained at 4 °C. Two million of CTH cells, five million cells of uninfected HMECs, two million of MCF-7 cells and MDA-MB-231 cells suspended in 100 µl of serum-free Dulbecco's modified Eagle's medium together with 100 µl of Matrigel at the day 0, were injected into the mammary fat pad of NSG mice. Mice were checked twice in a week for tumor growth. Once tumor development was detectable by eye, tumor dimensions were measured using vernier caliper. Tumor volume was calculated using the formula: volume (cm³) = (d × d × D)/2 where d is the shortest diameter and D is the longest diameter. On day 37 post injection, mice were killed according to the guidelines of the animal ethical committee. Tumors were retrieved from the mice and analyzed for the presence of MIEP and lncRNA4.9 sequences from HCMV-DB using PCR.

2.13. Immunohistochemistry of Mice Tumors

Formalin-fixed tumors retrieved from the mice were embedded in wax and sections (5 µm) were prepared using standard methods and stained with eosin and hematoxylin. Sections were processed and stained individually for hematoxylin and eosin staining, ER (1:1, Roche), PR (1:1, Roche), HER2 (1:1, Roche), vimentin (1:400, Leica), E-cadherin (1:50, Dako), CK5/6 (1:50, Dako), GATA3 (1:100, Cell Marque), CK20 (1:5000, Biocare), and GCDPF (1:1000, Cell Marque) before observation by microscopy.

2.14. Detection of HCMV lncRNA 4.9 Genomic Sequence in CTH Cells, Xenografted Mice Tumor and Human Breast Cancer Tissue

Total DNA from uninfected HMEC, HCMV-DB infected HMEC, CTH cells and MRC5 cultures was isolated. The retrieved mice tumors were grinded in liquid nitrogen and DNA was isolated using QIAamp DNA mini kit (Qiagen, Valencia, CA) as per manufacture's guidelines. Genomic DNA isolated from patient breast tumor biopsies and from healthy human breast tissue was provided by the regional tumor bank (BB-0033-00024 Tumorothèque Régionale de Franche-Comté).

Presence of HCMV was determined by qualitative and quantitative PCR where specified using a set of HCMV-DB lncRNA 4.9 gene primers (sense, 5'-GTGAACCGATACGGGTGAG-3'; antisense, 5'-CATTTGAACAGAGAAAGTGG-3'). An amplicon of 126 bp corresponding to HCMV-DB lncRNA4.9 gene was amplified as confirmed by Sanger's sequencing (Genoscreen, Lille, France and GATC, Köln, Germany). We also screened CTH cells, mice tumors and human breast biopsies (tumor and healthy tissue) for the presence of MIEP sequence using PCR assay (sense, 5'-TGGGACTTCTACTTGG-3'; antisense, 5'-CCAGGCGATCTGACGGTT-3'). As a positive control, DNA isolated from HCMV-DB infected HMECs and from HCMV-DB viral stock was included in the study. Equal amount of DNA was analyzed by PCR using HCMV-DB MIEP and lncRNA 4.9 primers. As equal loading control β-globin gene was amplified (sense, 5'-TCCCCCTCTACCCCTACTTCTA-3'; antisense, 5'-TGCTGGACTAATC

Table 1
Phylogenetic analysis based on UL144 gene was determined among several HCMV strains.

Strain name	Abbreviation	Accession number
1 HHV5 strain DB, complete genome	DB	KT959235
2 HHV5 transgenic strain Towne, complete genome	Towne	GQ121041
3 HHV5 strain AD169, complete genome	AD169	FJ527563
4 HHV5 strain TB40/E clone Lisa, complete genome	TB40/E	KF297339
5 HHV5 transgenic strain Merlin, complete genome	Merlin	GU179001
6 HHV5 strain Toldeo, complete genome	Toldeo	GU937742
7 HHV5 strain VR1814, complete genome	VR1814	GU179289
8 HHV5 strain Davis, complete genome	Davis	JX512198
9 HHV5 strain JP, complete genome	JP	GQ221975
10 HHV5 strain TR, complete genome	TR	KF021605
11 HHV5 PH-BAC isolate, complete genome	PH	AC146904

TGCAAGAG-3'). Amplified product was electrophoresed in 2% agarose gel stained with Sybr green I nucleic acid stain.

2.15. Phylogenetic Analysis

Phylogenetic analysis was determined among several HCMV strains (described in Table 1) with respect to the UL144 gene as previously reported (Waters et al., 2010). Multi-sequence alignments (MSA) were performed using CLUSTAL W with following parameters: a gap opening penalty of 15 and gap extension penalty of 6.66. Phylogenetic tree was constructed using the neighbor-joining method. Each clustering was confirmed by the bootstrap method with 1000 replicates. The analysis was conducted using MEGA7 (<http://www.megasoftware.net/>).

2.16. Statistical Analysis

The reported values are the means and SD of independent experiments. Statistical analysis was performed using Mann Whitney U test, and differences were considered significant at a value of P < 0.05. Microsoft Excel was used to construct the plots.

3. Results

3.1. HCMV Permissively Infects and Completes its Life Cycle in HMECs

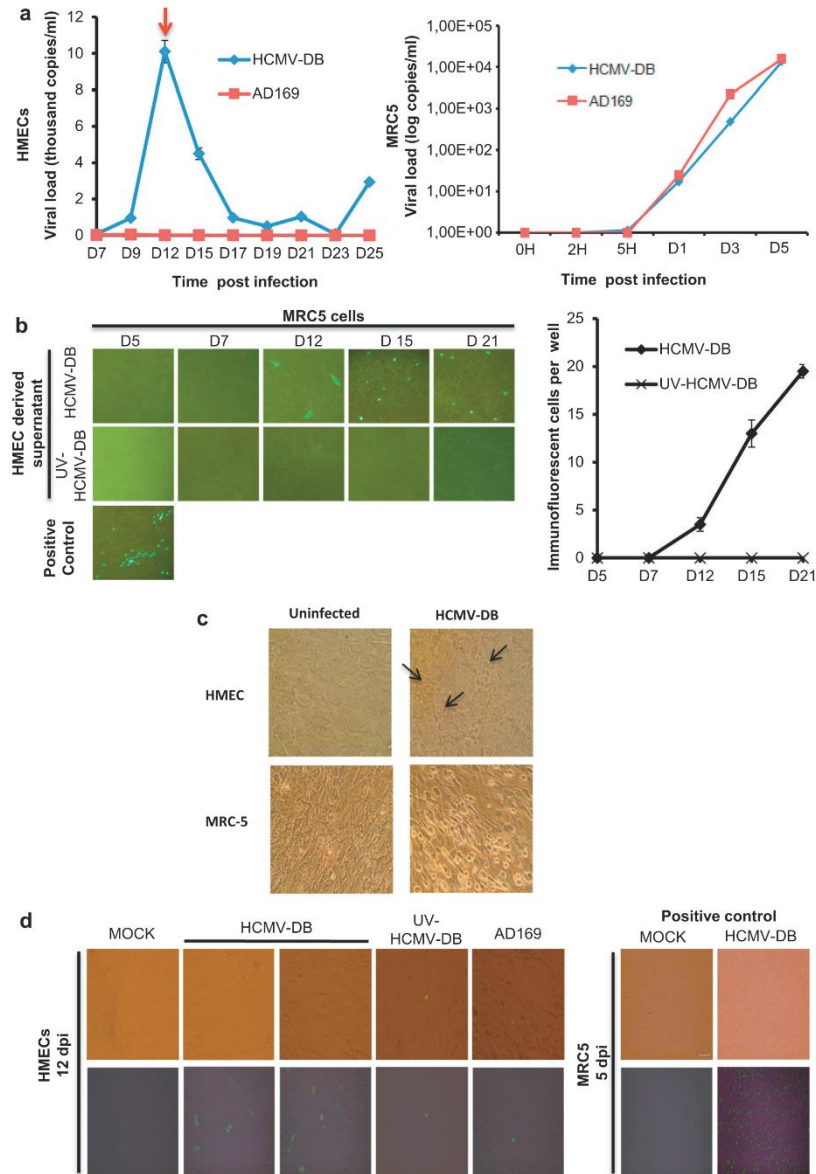
Although HCMV proteins and DNA have been reported in breast cancer tissue (Harkins et al., 2010; Taher et al., 2013), so far only few attempts have been made to investigate HCMV replication in HMECs *in vitro* (Twite et al., 2014). We infected HMECs with two strains of HCMV, HCMV-DB a clinical isolate which is macrophage-tropic, close to PR and TH strains and belongs to UL144 genotype C (Suppl. Fig. S1) (Khan et al., 2009), and the extensively passaged laboratory strain AD169 that has lost the ability to efficiently replicate in endothelial and epithelial cells (Sinzger et al., 2008; Wang and Shenk, 2005). We observed productive replication of HCMV-DB in infected HMECs with peak viral titer at day 12 post infection (Fig. 1a, left panel). AD169 did not replicate in HMECs (Fig. 1a, left panel). The level of productive infection of the HCMV-DB strain in HMECs was much lower than what was

Fig. 1. HCMV-DB infects and replicates in HMECs. a. Growth kinetics of HCMV in HMECs and MRC5 cells. Left panel. HMECs were infected individually with two HCMV strains (HCMV-DB and AD169) (MOI = 1). At 2 h post infection inoculum was removed and cells were washed three times with 1 × PBS followed by three washes with HMEC media. At several time points (up to day 25) supernatants were collected and viral growth kinetics were determined by real-time PCR. Right panel. MRC5 cells were infected with HCMV-DB and AD169 (MOI = 1) and supernatant was collected at several time points (up to day 5). Viral growth kinetics was determined using real time qPCR. Results represent means (±SD) of three independent experiments. b. Replicative virus is detected in supernatants harvested from HMECs infected with HCMV-DB. Supernatants collected from HMECs infected with HCMV-DB at several time points (up to day 21) were used to infect MRC5 cells and at day 5 post infection cells were fixed. Expression of IE1 antigen was determined by immunofluorescence as described in Materials and Methods. Direct infection of MRC5 cells by HCMV-DB (MOI = 1) was used as a positive control. Supernatants harvested from HMECs infected with UV-inactivated HCMV-DB (MOI = 1) were used as a negative control. Right panel. The curves represent the number of IE1 positive foci detected in MRC5 cultures treated with supernatants harvested from HMECs infected with HCMV-DB and UV-inactivated HCMV-DB respectively. Results represent means (±SD) of three independent experiments. c. Detection of a typical cytopathic effect at day 15 post-infection in HMECs infected with HCMV-DB (MOI = 1) (Upper right panel). MRC5-infected with HCMV-DB were used as a positive control (Lower right panel). Mock-infected HMECs and MRC5 cells were included as negative controls. Magnification 100×. d. Detection of IE1 antigen in HMECs infected with HCMV-DB at day 12 post infection. Presence of IE1 antigen was assessed in HMECs cells infected with HCMV-DB (two positive cultures are shown) and AD169 strains at day 12 post infection by immunofluorescence assay as described in Materials and Methods. As controls, uninfected HMECs and MRC5 cells, HMECs infected with UV-treated HCMV-DB and MRC5 cells infected with HCMV-DB are shown. Magnification 20×.

observed in MRC5 fibroblasts, using a PCR assay (Fig. 1a, right panel). To determine whether HCMV DNA detection by PCR assay represents genuine replicative virus, we harvested supernatants of HMECs infected with HCMV-DB strain up to day 21 post infection. Harvested supernatants were used to infect MRC5 cells and the number of infected cells was determined by using IE1 immunofluorescence staining (Fig. 1b). Using harvested supernatants to infected MRC5 cells, we observed low levels of HCMV-DB replication in HMECs (Fig. 1b). In contrast

supernatants of HMECs infected with UV-inactivated HCMV-DB failed to replicate in MRC5 cells (Fig. 1b). MRC5 cells directly infected with HCMV-DB were used as a positive control (Fig. 1b).

Although limited, a typical cytopathic effect was observed in HMEC cultures directly infected with HCMV-DB (Fig. 1c). In contrast to AD169, HCMV-DB was able to start its life cycle in HMECs as evidenced by expression of IE1 genes determined by immunofluorescence assay (Fig. 1d). We detected both viral transcripts and proteins of various



phases of HCMV replication cycle (immediate early to late phases) in HMECs infected with HCMV-DB but not in AD169 infected cultures. We observed the transcripts of IE1, US28 and UL82 at two time points, day 1 and day 3 of post infection (Fig. 2a). The immediate early proteins (IE1 and IE2), the tegument protein pp65 and the late antigen pp85 were detected in HMECs infected with HCMV-DB up to 5 days post infection using western blotting (Fig. 2b). The detection of the pp65 tegument protein at early time-points post infection is most likely due to the translocation of pp65 after virus entry as previously reported (Hensel et al., 1995). Of note, in contrast to AD169 we found that HCMV-DB has an intact ULb' region that has been shown to govern tropism of HCMV strains, especially for efficient infection of epithelial cells (Fig. 2c). Taken together our data indicate a tropism of the clinical isolate HCMV-DB for HMECs.

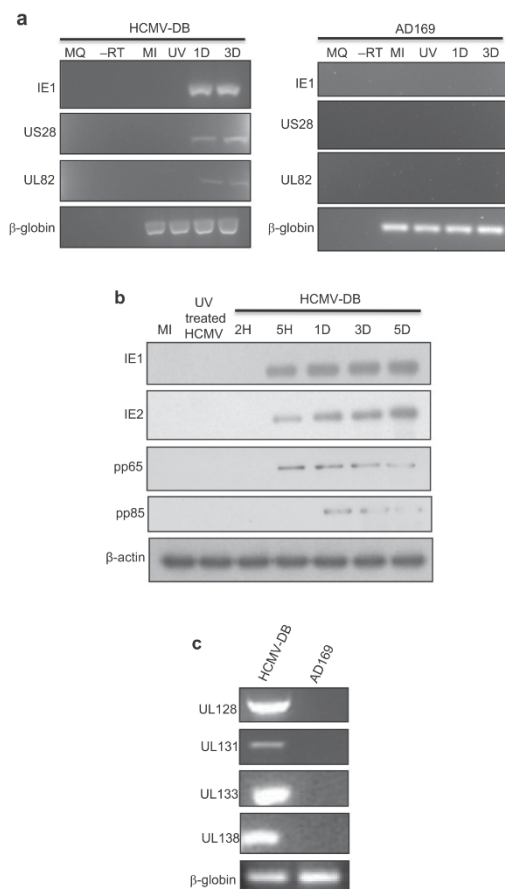


Fig. 2. Detection of HCMV transcripts and proteins in infected HMECs. **a.** Detection of IE1, US28 and UL82 transcripts in HMECs infected with HCMV-DB, but not in HMECs infected with AD169, at day 1 and day 3 post infection using RT-PCR. Beta-globin was used as an internal control. Results are representative of three independent experiments. **b.** Detection of immediate early (IE1 and IE2) and late (pp65 and pp85) viral proteins in HMECs infected with HCMV-DB using western blotting. Results are representative of three independent experiments. **c.** Determination of an intact ULb' locus in HCMV-DB, but not in AD169, using conventional DNA PCR. Results are representative of three independent experiments.

3.2. HCMV Infection Promotes Oncogenic Environment in HMECs

A limited set of growth-deregulating changes is required for oncogenic conversion of HMECs. These changes minimally involve the inactivation of the p53 and retinoblastoma protein (Rb) pathways, telomere maintenance conferred by the *hTERT* gene, and acquisition of a constitutive mitogenic signal provided by oncogenic H-Ras (Elenbaas et al., 2001).

HCMV is known to upregulate the cellular levels of p53 in infected cells (Hannemann et al., 2009). We also observed the upregulation of p53 protein in HMECs infected with the HCMV-DB strain as measured by western blot (Fig. 3a). In addition, we observed the binding of p53 to HCMV-IE2 viral protein in infected HMECs by co-immunoprecipitation assay (Fig. 3b), indicating a potential inhibitory effect of IE2 on p53 transcriptional activity as previously reported (Hsu et al., 2004). In HMECs infected with HCMV-DB, we observed decreased binding of p53 to endogenous p53-responsive promoters of *p21* and *MDM2* genes as shown by chromatin immunoprecipitation (Fig. 3c).

Sustained human telomerase reverse transcriptase (hTERT) activity is one of the prerequisite for the immortalization and potentially transformation of HMECs *in vitro* (Elenbaas et al., 2001). HCMV has been reported to activate telomerase in normal human fibroblasts and human malignant glioma cell lines (Strååt et al., 2009). Therefore, we determined the expression of hTERT mRNA and telomerase activity in HMECs infected with HCMV-DB. We observed overexpression of hTERT mRNA and enhanced telomerase activity in HMECs infected with HCMV-DB as compared to uninfected HMEC cells (Fig. 4a and b). Overexpression of hTERT mRNA and enhanced telomerase activity observed in HMECs infected with HCMV-DB could contribute actively to the pro-oncogenic environment in HCMV-DB infected cells.

DNA tumor viruses encode gene products which can functionally inactivate Rb, promoting cellular proliferation and viral DNA synthesis (Helt and Galloway, 2003). The inactivation of the Rb protein results from direct degradation of the Rb protein in the 26S proteasome by HCMV pp71 and/or from its hyperphosphorylation by UL97 protein which inhibits the tumor suppressor activity of Rb (Hume et al., 2008; Kalejta and Shenk, 2003). We observed that Rb is hyperphosphorylated at Ser 780 residue after infection of HMECs with HCMV-DB (Fig. 5) and that Rb expression is downregulated at day 3 post infection (Fig. 5) which is consistent with previous findings (Hume et al., 2008; Kalejta and Shenk, 2003). Thus, both p53 and Rb could be inactivated in HMECs infected with HCMV-DB therefore potentially abolishing cellular senescence and hence promoting unchecked cell division (Dimri et al., 2005).

The acquisition of a mitogenic signal provided by the proto-oncogene *Myc* and/or activated Ras has been reported to be critical to observe complete transformation of HMECs *in vitro* (Elenbaas et al., 2001; Wang et al., 2011). We observed overexpression of c-Myc protein and transcript in HMECs infected with HCMV-DB by western blotting and RT-PCR microarray, respectively (Fig. 5, Suppl. Fig. S2). In HMECs infected with UV-treated HCMV-DB, we also observed higher levels of c-Myc protein as compared to uninfected controls (Fig. 5). In addition, we observed higher expression of activated Ras (GTP-Ras) on day 3 postinfection in HMECs infected with HCMV-DB (Fig. 5).

Upregulation of c-Myc and activated Ras coupled with constitutively activated PI3K/Akt pathway can lead to anchorage-independent growth and transformation of HMECs (Zhao et al., 2003). Therefore, we further assessed the PI3K/Akt signaling in HMECs infected with HCMV-DB. We observed that Akt undergoes serine-473 and threonine-308 phosphorylation in HMECs infected with HCMV-DB (Fig. 5). We did not observe any significant increase in the levels of Akt protein in HMECs infected with HCMV-DB as compared to controls using western blotting (Fig. 5).

In our previous study, we have observed the activation of JAK/STAT3 axis in primary human hepatocytes and HepG2 cells infected with HCMV (Lepiller et al., 2013). In addition STAT3 activation has been

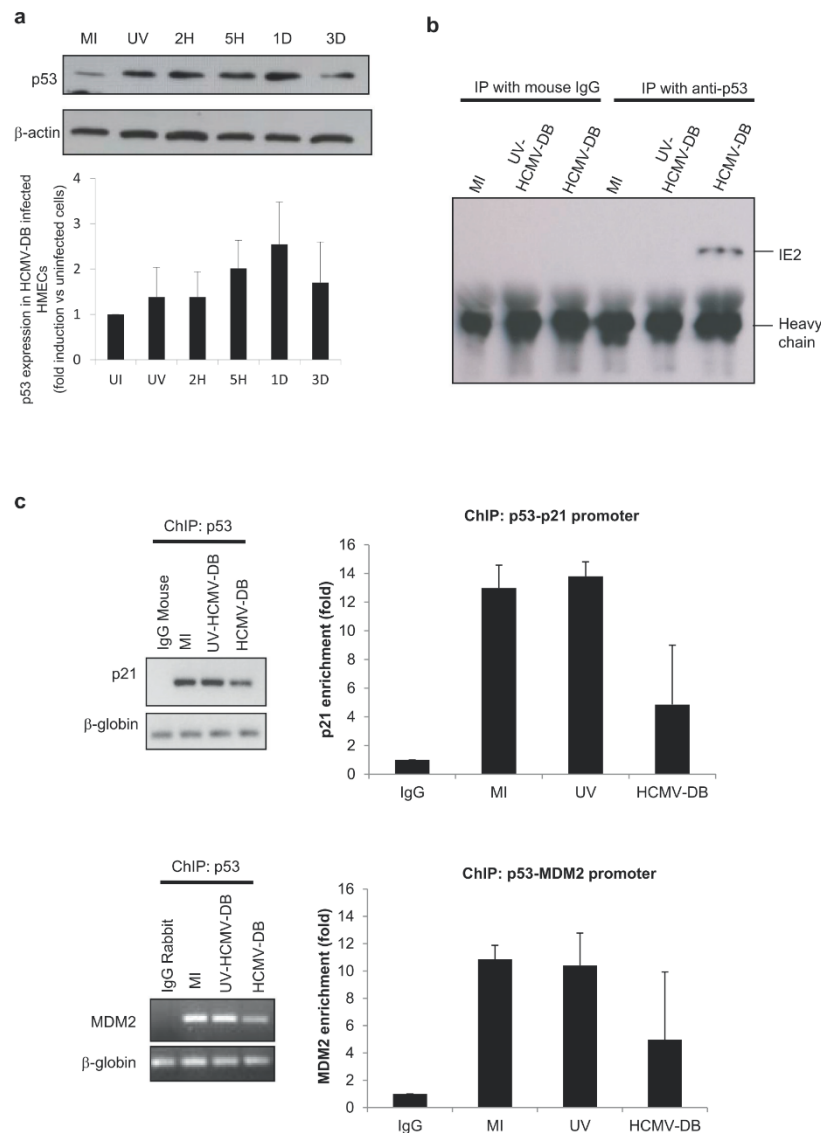


Fig. 3. Functional inactivation of p53 in HMECs infected with HCMV-DB. a. HCMV-DB upregulates the expression of the p53 protein in infected HMECs. HMECs cells were left uninfected or infected with either HCMV-DB or UV-inactivated HCMV-DB (MOI = 1). Cell lysates were prepared at several time points and expression of the p53 protein was determined using western blotting. Beta-actin was included as an internal control. Results are representative of three independent experiments. The histogram represents means (\pm SD) of three independent experiments. b. The interaction between HCMV-DB IE2 protein and p53 was detected by co-immunoprecipitation as mentioned in Materials and Methods. Results are representative of two independent experiments. c. Decreased binding of p53 to its target genes promoters p21 and MDM2 in HMECs infected with HCMV-DB. p53 ChIPs were assessed by semi-quantitative as well as by quantitative PCR for p21 and MDM2 promoter sequences as described in Materials and Methods. Results represent mean (\pm SD) of three independent experiments.

described in breast cancer biopsies (Diaz et al., 2006). We assessed the expression of STAT3, phospho-STAT3 and cyclin D1 in HMECs infected with HCMV-DB. We observed the enhanced phosphorylation of STAT3 protein (P-STAT3) and the upregulation of cyclin D1 protein and its transcript in HMECs infected with HCMV-DB as compared to uninfected

and UV-treated controls (Fig. 5, Suppl. Fig. S2). Our data indicate that HCMV-DB activates STAT3 in HMECs that leads to upregulation of cyclin-D1, a key regulator of cell proliferation. Therefore, we assessed the proliferation of HMECs infected with HCMV-DB. We measured the expression of the nuclear antigen Ki67, a hallmark of cell proliferation

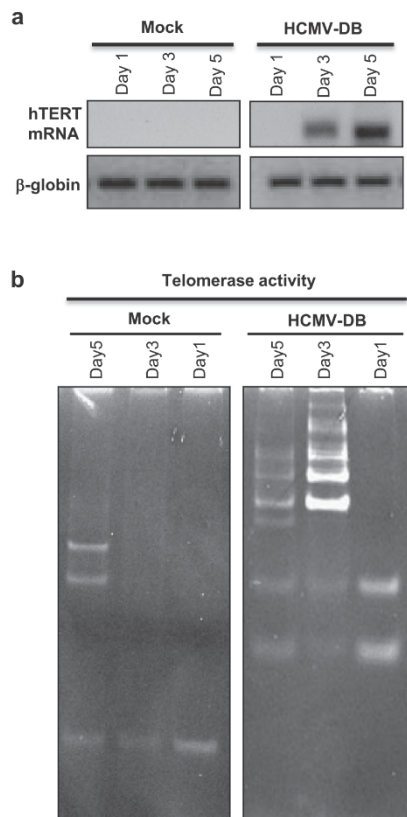


Fig. 4. Upregulation of telomerase transcript and telomerase activity in HMECs infected with HCMV-DB. Upregulation of telomerase mRNA (a) and telomerase activity (b) in HMECs infected with HCMV-DB compared to mock infected HMECs. HMECs were infected with HCMV-DB (MOI = 1) and cells were harvested at different time points. Mock infected cells were included as a negative control. a. RNA was isolated from the cells and expression of hTERT mRNA was determined by RT-PCR as described in Materials and Methods. b. Increased telomerase activity in HMECs following infection with HCMV-DB. Telomerase activity was determined using TRAPEZE Telomerase detection kit as described in Materials and Methods. Results are representative of three independent experiments.

by flow cytometry. We observed significant increase in Ki67 Ag expression upon HCMV-DB infection of HMEC cells (Fig. 6). As an additional control, viral supernatants were filtered through 0.2 μ m filter and were added to HMECs (Fig. 6a). The increase in Ki67 antigen expression upon HCMV-DB infection of HMEC cells was abolished by UV-inactivation of the virus (Fig. 6b). The cellular proliferation was observed primarily in infected HMECs labelled with an anti-pp71 HCMV antibody compared to uninfected (pp71-negative) cells (Fig. 6c).

Altogether, our results indicate that, in HMECs infected with the clinical isolate HCMV-DB, the molecular requirements for oncogenic immortalization and transformation of primary HMECs *in vitro* are fulfilled, namely inactivation of p53 and Rb, telomere maintenance, acquisition of constitutive mitogenic signals provided by Ras/cMyc, Akt activation, STAT3 activation and cyclin D1 overexpression with enhanced cellular proliferation.

3.3. Cellular Transformation of HMECs by HCMV-DB

Above-mentioned observations indicate that HCMV can challenge HMECs to proceed towards oncogenic transformation *in vitro*, preferentially with the clinical isolate HCMV-DB. Thus to further test whether these observations can be translated into malignant transformation of HMECs, we proceed to perform soft agar assay which is the most stringent *in vitro* assay for the detection of transformation. HMECs were infected individually with HCMV-DB and AD169 and at day 1 post infection HMECs were seeded in soft agar medium for 14 days. In parallel, uninfected cells and cells infected with heat-inactivated HCMV and UV-treated HCMV were seeded as negative controls, and tumoral MCF-7 cells and MDA-MB-231 cells were seeded as positive controls. As additional controls, viral supernatants were filtered through 0.2 μ m filter and were used to infect HMECs. In addition, HMECs were treated with ganciclovir followed by HCMV infection. After 14 days of culture (i.e. on day 15 post-infection), we observed the formation of colonies in soft agar that had been seeded with HMECs infected with HCMV-DB (Fig. 7). Colonies were also observed for established representative breast cancer cell lines such as MCF-7 cells and MDA-MB-231 cells (Fig. 7). We did not observe the formation of colonies in soft agar that had been seeded with HMECs infected with AD169 (Fig. 7) or with MRC5 cells infected with HCMV-DB or AD169 (data not shown). Additionally, we isolated DNA from soft agar colonies at day 15 post infection (after 14 days of culture in soft agar) and assessed the presence of HCMV DNA, namely the major immediate early promoter (MIEP) sequence, using a PCR assay. Surprisingly, we did not detect HCMV MIEP DNA in colonies grown in soft agar seeded 14 days earlier with HMECs infected with HCMV-DB (Suppl. Fig. S3).

3.4. Emergence of Clusters of Spheroid Cells Termed “CMV-Transformed HMECs” or CTH Cells in HCMV-DB-Infected HMEC Cultures

HMEC cultures were individually infected with HCMV-DB, Epstein-Barr virus (EBV), herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV). As negative controls uninfected HMECs, UV-inactivated HCMV-DB and HCMV-DB supernatant passaged through 0.2 μ m were used to infect HMECs. Several clusters of spheroid cells called CMV-transformed HMECs or CTH cells were observed in HMECs infected with HCMV-DB around day 20 post infection in some of the cultures (Fig. 8a). No such cell clusters were observed in cultures of HMECs infected with other members of *Herpesviridae* family tested such as EBV, HSV-1 and VZV, and in negative controls such as HMEC cultures infected with HCMV-DB filtrate and UV-treated HCMV-DB (Fig. 8a and data not shown). These clusters of spheroid CTH cells were gently detached and cultured in HMEC Ready medium for several passages (currently up to >150 passages) (Fig. 8a) and suggested to have undergone immortalization. We detected the presence of a HCMV-DB IncRNA4.9 genomic sequence of 126 bp in CTH cells using both qualitative and quantitative PCR (Fig. 8b and c). The sequence of the amplified 126 bp fragment of HCMV-DB IncRNA4.9 DNA detected in CTH cells was confirmed to match the IncRNA4.9 gene sequence from HCMV-DB (Suppl. Fig. S4). We did not detect the 126 bp amplicon of IncRNA4.9 gene in uninfected HMEC and MRC5 cells (Fig. 8b and c). Using both qualitative and quantitative PCR assays, we did not detect the presence of HCMV-DB MIEP sequences in CTH cells (Fig. 8b and c), indicating that only part of the HCMV-DB genome is present in CTH cells.

3.5. Injection of CTH Cells in NSG Mice Results in Tumor Formation With a HCMV IncRNA4.9 Signature

With previously mentioned encouraging results, we sought to determine the oncogenic potential of CTH cells *in vivo*. We injected two million CTH cells in the mammary fat pad of NOD SCID Gamma (NSG) mice. As controls uninfected HMECs, MCF-7 and MDA-MB-231 cells were used. No tumor formation was observed in mice injected with

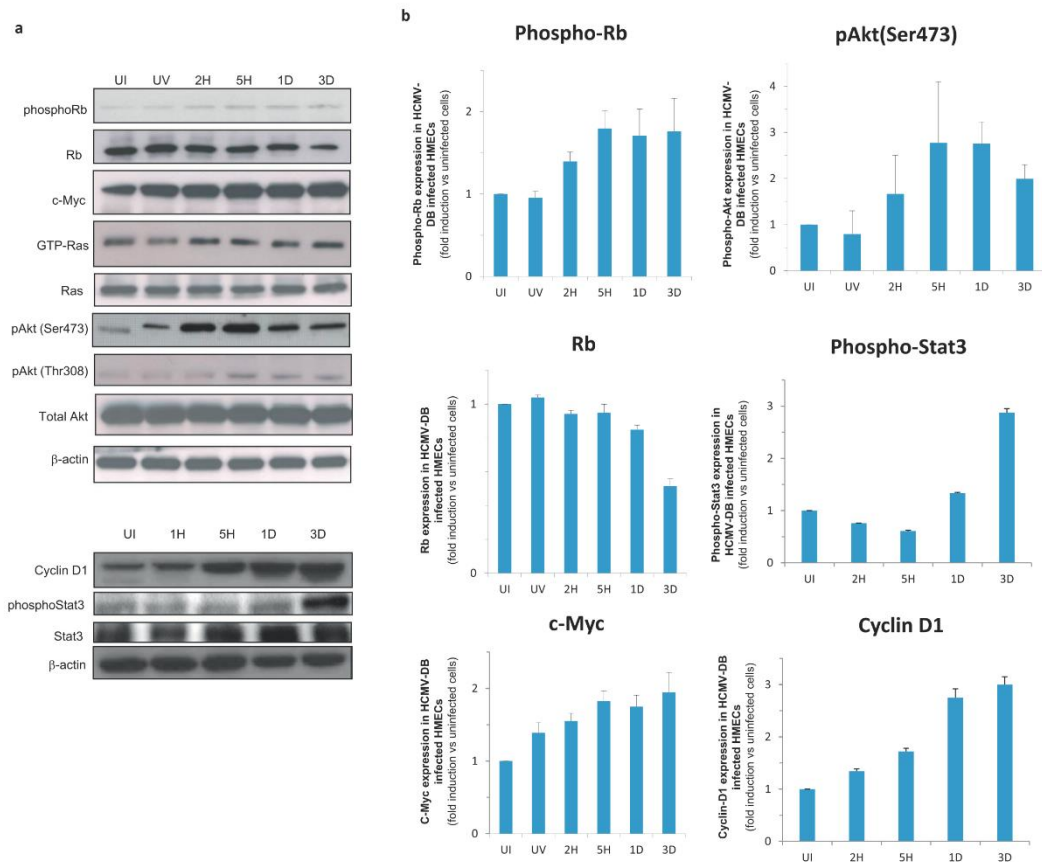


Fig. 5. Increased expression of phospho-Rb, c-Myc, phospho-Akt, phospho-STAT3 and cyclin-D1 in HMECs infected with HCMV-DB. a. Expression of phospho-Rb, c-Myc, phospho-Akt (Ser473 and Thr308), phospho-STAT3 and cyclin D1 is upregulated in HMECs infected with HCMV-DB as determined by western blotting. HMECs were infected with HCMV-DB and cells were harvested at described time points. As controls, HMECs were either left uninfected or infected with UV-inactivated HCMV-DB. β -actin was used as an internal control. To determine the active Ras (GTP-Ras) expression in HMECs, GST-pull down assay was performed as described in Materials and Methods. Results are representative of three independent experiments. b. Histograms represent the expression of phospho-Rb, Rb protein, c-Myc, phospho-Akt, phospho-Stat3, and cyclin-D1 quantified by densitometry using ImageJ 1.40 software. Results are the means (\pm SD) of three independent experiments.

uninfected HMECs (Fig. 9a and b). On the other hand, tumor begins to appear two weeks post injection in mice injected with CTH cells (Fig. 9a and b). Surprisingly, tumor growth arising from CTH cells was rapid as compared to tumor growth resulting from injection of MDA-MB-231 cells (Fig. 9a). As a negative control no tumor formation was observed in mice injected with MCF-7 cells in the absence of estrogen supplementation (Fig. 9a). Using immunohistochemistry staining, the tumor biopsies of mice injected with CTH cells displayed a triple negative phenotype with the absence of ER, PR and HER2 protein, were negative for E-cadherin, CK5/6, GATA3, CK20 and GCDFFP (Fig. 9c). Interestingly vimentin expression was upregulated in the tumor biopsies, indicating EMT traits (Fig. 9c). Interestingly, we detected the presence of lncRNA4.9 DNA, but not of MIEP DNA, in all tumor biopsies using PCR assay (Fig. 9d).

3.6. Detection of HCMV lncRNA4.9 DNA in Biopsies From Patients With Breast Cancer

Genomic DNA isolated from patient breast tumor and healthy breast tissue biopsies was provided by the regional tumor bank (BB-0033-

00024 Tumorothèque Régionale de Franche-Comté). Using qualitative PCR, we were able to detect HCMV sequences of lncRNA4.9, but not of MIEP, in the genomic DNA preparation from biopsies of patients with breast cancer (Fig. 9e). Neither lncRNA4.9 nor MIEP sequences were detected in biopsies of healthy breast tissue (Fig. 9f).

4. Discussion

Our results indicate that the clinical isolate HCMV-DB replicates productively in HMECs. Infection of HMECs with HCMV-DB results in a pro-oncogenic cellular environment with decreased p53 functional activity and Rb hyperphosphorylation, overexpression of *hTERT* mRNA and enhanced telomerase activity, upregulation of c-Myc and activation of Akt and STAT3, and upregulation of cyclin D1 leading to enhanced cellular proliferation. We also observed the potential of the clinical isolate HCMV-DB in transforming primary HMECs as assessed by colony formation in soft agar. Interestingly, we observed spheroid-cell clusters in HMEC cultures infected with HCMV-DB resulting in the growth of floating rapidly proliferating transformed cells which we named CTH cells.

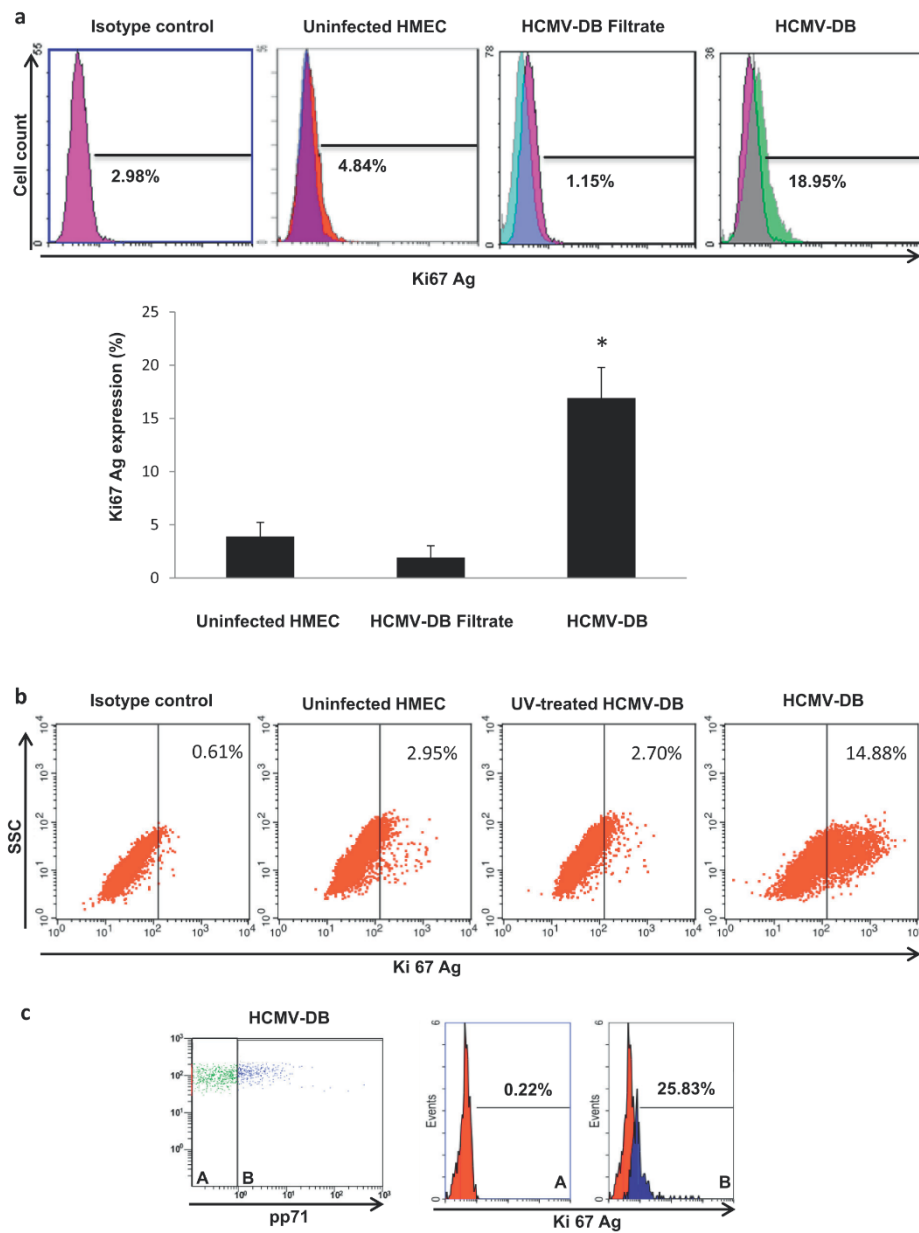


Fig. 6. Enhanced cellular proliferation following HCMV-DB infection of HMECs. **a.** HMECs were either left uninfected or infected with filtrate or wild type HCMV-DB. At day 3 post infection cells were harvested and expression of the Ki-67 antigen was determined using flow cytometry as described in Materials and Methods. The histogram represents means (\pm SD) of three independent experiments. * $P < 0.05$. **b.** HMECs were either left uninfected or infected with UV-treated or wild type HCMV-DB. At day 3 post infection cells were harvested and expression of the Ki-67 antigen was determined using flow cytometry as described in Materials and Methods. The results are representative of three independent experiments. **c.** Enhanced expression of the Ki-67 antigen in infected (pp71-positive) cells compared to uninfected (pp71-negative) cells. Results are representative of three independent experiments.

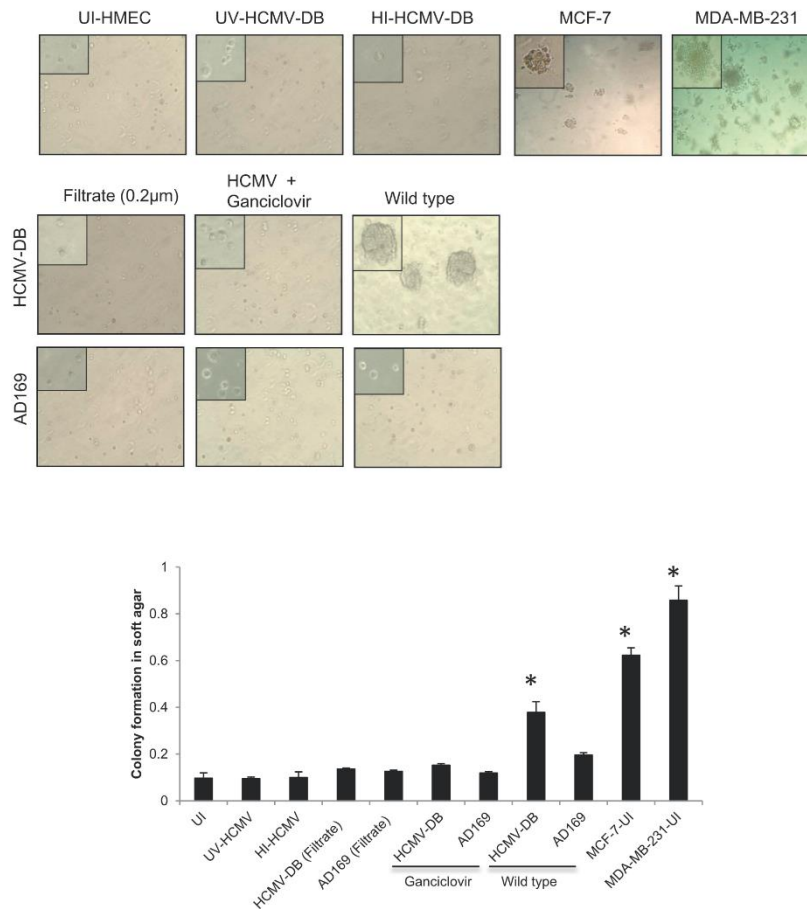


Fig. 7. Colony formation in soft agar seeded with HMECs infected with HCMV-DB. HMECs were either left uninfected or infected with UV-inactivated HCMV, heat-inactivated HCMV or wild-type HCMV strains (DB and AD169) at MOI = 1. As controls viral supernatants were passed through 0.2 μm filter and filtrates were used to infect HMECs. In addition HMECs were infected with ganciclovir-treated HCMV. At day 1 post infection, cells were seeded in soft agar as per manufacturer's instructions. After 14 days in soft agar (day 15 post infection) soft agar colonies were observed under an Olympus microscope (magnification 100× and 200×). As positive controls, breast cancer cell lines MCF-7 and MDA-MB-231 were used. Results are representative of three independent experiments. Histogram shows quantification of colony formation in soft agar seeded with HMECs at day 15 post infection. Histogram represents means (±SD) of three independent experiments. *P < 0.05.

We detected the presence of HCMV-DB lncRNA4.9 genomic sequence in CTH cells using both qualitative and quantitative PCR. The CTH cells when injected into mammary fat pad of NSG mice form fast growing triple-negative breast tumors *in vivo* which express the genomic sequence of HCMV-DB lncRNA4.9. In breast cancer biopsies from patients we also observed the presence of lncRNA 4.9 genomic sequence, indicating the potential *in vivo* relevance of our HCMV-DB model. Our results indicate that some HCMV strains such as HCMV-DB display oncogenic properties, transform HMECs, result in fast growing triple-negative tumors in NSG mice and could favor the appearance of human breast tumors.

We observed the most efficient and sustained replication of HCMV-DB in HMECs as compared to laboratory passaged strain AD169 (Fig. 1). We observed the complete life cycle of the clinical isolate HCMV-DB in HMECs as determined by the detection of early and late viral transcripts and proteins by RT-PCR and western blotting, respectively (Fig. 2). In

agreement with our results, laboratory adapted HCMV strains and low passage clinical strains show different cell tropism *in vitro*. Low passage clinical HCMV isolates have been shown to preferentially replicate in epithelial cells, endothelial cells and myeloid cells (O'Connor and Shenk, 2012; Wang and Shenk, 2005). In addition to infecting HMECs, the clinical isolate HCMV-DB (Accession number KT959235) infects macrophages and we demonstrated previously its ability to induce M2 phenotype and upregulation of proto-oncogene Bcl-3 (Khan et al., 2009). In contrast to laboratory adapted strains, some HCMV clinical strains including HCMV-DB but also PH and TR strains (Fig. S1), display a tropism for monocytes/macrophages (O'Connor and Shenk, 2012; Wang and Shenk, 2005). Thus, the macrophage-tropic HCMV strains might play a role in the development of tumor-associated macrophages (TAM), a marker of poor prognosis in human breast carcinomas and glioblastomas (Dziurzynski et al., 2011; Medrek et al., 2012). Recently, using high throughput sequencing and population genetics approaches,

a detailed map of HCMV *in vivo* evolution was built and provided evidence that viral populations can be stable or rapidly differentiate, depending on host environment, and ultimately could result in tissue compartment colonization (Renzette et al., 2013). Positive selection could be a strong driver of evolution associated with compartmentalization (Renzette et al., 2013). Taken together these data indicate that the tissue (tumor) environment can drive the viral fitness and that clinical isolates such as the macrophage-tropic HCMV-DB strain isolated from a pregnant women could be well adapted to this environment and in turn might ultimately favor the development of breast cancer.

Our data show that HCMV-DB not only productively infects HMECs but also shapes the cellular environment in favor of the transformation. We observed upregulation of p53 in HMECs infected with HCMV as previously reported in other cell types (Hannemann et al., 2009) (Fig. 3). In addition to IE2 binding to p53 in HMECs infected with HCMV as reported previously in human fibroblasts (Hsu et al., 2004), we observed decreased binding of p53 to its target gene promoters (p21 and MDM2) in the HMECs infected with the clinical isolate HCMV-DB (Fig. 3b and c). This could explain why p53 response mounted in HMECs upon HCMV-DB infection, failed to efficiently protect HCMV-infected cells against cell cycle promotion and cellular proliferation. Furthermore, recently it has been shown the role of viral proteins in inducing heterochromatin state in the region of p53 targets genes (Soria et al., 2010). In order to investigate the potential of HCMV-DB infection in inducing heterochromatin at the region of p21 and MDM2 promoter, we

performed a ChIP assay using anti-H3K9me3 antibody. However, we did not observe any significant change in lysates from HMECs infected with HCMV-DB compared to uninfected control lysates (data not shown).

Immortalization is prerequisite for the cancer development. Continuous cell growth without cellular senescence, one of the hallmarks of cancerous cells, is governed by telomerase activity which usually declines in the normal cells (Hahn and Meyerson, 2001). We observed that infection with HCMV-DB results in upregulation of telomerase activity in infected HMEC cells (Fig. 4) that usually leads to cell immortalization. Recently the role of STAT3 in activating catalytic subunit of telomerase has been shown (Chung et al., 2013) which could explain increased telomerase activity parallel to elevated levels of activated STAT3 in HMECs infected with HCMV-DB.

Several molecular mechanisms have been described contributing to oncogenesis including modifications in cell cycle progression and apoptosis (Alibek et al., 2013). It is a generalized notion that more than one proto-oncogene and/or deregulation of tumor suppressor proteins are involved in the tumor progression. In accordance with previous findings, we observed elevated levels of phosphoRb (at Ser 780 residue) upon infection of HMECs with HCMV-DB (Fig. 5) (Hume et al., 2008). The role of HCMV UL97 in phosphorylating and inactivating Rb family proteins and promoting cell cycle progression has been shown (Iwahori et al., 2017). Furthermore, the downregulation of retinoblastoma family of proteins by pp71 (pUL82) has been also reported

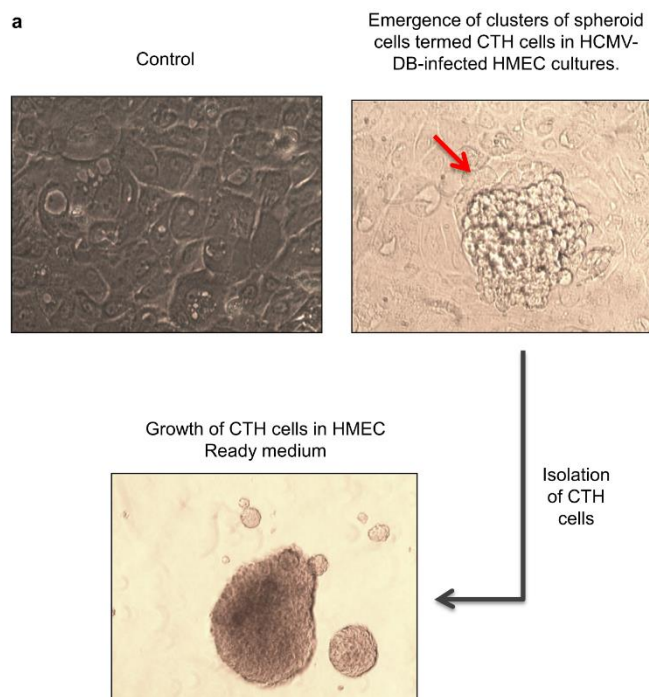


Fig. 8. Emergence of clusters of spheroid cells named CTH cells in HMECs infected with HCMV-DB at day 20 post infection which display a HCMV-DB IncRNA4.9 signature. a. Right panel. Clusters of spheroid cells appeared in cultures of HMECs infected with HCMV-DB (MOI = 1) at day 20 post-infection. Left panel. HMECs treated with HCMV-DB filtrate were used as a negative control. Magnification 200 \times . Lower panel. The clusters of spheroid CTH cells were gently detached and cultured in HMEC Ready medium (currently up to >150 passages). b., c. We detected the presence of a 126 bp HCMV-DB RNA4.9 DNA sequence, but not of MIEP sequence, in CTH cells using (b) qualitative PCR and (c) quantitative PCR. Uninfected HMECs and MRC5 cells were used as negative controls. HCMV-DB viral stock (HCMV-DB) and HMECs infected with HCMV-DB (HMEC + HCMV-DB) were used as positive controls. Beta-globin was used as an internal control. Results are representative of three independent experiments.

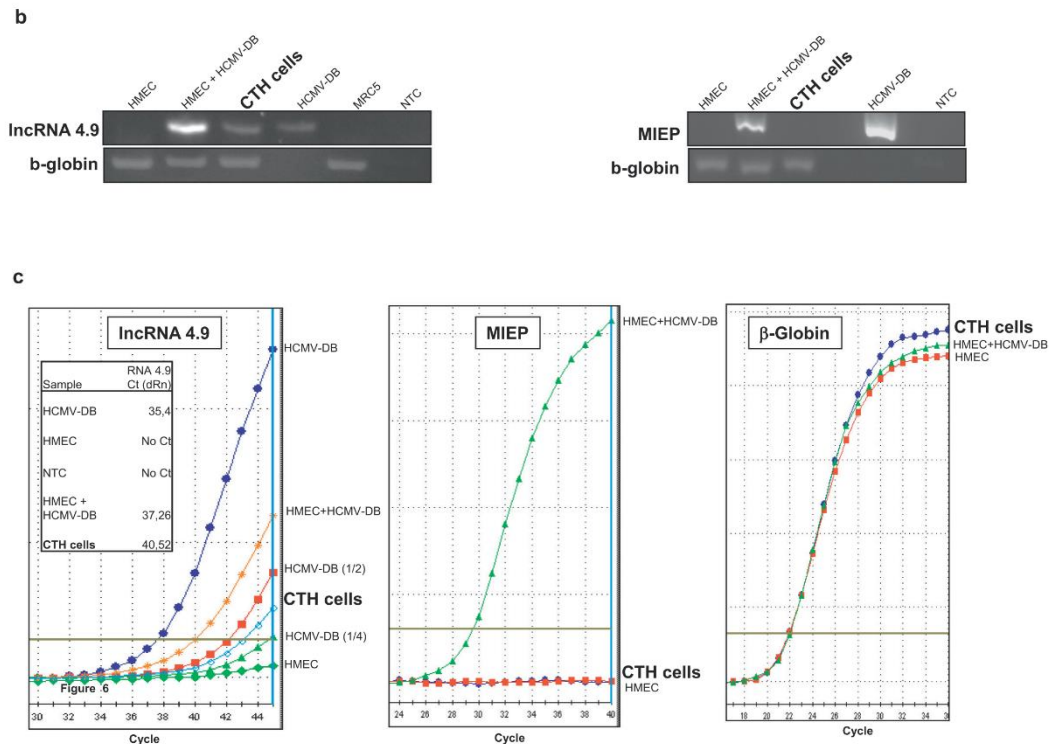


Fig. 8 (continued).

(Kalejta and Shen, 2003). In HMECs infected with HCMV-DB we observed the presence of the UL82 transcript at day 1 and day 3 (Fig. 2a) parallel to the decrease in the expression of Rb protein at day 3 (Fig. 5). We also observed the up-regulation of c-Myc, elevated levels of phospho-Akt, phospho-STAT3, and cyclin-D1 expression following infection of HMECs with HCMV-DB (Fig. 5) (Boldogh et al., 1991, 1990; Chan et al., 2009; Hagemeier et al., 1992; Slinger et al., 2010; Soroceanu et al., 2011). Data are consistent with previous reports where HCMV has been shown to induce the expression of proto oncogenes including c-Myc, c-Fos and c-Jun in human embryo lung cells (Boldogh et al., 1991, 1990). In addition, we observed increased expression of Ki67 antigen in infected HMECs upon HCMV-DB infection (Fig. 6). To rule out the possibility that the involvement of serum factors present in HCMV-DB supernatant resulted in enhanced proliferation, we passed HCMV-DB viral supernatant through 0.2 μ m filter and also exposed HMECs to the filtrate. We observed only marginal increase in Ki67 antigen expression with the filtrate as compared to HCMV-DB viral supernatant, linking the presence of HCMV-DB virus itself to increased expression of Ki67 antigen (Fig. 6).

Soft agar assay is widely recognized as the most stringent and reliable assay to determine the malignant transformation *in vitro* (Gilmore et al., 2001). We observed that HCMV-DB, but not AD169, was able to induce colony formation in soft agar seeded with infected HMECs (Fig. 7). In contrast, no colonies were observed neither in uninfected, heat-inactivated, UV-treated, ganciclovir-treated and filtered HCMV controls (Fig. 7) nor in HMECs infected with EBV, HSV-1 and VZV (data not shown). The proposition that HCMV has the potential to contribute to oncogenesis by inducing mutations in cellular genes

has been evoked previously indicating a close collaboration between HCMV IE1 and IE2 and the adenovirus E1A proteins to generate transformed foci of primary baby rat kidney cells (Shen et al., 1997). In addition, primary HCMV infection can cause chromosomal breaks or mutations (Fortunato et al., 2000). Accumulation of such mutations in infected cells may trigger tumor initiation.

We were not able to amplify the MIEP sequence in the colonies harvested from soft agar seeded with HMECs infected with HCMV-DB. At least two possibilities could account for the observed result. First, the HCMV genome is not present in colonies after 14 days in soft agar cultures and the sustained cellular transformation does not require anymore the presence of viral genes/proteins at this time. Second, only part of the HCMV-DB genome excluding the MIEP sequence is still present in the soft agar transformed HMECs after 14 days in culture, and is sufficient for the sustained transformation of the infected HMECs. This latter hypothesis seems to be the most plausible since we detected HCMV IncRNA4.9 sequence, but not MIEP sequence, in CTH cells. Our results are in agreement with the transformation of keratinocytes by the human papilloma virus (HPV) where only a few viral genes (e.g. E6 and E7 genes) are detected and required for the sustained transformation of infected cells (Zur Hausen, 2009).

To confirm the results of soft agar assay, HMECs infected with HCMV-DB were cultured for a prolonged period of time. On the 20th day of infection we observed several spheroid-cell clusters (Fig. 8a). These spheroid-cell clusters were absent in uninfected HMECs cultures and cultures infected with HCMV-DB filtrate and UV-inactivated HCMV-DB. These spheroid-cell clusters composed of CTH cells exhibit rapid growth as compared to the surrounding cells, were easily detached

and were cultured in HuMEC Ready Medium containing epidermal growth factor, hydrocortisone, isoproterenol, transferrin, insulin and bovine pituitary extract (Fig. 8a). These CTH cell clusters grow in suspension and fails to attach the surface (Fig. 8a), a sign of anchor independent growth and epithelial mesenchymal transition (Larue and Bellacosa, 2005).

To demonstrate a close link between HCMV-DB infection and HMEC transformation it was critical to demonstrate that part of the HCMV-DB genome was present in CTH cells. In CTH cells we detected the lncRNA4.9 DNA sequence of HCMV-DB by both qualitative and quantitative PCR that amplified a 126 bp region of the lncRNA 4.9 gene from HCMV-DB, as confirmed by Sanger's sequencing (Fig 8b and c, Suppl. Fig. S4). Since we did not detect the MIEP sequence in CTH cells, the presence of a limited region of the HCMV-DB genome in CTH cells

including the 126 bp lncRNA4.9 sequence might be a critical factor for the sustained transformation of these cells during time. Interestingly, a small number of loci of HCMV, centered on long non coding RNA including lncRNA4.9 show evidence of selective sweeps and these loci are targets of positive selection indicating a strong conservation within HCMV (Renzette et al., 2016). The detection of HCMV-DB lncRNA sequence in CTH cells rules out the possibilities of spontaneous generation of transformed cells (Soule et al., 1990).

Our data indicate the potential of a clinical HCMV strain, namely the HCMV-DB isolate, to fulfill all requirements to transform HMECs *in vitro* possibly through limited viral gene expression including the lncRNA4.9 gene. Lnc RNAs have recently gained increased interest in cancer and in viral infections (Huarte, 2015; Fortes and Morris, 2016). Interestingly, HCMV lncRNA4.9 has been reported to be involved in HCMV latency

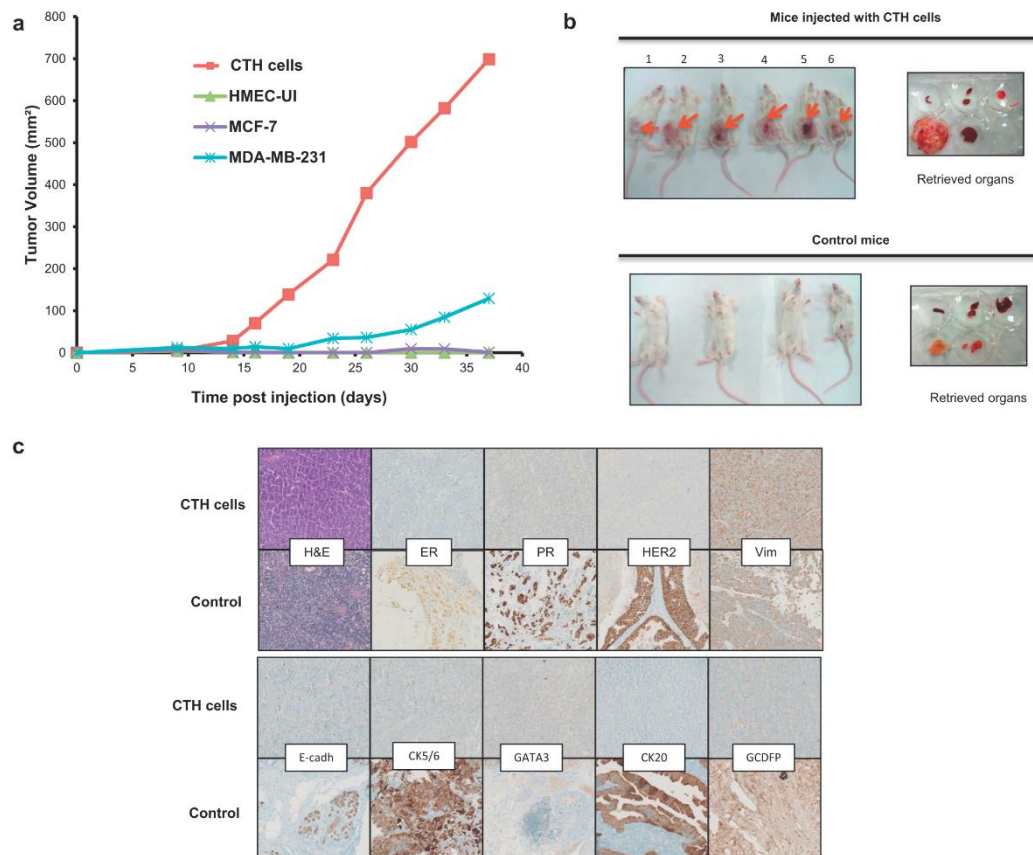


Fig. 9. Appearance of tumors with HCMV lncRNA4.9-signature in NSG mice injected with CTH cells and detection of the HCMV lncRNA4.9 gene in tumor biopsies of human breast cancer. **a.** Injection of CTH cells in NSG mice results in tumor formation. Two million of CTH cells, five million of uninfected HMECs and two million of MCF-7 and MDA-MB-231 cells were injected in the mammary fat pad of NSG mice in the absence of hormone supplementation. Uninfected HMECs, MCF-7 cells and MDA-MB-231 cells were used as controls. Tumor dimensions were recorded as shown in the figure. Results are means of experiments with six mice in each group. **b.** After mice sacrifice, tumors and organs (liver, lung, kidney, spleen) were retrieved. **c.** Using immunohistochemistry staining, tumor biopsies display a triple negative phenotype with the absence of ER, PR and HER2 protein, are negative for E-cadherin, CK5/6, GATA3, CK20 and GCDFF, but positive for vimentin. **d.** Detection of the presence of lncRNA4.9 DNA, but not of MIEP DNA, in all tumor biopsies from mice injected with CTH cells using PCR assay ($n = 6$). DNA extract of HMECs infected with HCMV-DB (Control HMEC+HCMV-DB) was used as a positive control. Beta-globin was used as an internal control. **e.** Tumor biopsies of patients with breast cancer display a lncRNA4.9 signature. Using qualitative PCR, we were able to detect HCMV sequences of lncRNA4.9 gene, but not of MIEP, in the genomic DNA preparation from tumor biopsies of patients with breast cancer. DNA extract of HCMV-DB viral stock (Control HCMV-DB) was used as a positive control. **f.** lncRNA4.9 and MIEP DNA sequences were not amplified from human healthy breast tissue. Beta-globin was used as an internal control.

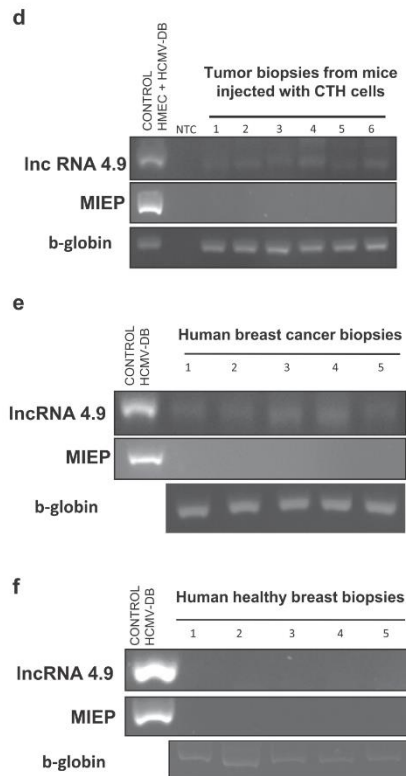


Fig. 9 (continued).

and also to interact with the polycomb repressive complex (PRC), especially the PRC2 complex which is abundant in highly proliferative cells including cancer cells (Rossetto et al., 2013; Van Damme and Van Loock, 2014).

We also assessed the expression of HCMV IncRNA4.9 gene in the genomic DNA obtained from human breast cancer biopsies. In contrast to the absence of the MIEP sequence, we detected the presence of the IncRNA4.9 gene (126 bp amplicon) in the tumors isolated from women with breast cancer (Fig. 9e). We did not detect IncRNA4.9 and MIEP sequences in biopsies of healthy human breast tissue (Fig. 9f). Altogether our results point to a critical role of HCMV in the appearance and development of breast cancer. Nevertheless additional studies are required to decipher the exact role of IncRNA4.9 and/or other yet to identified viral genes and proteins in the pathophysiology of the disease.

Finally, other HCMV strains especially low passaged clinical isolates may also activate oncogenic pathways in HMECs. Interestingly, the TB40/E strain isolated from a throat wash of a bone marrow transplant recipient, a low passage clinical isolate, enters into HMECs (data not shown), overexpresses hTERT mRNA and enhances the telomerase activity in infected HMECs as compared to uninfected HMECs (Suppl. Fig. S5a and b), and ultimately favors the apparition of colonies in soft agar (Suppl. Fig. S5c). The enhancement of telomerase activity and the appearance of colonies in soft agar is less pronounced for HMECs infected with TB40/E strain compared to cells infected with HCMV-DB. We are currently screening other primary HCMV isolates for their ability

to activate oncogenic pathways in HMECs *in vitro* and potentially to lead to the appearance of tumors *in vivo*.

5. Conclusion

In conclusion, our data indicate that the infection of HMECs with HCMV-DB results in a pro-oncogenic cellular environment with decreased p53 functional activity and Rb hyperphosphorylation, overexpression of hTERT mRNA and enhanced telomerase activity, upregulation of c-Myc and activation of Akt and STAT3, and upregulation of cyclin D1 leading to enhanced cellular proliferation. We also observed the potential of the clinical isolate HCMV-DB in transforming primary HMECs as assessed by colony formation in soft agar. We observed the appearance of transformed cells in HMEC cultures infected with HCMV-DB, namely the CTH cells which display a IncRNA4.9 viral signature. The injection of CTH cells in NSG mice resulted in the appearance of triple-negative breast tumors. Finally the HCMV IncRNA4.9 signature is present in the tumor biopsies of human breast cancer *in vivo*. Future studies will decipher the molecular mechanisms involved in the transformation of HMECs infected with HCMV-DB and to determine the exact relevance of such a model in the pathogenesis of breast cancer.

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Conflicts of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

The data sets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author Contributions

AK, MKT, WA, KAK, FAM, SP, LC, LR, MPA, SVD, OA performed research; GH and SMB designed research; AK and GH wrote the paper.

Consent for Publication

Not applicable.

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The transcriptome of human mammary epithelial cells infected with the HCMV-DB strain displays oncogenic traits

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Increasing evidence indicates that human cytomegalovirus (HCMV) populations under the influence of host environment, can either be stable or rapidly differentiating, leading to tissue compartment colonization. We isolated previously from a 30-years old pregnant woman, a clinical isolate of HCMV, that we referred to as the HCMV-DB strain (accession number KT959235). The HCMV-DB clinical isolate demonstrated its ability to infect primary macrophages and to upregulate the proto-oncogene Bcl-3. We observed in this study that the genome of HCMV-DB strain is close to the genomes of other primary clinical isolates including the Toledo and the JP strains with the later having been isolated from a glandular tissue, the prostate. Using a phylogenetic analysis to compare the genes involved in virus entry, we observed that the HCMV-DB strain is close to the HCMV strain Merlin, the prototype HCMV strain. HCMV-DB infects human mammary epithelial cells (HMECs) which in turn display a ER-/PR-/HER2- phenotype, commonly referred to as triple negative. The transcriptome of HCMV-DB-infected HMECs presents the characteristics of a pro-oncogenic cellular environment with upregulated expression of numerous oncogenes, enhanced activation of pro-survival genes, and upregulated markers of cell proliferation, stemcellness and epithelial mesenchymal transition (EMT) that was confirmed by enhanced cellular proliferation and tumorsphere formation *in vitro*. Taken together our data indicate that some clinical isolates could be well adapted to the mammary tissue environment, as it is the case for the HCMV-DB strain. This could influence the viral fitness, ultimately leading to breast cancer development.

Breast cancer, the most common cancer diagnosed among women, exhibits heterogeneous molecular characteristics. Several types of breast cancer have been identified based on the differential gene expression patterns. These groups include among others the normal breast epithelial-like, the luminal epithelial type A and type B, the basal-like and the claudin low groups¹. Genetic risk factors and environmental risk factors are some of the etiologic factors involved in breast cancer². Of all worldwide cancers close to one-fifth could involve infectious agents including viruses, as part of the environmental risk factors^{3–5}.

The *Betaherpesviridae* human cytomegalovirus (HCMV) is known to cause, in the immunocompetent host, an infection that usually ranges from asymptomatic to mild. Serious complications can however be the result of the infection of immunocompromised hosts⁶. In opposition to laboratory strains of HCMV, which growth appears to be restricted to fibroblasts only, clinical isolates are able to infect and grow in several types of cells including epithelial cells, endothelial cells, monocytes, macrophages, fibroblasts, stromal cells, hepatocytes, smooth muscle cells, and neural stem/progenitor cells^{7–10}. Recently, a detailed *in vivo* evolutionary map of HCMV was built by combining population genetics methods and high throughput sequencing. This map provided evidence that viral populations under the influence of host environment can either be stable or rapidly differentiating, leading to tissue compartment colonization¹¹.

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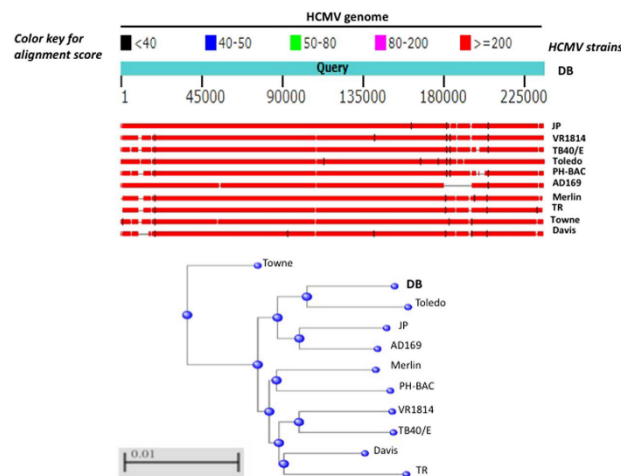


Figure 1. Comparison of the genomic sequence of HCMV-DB with the genomic sequences of other clinical and laboratory adapted HCMV strains. The upper panel shows the genomic sequence of HCMV-DB as compared to two laboratory adapted strains (AD169 and Towne) and eight low passages clinical isolates (Merlin, Toledo, TR, PH, VR1814, Davis, JP, and TB40/E). The lower panel shows the alignment tree related to the whole viral genome with comparison between HCMV-DB genome and the genome of ten other HCMV strains.

Several research groups focusing on inflammatory diseases and on cancer addressed the role played by HCMV in these diseases^{12–14}. Tumor tissues from several cancers, including brain, colon, prostate, liver and breast cancer, have been found positive for HCMV DNA or antigens^{15–20}. In the paradigm of oncomodulation, oncogenesis mechanisms could be amplified by HCMV acting as a cofactor, when infecting the tumor tissue²¹. In addition, monocytes and macrophages, respectively located in the blood and in the tissues, may act as sites for the establishment of latency, given their role as HCMV cellular reservoirs which are responsible for the dissemination of the virus^{8,22–24}. Finally, in breast carcinomas and glioblastomas, tumor-associated macrophages (TAM) are a marker of poor prognosis, and their development might be influenced by macrophage-tropic HCMV strains^{25–28}. Thus the quest of new HCMV isolates which target monocytes/macrophages and thereby might play a role in oncogenesis has shown increased interest.

In the present study, we found that the genomic sequence of the HCMV-DB strain isolated from a cervical swab specimen is close to the sequence of other primary clinical isolates, especially the Toledo strain, originally isolated in the urine of a child presenting a congenital infection by HCMV, and the JP strain, isolated from a glandular tissue, the prostate^{21,29}. Based on the analysis of genes involved in virus entry, our results indicate that the HCMV-DB strain is close to the Merlin strain. Given the scarcity of evidence for a direct role of HCMV in the cellular transformation of epithelial cells, we studied the transcriptome profile of HCMV-DB infected human mammary epithelial cells (HMECs). We observed that the transcriptome of HCMV-DB infected HMECs displays a triple negative ER[−]/PGR[−]/HER2[−] phenotype, presents some oncogenic traits, favors cell cycling and cell proliferation, and modulate angiogenesis and proteolysis. All these phenomena are potentially involved in tumor development. Finally, the infection of HMECs with the HCMV strain DB resulted in enhanced proliferation and tumorsphere formation *in vitro*.

Results

Genomic profile of the HCMV-DB strain. We previously isolated a novel HCMV strain, that we characterized and named HCMV-DB⁸. We compared its genomic sequence to that of ten HCMV strains, including two laboratory adapted strains (AD169, Towne) and eight clinical isolates with low passages in culture (Merlin, Toledo, TR, PH, VR1814, Davis, JP, TB40/E)^{30,31}. The complete genomic sequence of HCMV-DB has a total length of 235,512 bp (Fig. 1 and Table 1). In comparison the sequence of the Merlin strain has approximately the same size (235,645 bp), the laboratory adapted strain AD169 has the shortest sequence (229,354 bp) and the clinical strain TB40/E the longest sequence (237,683 bp) (Fig. 1 and Table 1). Clinical isolates with low passages in culture have a total length ranging from 229,700 bp for PH to 237,683 bp for TB40/E (Fig. 1 and Table 1).

Based on the sequence similarity between HCMV strains, the highest total scores by Blast were measured for JP and Toledo strains when compared to the DB strain (Table 1). The DB strain shares around 99% identity with JP and Toledo strains, by linear full genome alignment (Table 1). The other clinical isolates analyzed share 98% identity with the DB strain (Table 1). HCMV-DB strain is mutated in RL13 and UL9 genes, but not in the ULB'

HCMV strain	Clinical	Geographical	DNA	Passages	Nucleotide length	Genes Mutated	Max. score (e + 05)	Total score (e + 05)	Query cover	Identity	Accession
DB	Pregnant women	France	Clinical material on macrophage cultures	low passage on fibroblasts	235512	RL13, UL9			100	100	KT959235
Towne	Urine from a congenitally infected infant	USA	Fibroblast culture cells	many	235147	many: RL13, UL1, UL40, UL130, US1	1.316	3.929	95	99	FJ616285
AD169	Adenoid tissue	USA	Fibroblast culture	many	229354		1.238	4.036	92	98	XI7403.1
DAVIS	Liver biopsy from a congenitally infected infant	USA	Fibroblast culture	many	229768	RL5A, RL12, RL13, UL1, UL2, UL4, UL5, UL6, UL99, UL130	1.29	3.936	95	98	JX512198.1
JP	Post mortem prostate tissue from an AIDS patient	UK	Clinical material	low passage on fibroblasts	236375		2.838	4.129	98	99	GQ221975.1
Merlin	Urine from a congenitally infected infant	UK	Clinical material	low passage on fibroblasts	235646	RL13	2.841	4.034	97	98	AY446894
PH	Bone marrow transplant recipient	USA	Clinical material	low passage on fibroblasts	229700		1.545	3.856	95	98	AC146904
TB40/E	Throat wash of a bone marrow transplant recipient	Germany	Fibroblast culture	Few	237683	RL13, UL128, IRS1, US1, US2	1.555	3.962	96	98	KF297339
Toledo	Urine from a congenitally infected infant	USA	Fibroblast culture	several	235404	RL13, UL9, UL128	1.548	4.171	99	98	GU937742
TR	Vitreous humor from eye of HIV-positive male	USA	Fibroblasts	several	235681		1.539	3.955	96	98	KF021605.1
VR1814 (FIX)	Cervical secretions of a pregnant woman with a primary HCMV infection	Italy	Clinical material	low passage on fibroblasts	235233		1.564	3.995	97	98	GU179289

Table 1. Genomic comparison of HCMV-DB with other HCMV strains.

region (Table 1). Altogether our analyses indicate that the genomic sequence of HCMV-DB is highly similar to the genomic sequences of the clinical strains JP and Toledo.

Phylogenetic classification of the HCMV-DB strain based on genes involved in virus entry.

Since HCMV-DB is a highly macrophage-tropic strain⁸, we performed a phylogenetic analysis to compare the genes coding for the viral envelop that are involved in tropism (Fig. 2, Suppl. Table 1)^{32–34}. For entry into fibroblasts, HCMV only requires the minimal complex of gB:gH/gL^{32,35}. For entry into monocytes, macrophages, epithelial cells, and endothelial cells, HCMV requires, in addition to gB, a pentameric complex formed by glycoproteins gH and gL and proteins pUL128, pUL130 and pUL131^{7,36–41}. We observed that the gB:gH/gL sequences from DB are close to those of VR1814, JP and Merlin, respectively (Fig. 2A). The HCMV-DB pentameric complex was close to that of JP for gH, and to that of Merlin for gL, UL128, UL130 and UL131 (Fig. 2A,B). For gB genotype, the DB strain is close to the VR1814 strain (Fig. 2A). Taking into account the sequences of the genes involved in virus entry, altogether our phylogenetic analysis indicates that the HCMV-DB strain is close to the Merlin strain. The UL144 gene, situated in the ULB' region of the viral genome, is commonly used for genotyping HCMV and we previously reported that HCMV-DB belongs to the UL144 genotype C²⁹.

HCMV-DB infects HMECs *in vitro*. To determine whether HMECs are permissive to HCMV-DB, HMECs were infected with HCMV-DB. We harvested the supernatants of infected HMECs 17 days post infection and added it to MRC5 cells. We observed a cytopathic effect (CPE) typical of HCMV in MRC5 cultures infected with these supernatants (Fig. 3A). We did not detect any CPE in HMECs directly infected with Epstein-Barr virus (EBV) and varicella zoster virus (VZV) (Fig. 3B). In contrast, direct infection of HMECs with herpes simplex virus type 1 (HSV-1) resulted in the appearance of clusters of round refringent cells (Fig. 3B). To further confirm the direct infection of HMECs by HCMV-DB we detected the early protein pUS28 using western blotting in HCMV-DB infected HMECs after 2 hours and up to 5 days post infection (Fig. 3C). Our results confirm our previously published data that indicate a full replicative viral cycle of HCMV-DB in HMECs²⁹.

The transcriptome of HCMV-DB infected HMECs displays a triple negative basal-like phenotype.

Uninfected HMECs and HCMV-DB-infected HMECs at low and high MOIs (1 or 10) were used one day after infection for the screening with oncogenes/tumor suppressor genes and human breast cancer genes RT² profiler PCR assays (PAHS-502Z and PAHS-131Z, respectively). Positive controls included MCF-7 and MDA-MB231

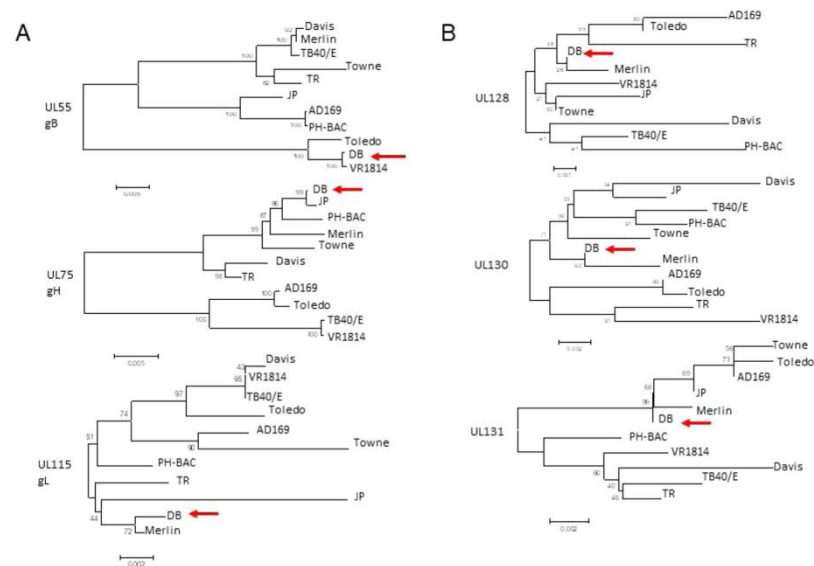


Figure 2. Phylogenetic analyses comparing HCMV-DB with several HCMV strains for genes involved in viral entry. (A) Phylogenetic analysis of genes coding for viral glycoproteins required for the entry into cells: UL55 (gB), UL75 (gH), and UL115 (gL). (B) Phylogenetic analysis of genes coding for UL128, UL130, and UL131 required in addition to the envelop glycoproteins for the viral entry into monocytes, macrophages, epithelial cells, and endothelial cells.

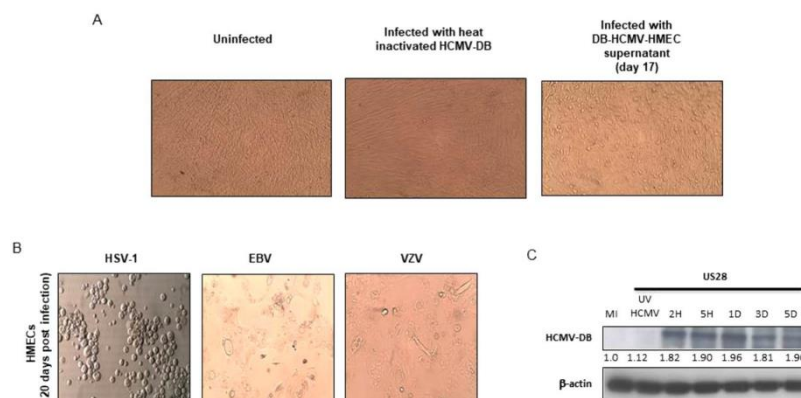


Figure 3. HCMV-DB infects HMECs. (A) HCMV cytopathic effect (CPE) in MRC5 cultures infected with supernatants harvested from HCMV-DB infected HMECs. Heat inactivated HCMV-DB is used as a negative control. (B) Clusters of round cells in HMECs infected with HSV-1. No CPE detected with the direct infection of HMECs with EBV and VZV. (C) Western blotting showing the time-course expression of pUS28 in HCMV-DB infected HMECs.

breast cancer cell lines as luminal and basal-like phenotypes, respectively. Each of the RT² Profiler PCR arrays profiles the expression of 84 key genes -which overlap sometimes in the two assays- commonly involved in tumor classification, signal transduction, and other commonly affected pathways such as angiogenesis, adhesion, proteolysis, cell cycle, and apoptosis (Suppl. Tables 2 and 3).

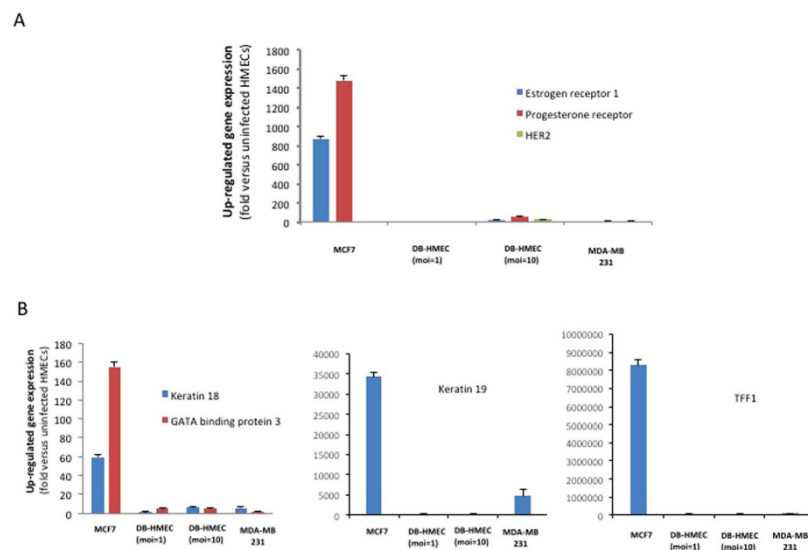


Figure 4. Transcriptome analysis of HMECs infected with HCMV-DB displays a triple negative basal-like phenotype. (A) HMECs infected with HCMV-DB and MDA-MB-231 cells show a similar pattern of ER[−]/PGR[−]/HER2[−] transcripts, in contrast to the ER⁺/PGR⁺/HER2⁺ transcripts detected in MCF-7 cells. (B) Low levels of gene expression of luminal markers (KRT18, KRT19, GATA3, TFF1) in both HCMV-DB infected HMECs and MDA-MB-231 cells as compared to the luminal MCF-7 cells.

In regard to the expression of ER/PGR/HER2 transcripts, HCMV-DB infected HMECs and MDA-MB231 cells showed a similar transcriptome that was clearly distinct from the transcriptome of MCF-7 cells (Fig. 4A). Although the triple negative ER[−]/PGR[−]/HER2[−] phenotype was present in both infected and uninfected HMECs, the levels of estrogen receptor (ESR1), progesteron receptor (PGR) and HER2 (ERBB2) transcripts were higher in HCMV-DB infected HMECs in comparison to uninfected HMECs (Fig. 4A). The gene expression of luminal markers (KRT19, KRT18, GATA3, TFF1) was low in HCMV-DB-infected HMECs, similar to the phenotype of MDA-MB231 cells (Fig. 4B, Suppl. Tables 2 and 3). In contrast, MCF-7 cells expressed high levels of luminal markers transcripts (Fig. 4B).

The transcriptome of HMECs infected with HCMV-DB presents oncogenic traits with enhanced cellular proliferation.

The gene expression of the oncogenes Myc (MYC), Fos (FOS), Jun (JUN), KRas (KRAS), HRas (HRAS) and NRas (NRAS) is upregulated in HCMV-DB-infected HMECs in comparison to uninfected HMECs (Fig. 5A). We also observed the upregulation of transcripts of numerous other oncogenes (KITLG, MCL1, MET, MYB, NFKB1A, PIK3CA, PML, PRKCA, RAF1, RARA, ROS1, RET, ABL1, ETS1, RUNX1, RUNX3) (Suppl. Tables 2 and 3). The gene expression of the tumor suppressor genes coding for the retinoblastoma protein (RB1) and the p53 protein (TP53) was upregulated in HCMV-DB-infected HMECs, as reported previously for basal-like triple negative cell lines such as MDA-MB231 cells (Fig. 5B and Suppl. Table 3)^{42,43}. Similarly, the expression of several other tumor suppressor genes (TP73, FHIT, VHL, SMAD4, TGFB1, STK11, TSC1) is also upregulated in HCMV-DB-infected HMECs, mostly at low moi (moi = 1) compared to uninfected HMECs (Fig. 5B).

The IL-6/JAK-STAT3/Cyclin D1 axis is activated in biopsies from breast cancer patients⁴⁴. We observed the upregulation of IL-6, IL-6 receptor and JAK2 gene expression in HCMV-DB infected HMECs compared to uninfected HMECs especially at high moi (moi = 10), with stable levels of STAT3 gene expression (Fig. 5C). The gene expression of cyclin dependent kinase 4 (CDK4) and of several cyclins (CCNA1, CCNA2, CCND1, CCNE1) especially cyclin D1 which function as regulator of CDKs is up-regulated in HCMV-DB-infected HMECs (moi = 10) compared to uninfected controls (Fig. 5D, left panel). The gene expression of CDK inhibitors (CDKN) 1A (p21), 2A (p16), 2B (p15), and 3 (CDKN1A, CDKN2A, CDKN2B, CDKN3) is up-regulated by 5 folds in HCMV-DB-infected HMECs compared to uninfected cells (Fig. 5D, right panel).

In agreement with activation of the IL6/JAK/cyclin D1 pathway in HCMV-DB-infected HMECs, we observed the upregulation expression of proliferation marker genes such as the Ki67 antigen (MKI67) and the topoisomerase 2 gene (TOPO2A) when compared to uninfected HMECs (Fig. 5E). The expression of genes involved in cell survival (NFKB1, REL, AKT1, PIK3C2A, BCL-2) is increased in HCMV-DB-infected HMECs compared to uninfected HMECs, indicating a prosurvival signal in infected cells (Fig. 5F). In agreement with the induction

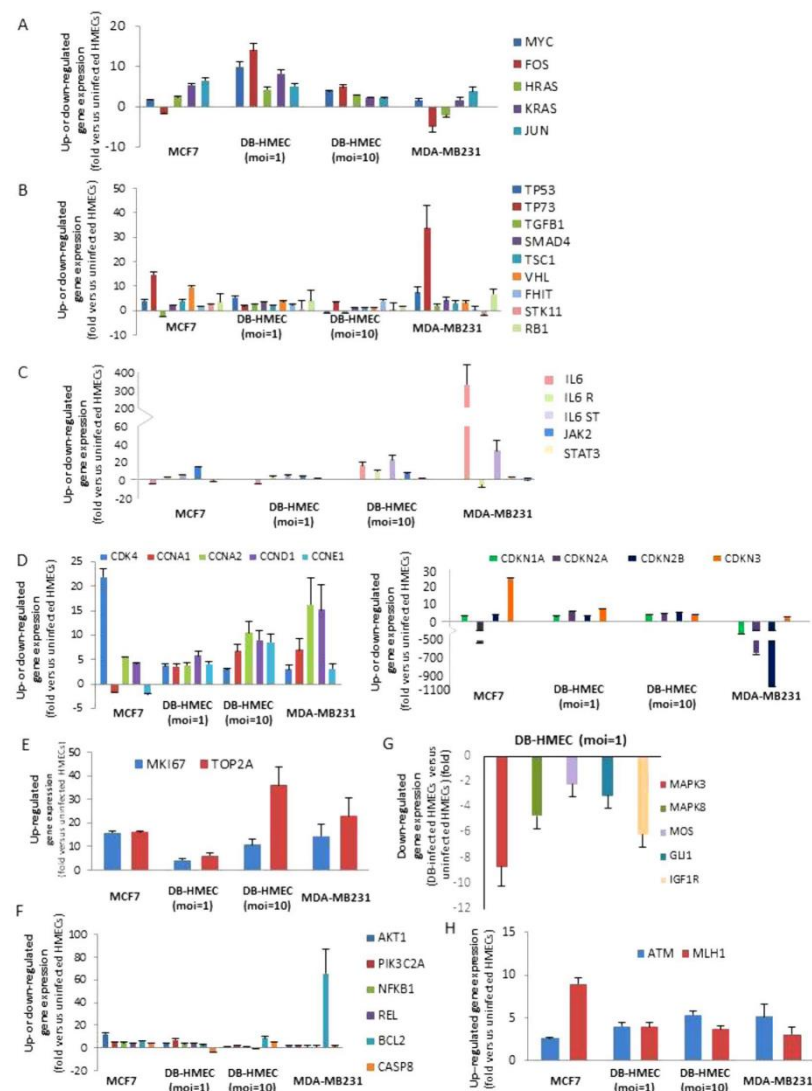


Figure 5. The transcriptome of HMECs infected with HCMV-DB displays oncogenic traits. (A) Upregulation of the gene expression of oncogenes: Myc (MYC), Fos (FOS), Jun (JUN), KRas (KRAS), HRas (HRAS) and NRas (NRAS). (B) Upregulation of the gene expression of tumor suppressor genes: TP53, TP73, FHIT, VHL, SMAD4, TGFB1, STK11, TSC1, RB1. (C) Expression of genes from the IL-6/JAK-STAT3 axis. (D) *Left panel*, Upregulation of genes coding for the cyclins: CCNA1, CCNA2, CCND1, CCNE1 and the cyclin dependent kinase 4 CDK4. *Right panel*, Upregulation of genes coding for the cyclin dependent kinase inhibitors: CDKN 1 A, CDKN 2 A, CDKN 2 B and CDKN 3. (E) Upregulation of the expression of genes coding for proliferation markers, the Ki67 antigen (MKI67) and the topoisomerase 2 (TOPO2A). (F) Upregulation of the expression of genes involved in cell survival (NFKB1, REL, AKT1, PIK3C2A, BCL-2) and downregulation of the expression of caspase 8 (CASP8). (G) Downregulation of gene expression of the members of the MAPK cascade (MAPK3, MAPK8, MOS, GLI1, IGF1R). (H) Upregulation of the expression of genes involved in DNA reparation: the ataxia telangiectasia mutated (ATM) and human MutL homolog (MLH1). The up- and down-regulation were measured in HMECs infected with HCMV-DB (moi, 1 and 10) as compared to uninfected HMECs.

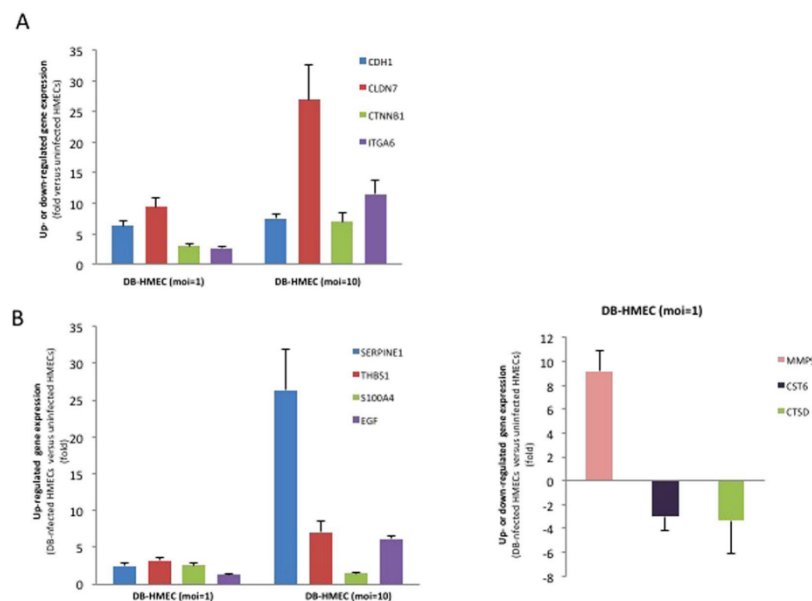


Figure 6. Modification of the transcriptome of genes involved in cell adhesion, angiogenesis and proteolysis in HMECs infected with HCMV-DB. (A) Upregulated gene expression of E-cadherin (CDH1), claudin 7 (CLDN7), beta-catenin (CTNNB1), alpha 6 integrin (ITGA6); (B) Upregulated expression of genes involved in angiogenesis (SERPINE1, THBS1, S100A4, and EGF) and proteolysis (MMP9), and downregulated expression of genes involved in proteolysis: CST6 and CTSD. The up- and down-regulation were measured in HMECs infected with HCMV-DB (moi, 1 and 10) as compared to uninfected HMECs.

of several prosurvival genes in infected cells compared to uninfected controls, the down-regulation of caspase 8 transcript (CASP8) is observed in HCMV-DB-infected HMECs at moi 1 compared to uninfected cells (Fig. 5F).

The transcriptome of HCMV-DB infected HMECs displays modifications in cell signaling, angiogenesis and proteolysis. The gene expression of the members of the MAPK cascade (MAPK3, MAPK8, MOS, GLI1, IGF1R) is down-regulated in HCMV-DB-infected HMECs (Fig. 5G). The gene expression for the transcription factors, tumor suppressor gene hypermethylated in cancer 1 (HIC1) and forkhead box D3 (FOXO3) is up-regulated in HCMV-DB-infected HMECs at high moi (Suppl. Figure 1). In HCMV-DB-infected HMECs, we observed the upregulation of the expression of the ataxia telangiectasia mutated (ATM) and human MutL homolog (MLH1) genes, both involved in DNA repair (Fig. 5H).

The gene expression of E-cadherin (CDH1), claudin 7 (CLDN7), beta-catenin (CTNNB1) and alpha 6 integrin (ITGA6) is upregulated in HCMV-DB-infected HMECs at high moi (moi = 10) (Fig. 6A). In HCMV-DB-infected HMECs, we observed both the up- and downregulation of genes involved in angiogenesis (upregulation: IL-6, SERPINE1, THBS1, S100A4, EGF; downregulation: SLIT2) and proteolysis (upregulation: MMP9; downregulation: CST6, CTSD) (Fig. 6B, Suppl. Table 3).

The transcriptome of HMECs infected with HCMV-DB reveals a global hypomethylation state.

Since epigenetic regulation of chromatin has been associated with breast cancer classification and prognosis, we performed a Human Epigenetic Chromatin Modification Enzymes RT² Profiler PCR Array (PAHS-085A) which profiles the expression of 84 key genes encoding enzymes known or predicted to regulate chromatin accessibility, and therefore gene expression, by modifying genomic DNA and histones (Suppl. Tables 4 and 5). Considering a standard two-fold increase or decrease of RNA expression between HCMV-DB-infected HMECs and uninfected HMECs to be biologically significant, performed analysis indicated the down-regulation of most (n = 50) of the genes including DNA methyltransferase genes (DNMT3A, DNMT3B), histone methyltransferase genes (PRMT1, PRMT2, PRMT8, SMYD3, SUV39H1), genes coding for SET domain proteins with a histone methyltransferase activity (ASH1L, MLL3 (KMT2C), MLL5 (KMT2E), NSD1, SETD1A, SETDB1, SETDB2, SETD2, SETD3, SETD5, SETD6, SETD7, SETD8, SUV420H1, WHSC1), histone acetyltransferase genes (ATF2, CIITA, CSRP2BP, KAT2A, KAT6B, NCOA1), histone kinase genes (AURKA, AURKB, AURKC, NEK6, RPS6KA3), genes involved in histone ubiquitination (MYSM1, RNF2, RNF20, UBE2A, UBE2B, USP21, USP22), DNA/histone demethylase genes (KDM1A, KDM5C, KDM4C, KDM6B) and histone deacetylase genes (HDAC2, HDAC4,

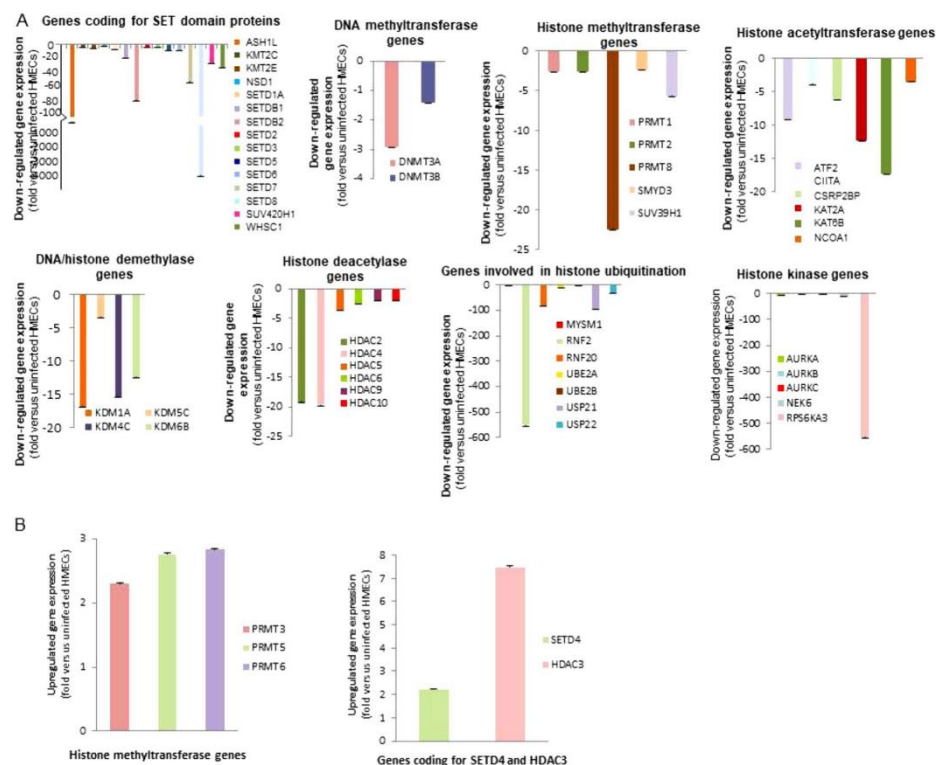


Figure 7. A general hypomethylation state was observed in HMECs infected with HCMV-DB. (A) Downregulation of the gene expression of DNA methyltransferase genes (DNMT3A), histone methyltransferase genes (PRMT1, PRMT2, PRMT8, SMYD3, SUV39H1), genes coding for SET domain proteins with a histone methyltransferase activity (ASH1L, MLL3 (KMT2C), MLL5 (KMT2E), NSD1, SETD1A, SETDB1, SETDB2, SETD2, SETD3, SETD5, SETD6, SETD7, SETD8, SUV420H1, WHSC1), histone acetyltransferase genes (ATF2, CIITA, CSRP2BP, KAT2A, KAT6B, NCOA1), histone kinase genes (AURKA, AURKB, AURKC, NEK6, RPS6KA3), genes involved in histone ubiquitination (MYSM1, RNF2, RNF20, UBE2A, UBE2B, USP21, USP22), DNA/histone demethylase genes (KDM1A, KDM5C, KDM4C, KDM6B) and histone deacetylase genes (HDAC2, HDAC4, HDAC5, HDAC6, HDAC9, HDAC10). (B) Upregulation of the gene expression of histone methyltransferase genes (PRMT3, PRMT5, PRMT6), genes coding for SET domain proteins with a histone methyltransferase activity (SETD4) and HDAC3. The up- and down-regulation were measured in HMECs infected with HCMV-DB (moi, 1 and 10) as compared to uninfected HMECs.

HDAC5, HDAC6, HDAC9, HDAC10) (Fig. 7A, Suppl. Table 4), and the upregulation of few genes ($n = 5$) including histone methyltransferase genes (PRMT3, PRMT5, PRMT6), a gene coding for SET domain proteins with a histone methyltransferase activity (SETD4) and the histone deacetylase HDAC3 (Fig. 7B, Suppl. Table 4). Altogether our results indicate an overall down-regulation of transcripts involved in methylation of DNA and histones in HCMV-DB-infected HMECs. In addition, transcripts of histone acetyltransferase and deacetylase genes are mostly downregulated in HCMV-DB-infected HMECs compared to uninfected controls.

Formation of tumorspheres from HMECs infected with HCMV-DB. Since it was previously showed that the activation of the IL-6/STAT3 axis signaling in cancer stem cells (CSC) can enhance proliferation and survival of cells and in turn favor the growth of tumors in mice, we decided to detect the presence of CSC in HCMV-DB-infected HMECs using a tumorsphere (mammosphere) formation assay^{45,46}. To determine whether the induction of CSC expansion could be the result of the HCMV-DB infection of HMECs, we infected them for 1 day and evaluated the proportion of stem-like cells by sphere formation assay. We found that HCMV-DB infected cultures formed tumorspheres (Fig. 8A,B). As expected MCF-7 cells and MDA-MB231 cells formed tumorspheres (Fig. 8A). In contrast, the use of UV-inactivated HCMV-DB to infect HMECs did not result in tumorspheres formation in cultures, neither did the use of uninfected HMECs (Fig. 8A,B).

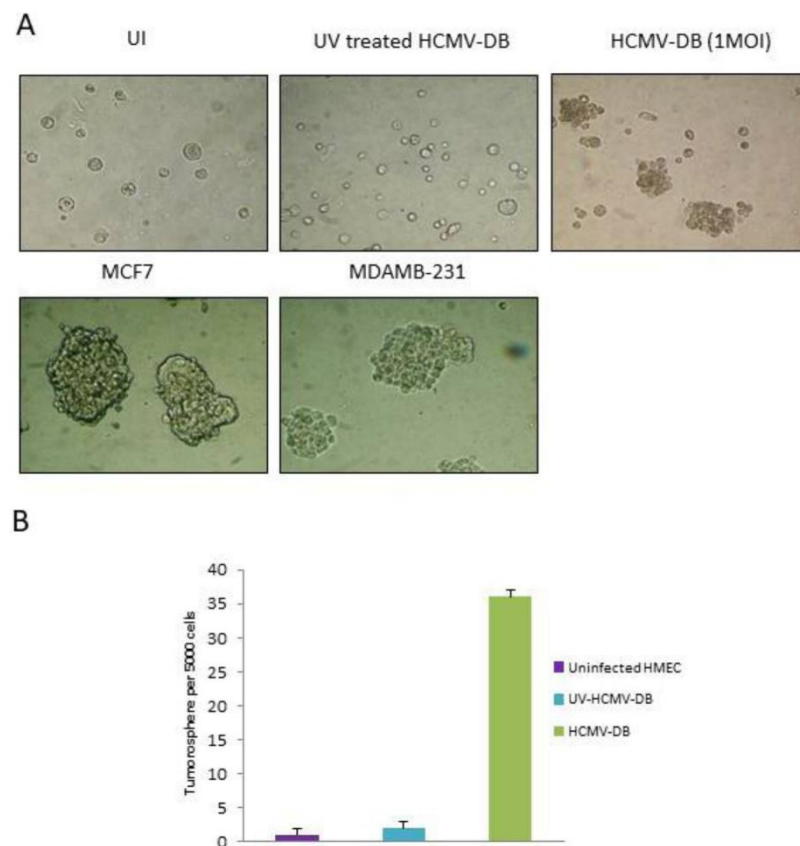


Figure 8. HMECs infected with HCMV-DB show tumorspheres formation. (A) Tumorsphere formation in HMECs infected with HCMV-DB as compared to uninfected HMECs (UI) and HMECs infected with UV-treated HCMV-DB. MCF-7 and MDA-MB-231 cells are used as positive controls. (B) The histogram represents quantification of tumorsphere formation in the cultures of HMECs infected with HCMV-DB. Uninfected HMECs and HMECs infected with UV-inactivated HCMV-DB were used as negative controls. Results are means (\pm SD) of three independent experiments.

Discussion

Our results indicate that, based on its genomic profile, the HCMV-DB strain is similar to clinical HCMV strains especially the JP and Toledo strains. Based on phylogenetic analysis of genes involved in virus entry, the HCMV-DB strain is close to the Merlin strain. In addition, we observed that HCMV-DB infects HMECs which display a triple negative phenotype. The transcriptome of HCMV-DB-infected HMECs shows the characteristics of a pro-oncogenic cellular environment with upregulated expression of numerous oncogenes, enhanced activation of pro-survival genes, and upregulated markers of cell proliferation, stemcellness and EMT that was confirmed by enhanced tumorsphere formation *in vitro*.

HCMV clinical isolates can infect among others epithelial cells, endothelial cells, monocytes, macrophages, fibroblasts, stromal cells, hepatocytes, smooth muscle cells, and neural stem/progenitor cells. In opposition to this broad cellular tropism, HCMV laboratory strains can only grow on fibroblasts⁷. Some clinical HCMV isolates infect the blood monocytes and tissue macrophages, induce a distinct macrophage polarization and establish latency in monocytes/macrophages^{8,22,24,47}. The tropism of HCMV strains could have a major role in M1/M2 macrophage activation and enhance the viral fitness, ultimately favoring breast cancer promotion^{26,48,49}. Previously, we isolated the HCMV-DB strain (KT959235)⁸. The HCMV-DB strain, given its characterization as highly macrophage-tropic, induces an M2 polarization of the macrophages and the upregulation of the proto-oncogene Bcl-3⁸. The ULb' region of the HCMV-DB strain is intact²⁹ and HCMV-DB show genetic similarities to other primary clinical isolates such as the JP strain which has been isolated post mortem from the prostate of an

HIV-infected patient^{30,31}. Interestingly, DB and JP strains infect mammary and prostate glands respectively which show numerous similarities with regard to appearance, physiology and pathology³⁰.

The HMECs infected with HCMV-DB exhibit a triple negative ER-/PR-/HER2- phenotype with low expression of luminal markers (KRT19, KRT18, GATA3, TFF1). Oncogenic conversion of HMECs requires a limited number of changes leading towards growth deregulation⁵¹. These changes include the inactivation of tumor suppressor pathways, mainly p53 and retinoblastoma protein (Rb)⁵², the establishment of telomere maintenance^{53,54}, the activation of the Akt pathway and activation of mitogenic signal delivered by oncogenes such as Myc and Ras^{51,55,56}. We observed the upregulation of Rb and p53 transcripts in HCMV-DB-infected HMECs. Increased p53 transcript and protein expressions have been reported in fibroblasts infected with several HCMV strains⁵⁷. The functional inactivation of p53 required for cell transformation, could be explained by the previously reported potential inhibition of the transcriptional activity of p53 by HCMV-IE2-86⁵⁸. In agreement with the upregulation of the p53 transcript, we previously reported that, in HCMV-DB infected HMECs, the p53 protein expression was enhanced, as well as its binding to IE2 and therefore could explain its functional inactivation²⁹. The gene expression of retinoblastoma protein (pRb) is 7 fold higher in HCMV-DB-infected HMECs in comparison to uninfected HMECs at day 1 post infection. We also observed the upregulation of the Rb transcript in MDA-MB231 cells in agreement with the upregulation of the pRb protein in MDA-MB231 cells reported previously⁴². Although the expression of the gene Rb is increased in HCMV-DB-infected HMECs, the post-translational phosphorylation of pRb has been reported to play a critical role in its inactivation⁵⁶. The HCMV UL97 protein (pUL97) exhibits similar activities to that of cellular cyclin-dependant kinase (CDK) complexes; thus it phosphorylates and inactivates the pRb protein. The pUL97 protein lacks the amino acid residues conserved in CDKs that allow for the attenuation of kinase activity, and is not inhibited by the CDK inhibitor p21. Therefore the activity of pUL97 is immune from normal CDK control mechanisms^{59,60}. Also the human cytomegalovirus pp71, encoded by UL82, has a role in the degradation of the retinoblastoma family of proteins⁶¹. In agreement with such an hypothesis, we reported previously the upregulation of UL82 transcript and protein (pp71) at day 1 and 3 post infection in HMECs infected with HCMV-DB²⁹. Thus, in HCMV-DB infected HMECs, both p53 and pRb proteins could be inactivated, most likely blocking cellular senescence, and therefore promoting unchecked cell division⁵¹. We observed, in infected cells, the activation of IL-6/STAT3 axis with expression of the pUS28 protein, which is in agreement with other studies that confirm the role of HCMV pUS28 in mediating proliferation through this axis activation^{62,63}.

The acquisition of a mitogenic signal, delivered by proto-oncogenes including c-Myc and Ras, could further allow the complete transformation of HCMV-DB-infected HMECs *in vitro*. In agreement with the enhanced expression of Myc and Ras transcripts in HCMV-DB-infected HMECs, we reported previously enhanced expression of Myc and Ras proteins starting at day 1 postinfection as measured by western blotting²⁹. Overexpression of other oncogene transcripts such as Fos and Jun could also participate in HMEC transformation induced by HCMV-DB^{64,65}. Interestingly, the *Myc* region on chromosome 8q24.21 is a known site of frequent human papillomavirus (HPV) integration and *Myc* is overexpressed in cervical carcinoma^{55,66,67}. In agreement with the upregulation of the *Myc* gene expression in HMECs infected with HCMV-DB, the activation of Fos and Myc is induced by the IE1 and IE2 proteins of HCMV⁶⁸. In addition, *MYB* gene is induced in HCMV-DB-infected HMECs similar to enhanced *MYB* gene expression in HPV-induced carcinoma⁶⁹. Altogether, the upregulation of oncogenes observed in HCMV-DB-infected HMECs is in part similar to the transcriptomic profile observed in HPV-induced carcinoma.

The PI3K/Akt pathway is activated in breast cancer and its activation is critical for the emergence of anchorage-independent growth, eventually leading to the transformation of HMECs^{70,71}. We observed enhanced expression of genes of the PI3K/Akt pathway, in agreement with phosphorylation of Akt in HCMV-DB-infected HMECs²⁹. In addition increased levels of transcripts of pro-survival genes such as Bcl-2, Akt, and NF- κ B was observed parallel to decreased levels of the caspase-8 transcript in HCMV-DB-infected HMECs at low moi (moi = 1). By contrast, at high moi (moi = 10), we observed increased levels of the caspase-8 transcript in HMECs infected with HCMV-DB. This apparent discrepancy between low and high moi, could be explained as follows. At early stages of the infection after viral entry, only a limited number of virions are present within the infected cell. At this initial step of the viral life cycle anti-apoptotic mechanisms have to be activated to favor cell survival and to fuel viral replication within the infected cell. Thus it is critical that early after infection (low moi) the infected cell survives to favor the completion of the viral life cycle and to allow the production of a maximum of new progeny virions. Later during infection, when numerous virions have been produced within the infected cells (high moi), the pro-apoptotic signals e.g. enhanced caspase-8 gene expression are activated and result in the lysis of the infected cells with release of the newly produced virions. Altogether, these results indicate that the transcriptome of HCMV-DB-infected HMECs displays a flexible adaptation to the viral life cycle and can be modulated differentially depending on the intracellular viral load and/or the amount of viral input.

We observed the upregulation of gene expression of proliferation markers such as the Ki67 antigen, the topoisomerase 2 and the transcription factor E2F1 in HCMV-DB-infected HMECs compared to uninfected HMECs. Our results are in agreement with the increased proliferation previously observed in cultures of HCMV-DB-infected HMECs²⁹. Parallel to enhanced cellular proliferation in HCMV-DB-infected HMECs, we noted the dysregulation of the cell cycling. In HCMV-DB-infected HMECs, we observed enhanced expression of cyclins genes (cyclin A1, D1, D2, E1) including cyclin D1, but also to a lesser extent of cell cycling inhibitors (CDKNs). In agreement with the induction of CDKN2A gene expression in HMECs infected with HCMV-DB, CDKN2A gene is strongly induced in HPV infection and is widely used as a surrogate marker for HPV carcinoma^{69,72}. Although we observed enhanced proliferation of HCMV-DB-infected HMECs, we also noticed a dysregulation of cell cycling which could lead to the inhibition of the cell cycle G1 progression and to the block of G1/S transition, as previously reported by other groups in fibroblasts infected with various HCMV strains⁷³. In agreement with an inhibition of the cell cycle proliferation in HMECs infected with HCMV-DB, the Ras

association domain-containing protein 1 (RASSF1) is upregulated in infected cells and was reported to inhibit the accumulation of cyclin D1 (Suppl. Table 3). An exquisite balance between enhanced cell cycling (enhanced cyclins gene expression) and enhanced cell cycling inhibition (enhanced CDKN gene expression), might ultimately result in enhanced cell proliferation as measured by increased Ki67Ag and TOP2A transcripts and enhanced Ki67 antigen detection in HMECs infected with HCMV-DB.

Decreased MAPK activation has been reported as pejorative in triple negative breast cancers⁷⁴. In agreement with this observation, decreased gene expression of members of the MAPK cascades (MAPK3, MAPK8) is observed in HCMV-DB-infected HMECs which exhibit a ER-/PR-/HER2- phenotype.

Several herpesviruses have been reported to lead to genome instability with activation of the DNA reparation pathway with increased expression of the *ATM* gene^{75–77}. We found that the *ATM* gene expression is upregulated in HCMV-DB-infected HMECs as reported previously in fibroblasts infected with other HCMV strains⁷⁷. Enhanced ATM expression could favor the reparation of DNA damages such as chromosomal breaks already reported in fibroblasts infected with HCMV^{78,79}, but could also lead to genome instability and thereby favor cellular transformation⁸⁰.

Several studies, including a comprehensive study by The Cancer Genome Atlas (TCGA) Network, have shown that, in opposition to luminal estrogen receptor-positive cancers, which exhibit the highest degree of hypermethylation, the triple negative breast cancers (TNBCs) are characterized by the most extensive hypomethylation^{81,82}. The expression of numerous histone methyltransferase genes and of genes coding for SET domain proteins with a histone methyltransferase activity is downregulated in HCMV-DB infected HMECs in comparison to uninfected HMECs. The gene expression of DNA methyltransferase 3A (DNMT3A) is also downregulated in infected HMECs.

Regarding mammary tumor progression, Suzuki *et al.*⁸³ reported that in the tumor progression from normal mammary epithelium to ductal carcinoma *in situ* (DCIS), there is a reduction of the histone acetylation levels. This change in histone acetylation was not present when comparing DCIS to invasive ductal carcinoma. A significant decrease in histone deacetylase (HDAC) protein levels during tumor progression was also described⁸⁴. This suggests an early role for histone acetylation modifications in breast tumor progression. We observed decreased expression of genes involved in histone acetylation (histone acetyl transferases) in HCMV-DB infected HMECs in comparison to uninfected HMECs. In addition, we observed the decreased gene expression of several histone deacetylases, including HDAC2, 4, 5, 6, 9 and 10 in HCMV-DB-infected HMECs in comparison to uninfected HMECs. Altogether, our data indicate that HCMV-DB-infected HMECs at day 1 post infection display already alterations of histone acetylation which have been evoked as an early event in breast tumor progression.

The transcriptomic analysis of HCMV-DB-infected HMECs indicates that genes involved in angiogenesis and proteolysis, and thereby potentially participating in the remodelling of the tumor microenvironment, are modulated in HCMV-DB-infected HMECs^{85,86}. We also observed the upregulation of IL-6-JAK gene expression in HCMV-DB-infected HMECs compared to uninfected HMECs which has been reported to play a role in the appearance of tumorspheres in cultures of hepatocytes infected with HCMV¹⁰. In addition, the gene expression of alpha 6 integrin (ITGA6), a marker of stemcellness, is upregulated in HCMV-DB-infected HMECs. Finally in HCMV-DB-infected HMECs, the proto-oncogene tyrosine-protein kinase Src⁸⁷, a gene involved in EMT, is upregulated compared to uninfected HMECs. Altogether our results indicate a potential trend towards the appearance of cancer stem cells (CSCs) in HMECs infected with HCMV-DB. CSCs are required for sustained tumor growth, invasiveness and metastasis formation⁸⁸. The expansion of CSCs can be measured by the formation of tumorspheres. In agreement with the activation of IL-6-JAK pathway, upregulation of alpha 6 integrin and src gene expression, we observed tumorsphere formation in cultures of HCMV-DB-infected HMECs.

In conclusion, we define here the genomic profile of a newly isolated clinical HCMV strain, namely HCMV-DB, and its close phylogenetic links with other HCMV strains, especially the JP, Toledo and Merlin strains. Further, we confirmed that the HCMV-DB strain infects HMECs which display a triple negative phenotype. Our data indicate that the transcriptome of HCMV-DB-infected HMECs presents traits of a pro-oncogenic cellular environment with upregulated expression of numerous oncogenes, enhanced activation of pro-survival genes, and upregulated markers of cell proliferation, stemcellness and EMT that was confirmed by enhanced tumorsphere formation *in vitro*.

Materials and Methods

Reagents. Anti-US28 and anti β -actin antibodies were respectively acquired from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich (St. Louis, MO).

Cell cultures. HMECs were purchased from Life Technologies (Carlsbad, CA, USA). MDA-MB231 and MCF-7 cells were provided by Institut Hiscia (Arlesheim, Switzerland). Cell culture and cell viability assay was performed as previously described⁸. Cultures were free of mycoplasma.

Infection of HMECs with HCMV. The clinical isolate HCMV-DB was previously isolated in our laboratory⁸. Cell-free virus stocks of HCMV-DB were grown in macrophages, as described previously⁸. Human fibroblasts (MRC5) were cultured as previously described^{6,8}. Cells infection by HCMV, heat-inactivated HCMV, UV-treated HCMV was performed as previously described²⁹. Virus titers were determined by plaque-forming assay in MRC5 as described previously⁸. Viral stocks purity and viral replication were assessed as previously described²⁹. Quantification of viral titer was performed by qPCR on cell culture supernatants as previously described⁸.

Western blotting. pUS28 and β -actin expression was examined by western blotting as described previously⁸, using infected or uninfected HMECs cell lysates.

RT² Profiler™ PCR Arrays. Total RNA was extracted from uninfected HMECs and HMECs infected with HCMV-DB at MOI of either 1 or 10 at day 1 post infection using TRIzol reagent (Life Technologies, Grand Island, NY), and the first strand cDNA synthesis was achieved using the RT² First Strand Kit (SABiosciences, Valencia, CA) following the manufacturer's instructions. Three RT² Profiler™ PCR Arrays for human breast cancer (PAHS-131ZA), oncogenes/tumor suppressor genes (PAHS-502Z) and human epigenetic chromatin modification enzymes (PAHS-085A) (all three from SABiosciences) were applied on the Strategene Mx3005P real-time PCR system (Agilent Technology, Santa Clara, CA) as per manufacturer's instructions. Housekeeping genes, contamination control, reverse transcription control and positive controls were included in each PCR according to manufacturer's instructions. Data analyses were performed using the web based analysis software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

Tumorsphere assay. Tumorsphere formation by uninfected HMECs or by HMECs infected using HCMV-DB or UV-inactivated HCMV-DB, was assayed as described previously¹⁰. MCF-7 and MDA-MB231 were used as positive controls. The number of tumorspheres larger than 60 microns was counted.

Genomic analysis of HCMV-DB. The analysis of HCMV-DB genome and its comparison to other HCMV strains: AD-169 and Towne (laboratory adapted strains) and Merlin, Toledo, TR, PH, VR1814, Davis, JP, and TB40/E (clinical strains with low passages in culture) was done using the NCBI nucleotide blast tool (<https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi>).

Phylogenetic analysis. Phylogenetic proximity was calculated among various HCMV strains (described in Table 1) for UL55, UL75, UL115, UL128, UL130, and UL131 genes. The phylogenetic analysis was performed as previously reported⁸⁹.

Statistical analysis. Numerical values are shown as the means and SD of independent experiments. Mann Whitney U test was performed for statistical significance and differences were considered significant at a value of $P < 0.05$. The plots were prepared using Microsoft Excel.

Availability of data and materials. The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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Author Contributions

F.A.M., A.K., S.P. and M.K.T. performed research; G.H., W.K. and M.D.A. designed research; F.A.M. and G.H. wrote the paper.

Additional Information

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Titre : Analyse moléculaire des cellules épithéliales mammaires humaines infectées par le cytomegalovirus humain

Mots clés : HCMV-DB – cancer du sein- HMEC- Transcriptome- génome

Resumé : Depuis plusieurs années, le rôle joué par le cytomegalovirus humain (HCMV) dans le développement des maladies inflammatoires et du cancer a été étudié par plusieurs groupes de recherche. Divers tissus tumoraux, ont montré la présence d'antigènes ou d'ADN du HCMV. Cette accumulation de preuves de l'implication de l'infection par le HCMV dans les maladies malignes de diverses entités cancéreuses a conduit au développement du concept d'«oncomodulation» effet du HCMV, sans toutefois aucun potentiel de transformation directe. HCMV-DB (KT959235) est un isolat clinique provenant d'un échantillon de col de l'utérus d'une femme enceinte de 30 ans, préalablement isolé dans notre laboratoire. Cette souche virale a montré sa capacité à infecter les macrophages primaires et a montré une réplication productive dans les cellules épithéliales mammaires humaines (HMECs). HCMV-DB a montré son potentiel pour transformer les HMECs primaires par test de formation de colonies sur gélose molle, avec l'émergence d'amas de cellules sphéroïdes, qui ont été désignées cellules CTH (HMECs transformées par le CMV). Nous avons caractérisé le profil génomique de la souche HCMV-DB et nous l'avons comparé à des souches soit cliniques soit de laboratoire. HCMV-DB a été caractérisée comme proche des génomes des souches Toledo et JP, et cette dernière est une souche clinique isolée à partir d'un tissu glandulaire, la prostate.

Nous avons également comparé les gènes impliqués dans l'entrée virale par des analyses phylogénétiques et nous avons observé la proximité de HCMV-DB avec la souche prototypique du HCMV, Merlin. En étudiant le profil transcriptomique des HMECs infectées par HCMV-DB, nous avons trouvé qu'elles présentent un phénotype basal-like triple négatif ER-/PR-/HER2-, ainsi que des caractéristiques oncogéniques, incluant une up-régulation de l'expression de plusieurs oncogènes, de gènes pro-survie (avec down-régulation de la caspase 8), et de marqueurs de la prolifération, du caractère souche des cellules et de la transition épithélio-mésenchymateuse (EMT). Le profil transcriptomique des HMECs infectées par HCMV-DB a également montré des modifications variées dans la signalisation cellulaire, l'angiogenèse et la protéolyse. Au niveau de la chromatine, les HMECs infectées par HCMV-DB ont révélé une hypométhylation globale. En cherchant la présence du génome de HCMV-DB dans les cellules CTH formées, nous avons détecté une signature du génome de HCMV-DB, à savoir le lncRNA4.9. Globalement, nos données ont montré que le transcriptome des HMECs infectées par HCMV-DB révèle clairement des traits pro-oncogéniques et la détection d'une partie du génome de HCMV-DB suggère que cette partie du génome viral peut être responsable de la transformation cellulaire obtenue.

Titre : Molecular Analysis of the Human Mammary Epithelial Cells Infected by the Human Cytomegalovirus

Keywords : HCMV-DB- Breast cancer- HMEC- transcriptome- genome

Abstract : Since several years, the role played by human cytomegalovirus (HCMV) in the development of inflammatory diseases and cancer has been extensively studied and addressed by different research groups. Various tumor tissues have shown to harbor either the antigen or the DNA of HCMV. These growing evidences about the implication of HCMV infection in malignant entities had led to the emergence of the concept of "Oncomodulation" effect of the virus, without any direct transformatory potential. HCMV-DB (KT959235) is a clinical isolate obtained from a cervical swab specimen of a 30-year-old pregnant woman previously isolated in our laboratory. This viral strain had shown its ability to infect the primary macrophages and to replicate productively in the human mammary epithelial cells (HMECs). HCMV-DB had shown its potential to transform the primary HMECs by colony formation on soft agar, with the emergence of clusters of spheroid cells that were named CTH cells (CMV Transformed HMECs).

We characterized the genomic profile of HCMV-DB strain and compared it to either clinical or laboratory strains. HCMV-DB was shown to be close to the genomes of Toledo and JP strains where the JP strain.

We also compared the genes that are involved in virus entry using phylogenetic analyses and we observed that HCMV-DB is close to the prototypic HCMV strain, Merlin. By studying the transcriptomic profile of HMECs infected with HCMV-DB, we found that it displays a triple negative basal-like phenotype, ER-/PR-/HER2-, and presents oncogenic characteristics with upregulated expression of several oncogenes, pro-survival genes (with a down-regulation of caspase 8), proliferation markers, stemcellness and epithelial mesenchymal transition (EMT). The transcriptomic profile of HMECs infected with HCMV-DB also displays variant modifications in cell signaling, angiogenesis and proteolysis. At the chromatin level, HMECs infected with HCMV-DB reveals a global hypomethylation state. By screening for the presence of HCMV-DB genome in the formed CTH cells, we detected a signature of the HCMV-DB genome, namely a lncRNA4.9. Taken together, our data showed that the transcriptome of HMECs infected with HCMV-DB clearly reveals a pro-oncogenic traits and the detection of part of the HCMV-DB genome suggests that this part of the viral genome might be responsible for the obtained cellular transformation.