MODULATION OF NATURAL KILLER CELL RESPONSE BY HUMAN CYTOMEGALOVIRUS

A thesis submitted in the candidature for the degree of

DOCTOR OF PHILOSOPHY

By

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SUMMARY

The Natural Killer (NK) cell activating receptor DNAM-1 (CD226) is stimulated through recognition of CD112 (nectin-2) and CD155 (nectin-like molecule 5; PVR) on target cells. HCMV UL141 elicits protection from NK-cells by down-regulating CD155 from the cell surface and sequestering it in the ER (Tomasec, 2005). Here, HCMV UL141 was shown to be involved in the down-regulation of CD112. Interestingly, UL141 appeared necessary but not sufficient to modulate CD112 expression. This thesis therefore focused on a hypothesis whereby UL141 was acting with one or more additional HCMV genes to target CD112 for degradation.

This project was the first to utilise an entire recombinant adenovirus (RAd) library expressing individual HCMV ORFs (RAd-HCMV-ORF library) to screen for function. The RAd-HCMV-ORF library clearly provided an extremely powerful tool for the screening of HCMV gene function as results were highly repeatable and robust. The co-infection of RAd-UL141 and RAd-US2 resulted in a single, clear, positive hit in the final screening process. This hit was further verified by immunoblot where CD112 appeared to be down-regulated in cells infected with both RAd-UL141 and RAd-US2, compared to controls. While a Hela-US2 cell line which stably expressed US2 also down-regulated CD112 when infected with RAd-UL141. A RCMVΔUS1-11 virus was constructed, which failed to down-regulate CD112 from the cell surface of RCMVΔSU1-11 infected cells.

The addition of proteasome inhibitors was able to partially restore CD112 expression in HCMV infected cells (Prod'homme et al., 2010). It therefore appeared that US2 and UL141 act to degrade CD112 via the proteasome during HCMV infection. CD112 downregulation may have the potential to prevent DNAM-1:CD112 interaction between HCMV infected targets and effector cells of the immune system, providing another facet to HCMV's ability to avoid the human immune response.

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ATCC Teddington, Middlesex,UK
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BD Pharmingen UK Ltd., Cowley, Oxford, UK.
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ABBREVIATIONS

°C	Degrees Centigrade
µM	Micromolar
µm	Micrometer
µg	Microgram
µl	Microlitre

A

aa	Amino Acid
Ad	Adenovirus
Ad5	Adenovirus Serotype-5
ADCC	Antibody Dependent Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection

B

B_2m	β_2-microglobulin
BAC	Bacterial Artificial Chromosome
BTLA	B and T lymphocyte attenuator
Bp	Base Pairs

C

CCMV	Chimpanzee Cytomegalovirus
CD	Cluster determinant
CNS	Central Nervous System
CPE	Cytopathic Effect
crs	Cis Repression Signal

D

DAPI	2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride
DB	Dense Bodies
DMEM	Dulbeccos Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dsDNA	Double Stranded DNA
ssDNA	Single Stranded DNA
DTT	Dithiothreitol

E

E	Early (gene expression in HCMV)
EBV	Epstein Bar Virus
ECM	Extracellular matrix
E.coli	Escherichia Coli
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
ERGIC	Endoplasmic Reticulum Golgi Intermediate Compartment

F

FACS	Fluorescence Activated Cell Sorter
FCS	Foetal Calf Serum
FSC	Forward Scatter

G

GAPDH	Glyceraldehydes-3-phosphate Dehydrogenase
gB	Glycoprotein B
gCI	Glycoprotein Complex I
gCII	Glycoprotein Complex II
gCIII	Glycoprotein Complex III
GFP	Green Fluorescent Protein
gH	Glycoprotein H
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
gO	Glycoprotein O
gp	Glycoprotein

H

h	Hours
HA	Haemagluttin
HCMV	Human cytomegalovirus
hCAR	Human Coxsackie Adenovirus Receptor
HFFF	Human Foetal Foreskin Fibroblast
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRP	Horse Radish Peroxidase
HSPG	Heparin Sulphate Proteoglycans
HSV-1	Herpesvirus type I
HSV-2	Herpesvirus type II
hTert	Human Telomerase Reverse transcriptase
HVEM	Herpes Virus Entry Mediator

I

IE	Immediate Early (expression in HCMV)
IE1	Immediate Early Gene 1
IE2	Immediate Early Gene 2
IFN	Interferon
IFNAR	IFNα Receptor
IFNGR	IFNγ Receptor
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IRS	Internal Repeat Short
IRL	Internal Repeat Long
ITAM	Intracellular Tyrosine-based Activatory Motif
ITIM	Intracellular Tyrosine-based Inhibitory Motif
IU	international Units

J

JAK1	Janus Kinase 1

K

kDa	Kilodaltons
KIR	Killer Cell Immunoglobulin-like Receptor
KSHV	Kaposi's Sarcoma Associated Herpesvirus

L

L	Litre
L	Late (gene expression in HCMV)
LB	Lauria Bertani Agar
LIR-1	Lectin-like Transcript 1

M

mAB	Monoclonal Antibody
MCMV	Murine Cytomegalovirus
MCP	Major Capsid Protein
mCP	Minor Capsid Protein
mCPBp	Minor Capsid protein binding protein
mg	Milligrams
MHC	Major Histocompatibility Complex
MICA	MHC Class I Chain-Related Gene A
MICB	MHC Class I Chain-Related Gene B
MIE	Major Immediate Early Promoter
mL	Millilitres
MOI	Multiplicity Of Infection
mRNA	Messenger Ribonucleic Acid

N

NCR	Natural Cytoxicity Receptor
ND-10	Nuclear Domain 10
Necl	Nectin-Like Molecule
NIEP	Non Infectious Envelope Particles
NK	Natural Killer Cell
NP40	Nonidet P-40

O

ORF	Open Reading Frame
oriLyt	Origin of Replication

P

P	Protein
PBMC	Peripheral Blood Mononuclear Cells
Pp	Phosphoprotein
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units

p.i.	Post Infection
PML	Promyelocitic Leukemia
PORT	Portal Protein
PRV	Porcine Rabies Virus
PVR	Polio Virus Receptor

R

RAd	Recombinant Adenovirus
RAd-HCMV-ORF	Recombinant Adenovirus expression a HCMV open reading frame
RANTES	Regulated upon Activations, Normal T-cell Expressed and Secreted
RCMV	Recombinant HCMV
RNA	Ribonucleic Acid
RNase	Ribonuclease
dsRNA	Double Stranded RNA
RNAI	RNA Interference
ncRNA	Non-Coding RNA
RPM	Rotations Per Minute
RT	Room Temperature

S

SCP	Smallest Capsid Protein
SDS	Sodium Dodecyl Sulphate
SGV	Salivary Gland Virus
Strep	Streptomycin

T

TAE	Tris acetic acid EDTA
TAP	Transporter Associated with Antigen Presentation
$TCIP_{50}$	Tissue Cultire Infectious Dose
Tet	Tetracycline
TNF	Tumor Necrosis Factor
TRL	Terminal Repeat Long
TRS	Terminal Repeat Short

U, V, W, X

UL	Unique Long
ULBP	UL16 binding protein
US	Unique Short
UV	Ultra Violet
V	Volts
vAC	Viral Assembly Compartment
VICA	Viral Inhibitor of Caspase Activation
vRC	Viral Replication Compartment
VZV	Varicella Zosta Virus
v/v	Volume to Volume Ratio
vMIA	Viral Mitochondrial Inhibitor of Apoptosis
w/v	Weight to Volume Ratio
X-Gal	5-bromo, 4-chloro-3-indolyl-β-D-galactopyranosidase

1.0. INTRODUCTION

1.1 HERPESVIRIDAE

Viruses can be defined as infectious particles that are incapable of reproduction in the absence of a living host cell. Viruses likely infect all living organisms and, like their hosts, show a wide diversity of structures, complexities and life cycles.

Herpesviruses represent a large family of enveloped double stranded DNA viruses that result in lifelong infection of the host. Herpesviruses fall into the order herpesvirales, which consists of three families (Herpesviridae, Alloherpesviridae and Malacoherpesviridae), three subfamilies and 17 genera (Davison, 2010). To date, approximately 150 members of the herpesvirales have been identified from a wide range of species including mammals, birds and fish which include 90 species and 58 tentative species and unassigned viruses (Roizman and Sears, 2001, Davison, 2010).

The specific architectural and biological properties of the herpesviridae distinguish members from other viruses and families of the double stranded DNA (dsDNA) group. Herpesviridae virions contain a linear dsDNA genome packaged in an icosahedral (T=16) capsid. The capsid is surrounded by an amorphous protein rich layer termed the tegument. The tegument, in turn, is enclosed in a glycoprotein containing lipid bilayer, referred to as the envelope. Herpesviridae capsids range from approximately 115 to 130nm in diameter, with the complete virion diameter ranging from 150-200nm, Figure 1.1

Based on biological criteria, the herpesviridae family has been classified into 3 subfamilies, alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae (Roizman and Sears, 2001). These biological classifications pre-date genome sequencing and were traditionally based on specific properties such as host species specificity, tissue tropism, serology and growth kinetics (Roizman et al., 1981, Roizman et al., 1973, Roizman and Sears, 2001). However, the increasing volume of sequence data has lead to the use of sequence homology in assigning herepsviruses to the relevant taxa. Lineages are defined by two criteria: (a) comparison of nucleotide or predicted amino acid sequences to conserved herpesvirus genes, and (b) identification of genes unique to a virus subset (Fauquet, 2005).

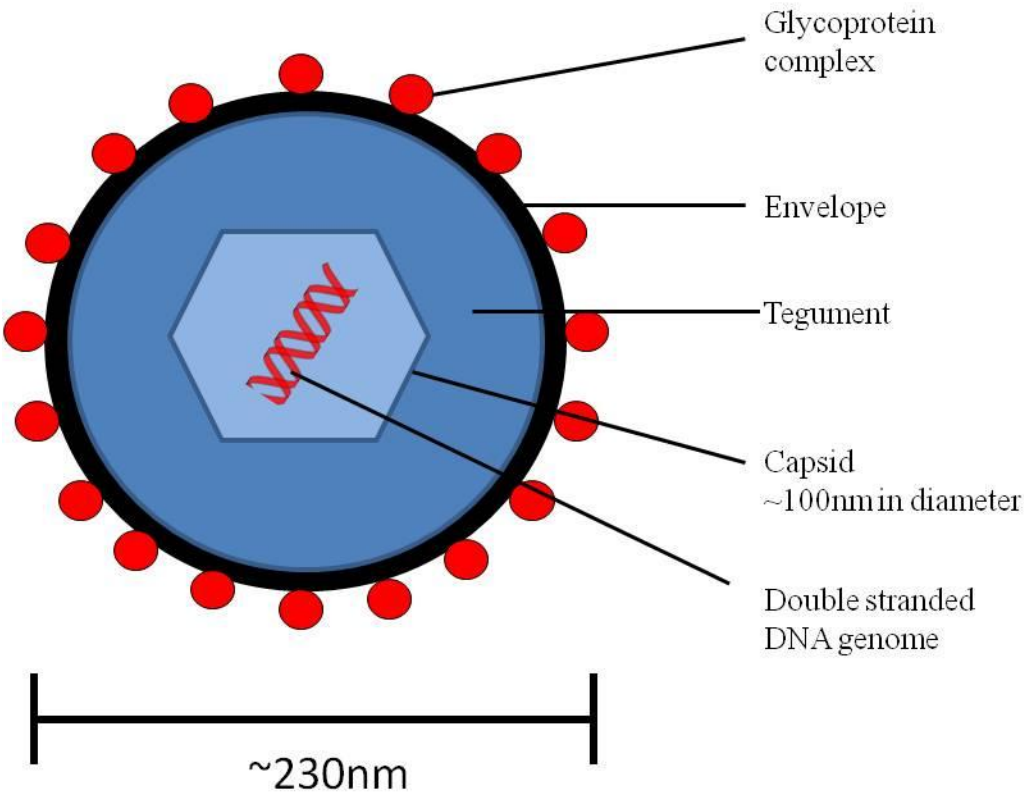


Figure 1.1. The HCMV virion.
A cartoon depicting the HMCV virion (not to scale). The double stranded DNA genome is surrounded by a capsid, which in turn is surrounded by an amorphous protein rich tegument. The tegument is in turn surrounded by a host derived envelope that contains various glycoproteins thought to be important in virus entry and immune evasion.

1.1.1. Human Herpesviruses

All human herpesviruses fall into the family Herpesviridae (Davison, 2010). To date, 9 human herpesviruses have been indentified, with representatives from all three subfamilies, Table1.1. The herpesviridae comprise some of the largest characterised human virus genomes, with Human cytomegalovirus (HCMV) being the largest characterised human virus to date, with a genome size of approximately 236kb, Figure 1.2 (Dolan, 2004). All herpesviruses result in lifelong infection and are associated with high seroprevalence within populations, although seroprevalence varies with important cofounders including geographical location and socio-economic status (Kangro et al., 1994, Cannon et al., 2010).

The alphaherpesvirinae are neurotrophic, have a relatively short reproduction cycle compared to other herpesviruses (~18hours) and spread rapidly in culture causing efficient destruction of infected cells (Roizman et al., 1981). A key attribute of the alphaherpesvirinae is their ability to establish lifelong latent infection in the peripheral nervous system of the host and are primarily, but not exclusively, found in sensory ganglia (Baringer and Swoveland, 1973). Members of this group include Herpes simplex type I (HSV-1, HHV-1) the causative agent of oral coldsores, Herpes simplex type II (HSV-2, HHV-2) the causative agent of genital herpes and Varicella Zoster virus (VZV, HHV-3) the causative agent of chicken pox and shingles.

The betaherpesvirinae contain the roseolviruses (HHV-6A, HHV-6B and HHV-7) and cytomegalovirus (HCMV, HHV-5). All betaherpesvirinae grow less efficiently compared to other herpesviruses with long reproductive cycles and slow spread of infection from cell to cell in culture (Roizman et al., 1981). Members are lymphotrophic, establishing latency in cells of the immune system. Roseolavirus latency is established in macrophages and T-lymphocytes, while HCMV latency is associated with $CD34^+$ progenitors (Kempf et al., 1997, Sinclair, 2008). In the immunocompetent population there is little clinical relevance of betaherpesvirinae infection. However, HCMV is able to cause severe morbidity in those with a weakened or compromised immune system.

The gammaherpesvirinae are also lymphotrophic and tend to be specific for either T or B lymphocytes, with some able to establish lytic infection in epithelial and fibroblast cells. The subfamily consists of Epstein Barr virus (EBV, HHV-4), and Kaposi's sarcoma associated herpesvirus (KSHV) (HHV-8). Both viruses are associated with malignant

Table 1.1. Members of the Human Herpesviridae family, which consist of the alphaherpesevirinae, betaherpesvirinae and gammaherpesvirinae subfamilies

Subfamily	Genus	Species	Common name	Acronym
Alphaherpesvirinae	Simplexvirus	Human herpesvirus 1 (HHV1)	Herpes simplex virus 1	HSV-1
		Human herpesvirus 2 (HHV-2)	Herpes simplex virus 2	HSV-2
	Varicellovirus	Human herpesvirus 3 (HHV-3)	Varicella-Zoster virus	VZV
Betaherpesvirinae	Cytomegalovirus	Human herpesvirus 5 (HHV-5)	Cytomegalovirus	HCMV
	Roseolovirus	Human herpesvirus 6 (HHV-6A)	-	-
		Human herpesvirus 6B (HHV-6B)	-	-
		Human herpesvirus 7 (HHV-7)	-	-
Gammaherpesvirinae	Lymphocryptovirus	Human herpesvirus 4 (HHV-4)	Epstein Barr virus	EBV
	Rhadinovirus	Human herpesvirus 8 (HHV-8)	Karpsoi's sarcoma associated Herpesvirus	KSHV

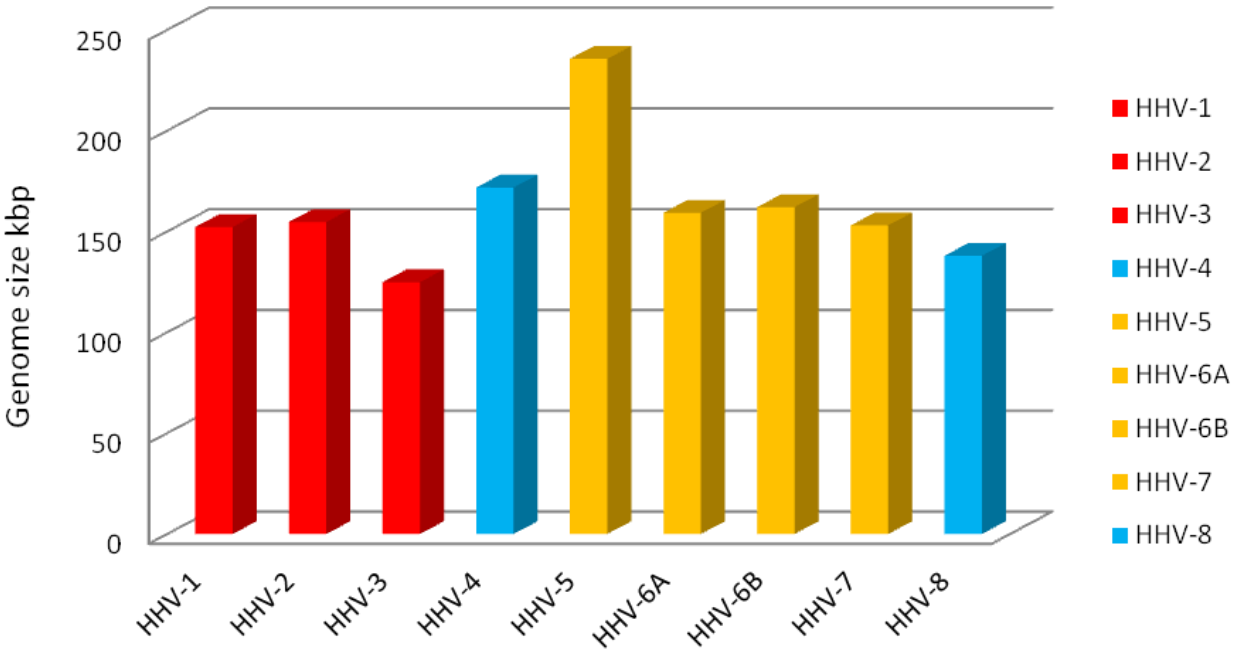


Figure 1.2. Comparison of the human herpesviridae family genome size.
Genome sizes were obtained from NCBI database. Alphaherpesviruses are designated Red and consist of HHV-1 (accession X14112), HHV-2 (accession Z86099), HHV-3 (accession X04370), Betaherpesviruses are found in yellow and consist of HHV-6A (accession X83413), HHV-6B (accession AF157706), HHV-7 (AF037218) and HHV-5 (AY446894). Gammaherpesviruses are designated blue and consist of HHV-4 (accession AJ507799) and HHV-8 (Accession AF148805).

transformation. EBV can lead to the development of lymphomas, such as Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL) or post transplant lymphoma. KSHV is present in all clinical variants of Karposi's sarcoma and the lymphoproliferative diseases: primary effusion lymphoma (PEL) and multicentric Calstlemans disease (MCD) (Dupin et al., 1999). *In-vivo* EBV latency is associated with resting B lymphocytes (Miyashita et al., 1997) In KSHV infection, productively infected lymphocytes transmit KSHV to other circulating cell types that are then recruited to tissues, and to resident endothelial cells that became latently infected (Blasig et al., 1997, Dupin et al., 1999, Sirianni et al., 1998, Sirianni et al., 1997, Ensoli et al., 2001).

1.2. HUMAN CYTOMEGALOVIRUS

A member of the betaherpesvirinae, HCMV is a ubiquitous pathogen with a worldwide distribution infecting up to 100% of the population in developing countries. HCMV seroprevalence in developed countries varies between 50-80%, which is generally attributed to a cleaner, more aseptic environment (Griffiths et al., 2000, Cannon et al., 2010).

The first observation of HCMV was by Ribbert (1904), who described an 1881 case where he noticed 'protozoan like cells' in kidney sections of a still born infant. The large cells had eccentric nuclei surrounded by a clear halo with clear cytoplasmic and nuclear inclusions. At the time this was not attributed to altered cell morphology as a result of viral infection but instead as a result of protozoa infection (Ribbert, 1904). Subsequently, the protozoan origin theory was dismissed when Goodpasture and Talbot suggested a viral aetiology (Goodpasture and Talbot, 1921). Shortly after, Cole and Kuttner (1926) showed that the causative agent of altered cell morphology in the submaxillary glands of guinea pigs was able to pass through filters that prevented the passage of protozoa. This confirmed that the causative agent of the altered cell morphology was viral and could not be due to protozoa (Cole and Kuttner, 1926).

The murine relative of the virus, murine cytomegalovirus (MCMV), was isolated and cultured *in-vitro* using primary murine fibroblasts in 1954 (Smith, 1954). Shortly after HCMV was isolated by three groups independently (Craig et al., 1957, Smith, 1956, Rowe et al., 1956). Smith (1956) isolated the virus from the submaxillary salivary gland of a

seven month old infant with adrenal cortical carcinoma and from the kidney of a one month old infant dying of generalised salivary gland virus (SGV) disease. Rowe et al (1956) isolated the virus from the adenoid tissues of three children; ages 6, 7 and 11, with the virus isolated from the 7 year old child to become the first laboratory strain of HCMV designated AD169. Craig et al (1957) isolated the virus from the liver and urine of an infected child. All three groups used primary human fibroblasts to isolate the virus and all three groups described almost identical cytopathic effects (cpe).

Initially these viruses were known as Salivary gland viruses (SGV) until the term 'cytomegalovirus' was eventually coined by Weller et al (1957) based on the typical cpe of infected cells involving characteristic nuclear and cytoplasmic inclusions and cell enlargement 'cytomegalia' (Weller et al., 1957). Human cytomegalovirus was later given the formal designation human herpesvirus 5 (HHV-5) by the committee on the taxonomy of viruses.

1.2.1. HCMV virion structure

The HCMV virion is comprised of four distinct structural components. The central core contains a double stranded DNA genome. The capsid surrounds the central core, which in turn is surrounded by an amorphous protein rich area known as the tegument. The tegument is itself surrounded with a lipid bilayer known as the envelope which contains various glycoproteins, Figure 1.1. The known virion proteins included 5 capsid proteins (UL46, UL48-49, UL80, UL85, and UL86), 14 tegument proteins (UL24, UL25, UL26, UL32, UL43, UL47, UL48, UL82, UL83, UL94, UL99, US22, US23, and US24), 11 glycoproteins (TRL10, UL22A, UL41A, UL55, UL73, UL74, UL75, UL77, UL100, UL115, and UL119), 12 proteins involved in DNA replication and transcription (IRS1, TRS1, UL44, UL45, UL54, UL57, UL69, UL72, UL84, UL89, UL97, and UL122), and 3 G-protein-coupled proteins (UL33, US27 and US28) (Varnum et al., 2004). Other virion constituents include UL5, UL38, UL50, UL71, UL79, UL93, UL96, UL103, UL132, US23, and US24, 9 of which are required for efficient growth in cultured cells (Varnum et al., 2004).

1.2.1.1. Capsid

The HCMV capsid displays icosahedral, T=16, symmetry, which consists of five core capsid proteins arranged in hexons, pentons and triplexes. The five capsid proteins are major capsid protein (MCP, pUL86), minor capsid protein (mCP, pUL85), minor capsid binding protein (mCPBp, pUL46), smallest capsid protein (SCP, pUL48A) and portal protein PORT (pUL104) (Chee et al., 1989, Baldick Jr and Shenk, 1996, Gibson et al., 1996a, Dittmer et al., 2005).

Capsid assembly is co-ordinated by the assembly protein precursor (pUL80.5) and the genetically related protease precursor (pUL80A), both of which are ultimately eliminated from the maturing particle. The major capsid protein, pUL86, is the most abundant protein component of the capsid and forms the pentons and hexons of the icosahedral capsid in the cytoplasm. The minor capsid proteins pUL85 and pUL46 interact with each other in the cytoplasm to form heterotrimers called triplexes, which are later incorporated between the pentons and hexons to form the correct structure. The pentons, hexons and triplexes are translocated to the nucleus and coalesce to form the procapsid, incorporating the portal protein complex (pUL104). The small capsid protein pUL48A decorates the hexons of the capsid and is essential for the assembly of infectious virus (Borst et al., 2001). Activation of pUL80A results in cleavage and elimination of both pUL80.5 and pUL80A from the procapsid resulting in the mature capsid. Elimination of the internal scaffold proteins, pUL80.5 and pUL80A, and incorporation of viral DNA appear to be concomitant, Figure 1.3.

There are three different capsid structures, termed A, B and C capsids formed within the infected cell. The fully mature capsids are termed C capsids and are the only form where DNA is incorporated (Pass, 2001). Type A capsids lack the scaffold protein UL80 and are unable to stably package the viral genome and hence just consist of the empty protein shell with no DNA (Irmiere and Gibson, 1983). Finally B capsids are an intermediate form containing the assembly protein UL80 and are predominately localised in the nucleus as precursors of mature capsids. All three forms are capable of acquiring envelopes but only C capsids are capable of producing mature infectious virus. Enveloped A and B capsids can be isolated from cytoplasmic and cell free preparations and are classified as non-infectious enveloped particles (NIEP).Therefore, NIEPs are enveloped B-capsids that closely resemble virions in structure and composition, but retain the internal scaffolding

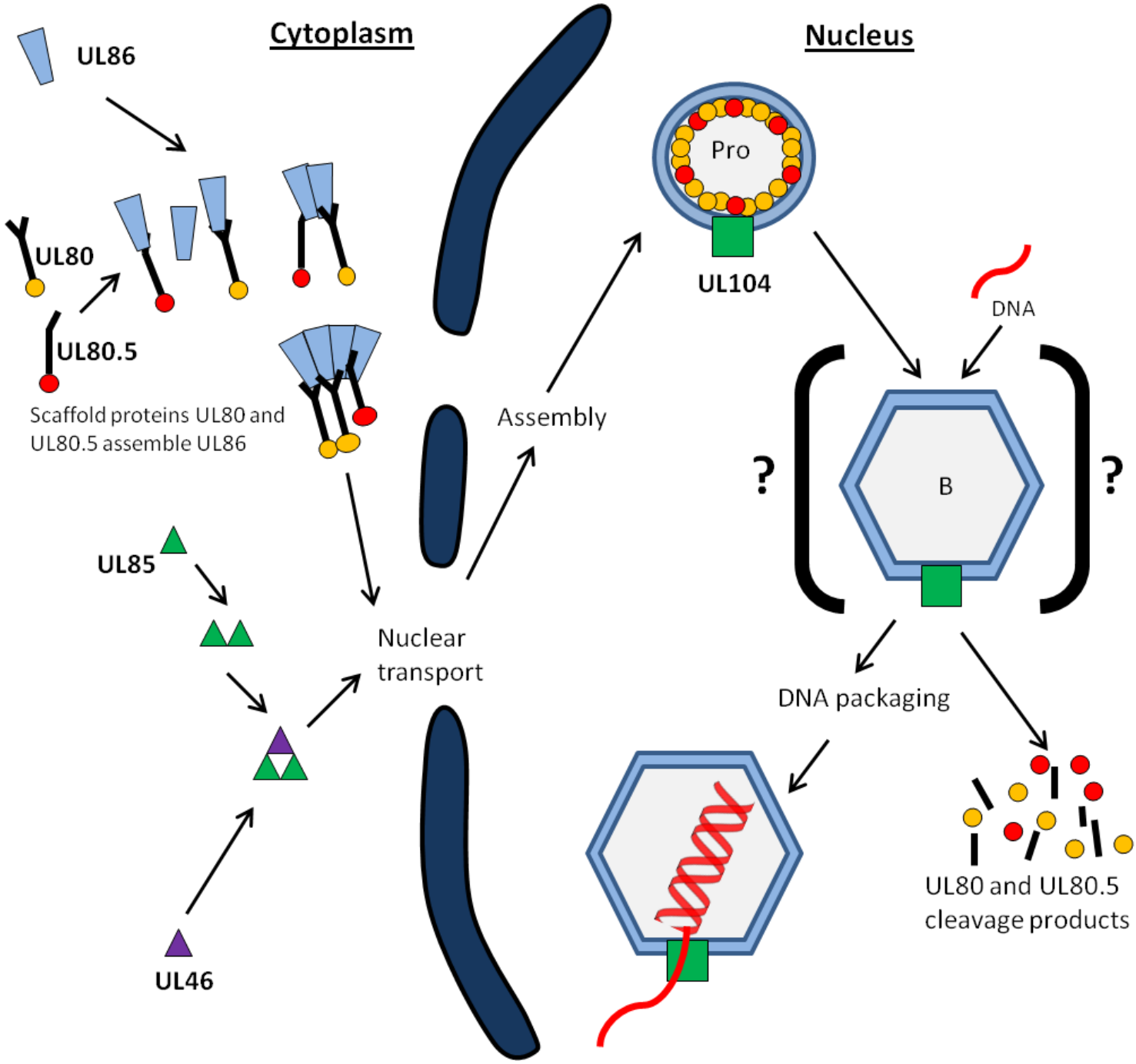


Figure 1.3. HCMV capsid assembly model.
A simplified illustration of the interactions between the five structural HCMV proteins during virion morphogenesis. The major capsid protein UL86, assembly protein precursor UL80.5 and protease precursor UL80.5 form complexes in the cytoplasm. The minor capsid protein UL85 and the minor capsid binding protein UL46 also interact with each other in the cytoplasm to form heterotrimers called triplexes. These two types of oligomers are translocated to the nucleus where the coalesce to form the procapsid (Pro), incorporating the portal protein UL104. Activation of the UL80.5 protease results in cleavage and elimination of the internal scaffold proteins UL80 and UL80.5 from the capsid. Brackets around the B-capsid (B) indicates uncertainty about the nature of putative intermediates between procapsids and DNA containing nucleocapsids. Modified from Shenk and Stinski (2008).

proteins. Dense bodies (DBs), which differ from NIEPs and virions by their larger and more heterogeneous size and absence of all nucleocapsid constituents, are solid spheroidal aggregates of a single predominant tegument species, pUL83, surrounded by an envelope so far undistinguished from that of the virion.

1.2.1.2. Tegument

The tegument is an amorphous proteinaceous layer that lies between the capsid and the lipid envelope and is thought to primarily consist of phosphoproteins (Roby and Gibson, 1986). More than half of the recognised virion proteins are believed to be located within the tegument region, ~22 in total (Varnum et al., 2004). Two major components of the tegument are the phosphoproteins ppUL32 (pp150) and ppUL83 (pp65) (Jahn et al., 1987). ppUL32 constitutes approximately 20% of the virion protein content and binds specifically to the nucleocapsid through its amino-terminus (Baxter and Gibson, 2001). ppUL83 is another abundant tegument protein, constituting approximately 15% of the HCMV virion, but is not essential for virion structure as ppUL83 is dispensable for growth *in-vitro* (Varnum et al., 2004, Schmolke et al., 1995). The amount of ppUL83 produced by low passage clinical isolates is much reduced compared to that produced by laboratory adapted strains and has been correlated to dense body formations during infection (Klages et al., 1989).

Tegument proteins have a tendency to be both phosphorylated and highly immunogenic. Antibody responses can be detected against UL55 (gB), UL75 (gH), UL44 (pp50) and UL98 (pp28). HCMV ppUL83 (pp65) also appears to be a major target of HCMV specific cytotoxic T-cells (Boppana and Britt, 1996).

Although the tegument appears amorphous it is known to participate in highly ordered structural interactions with both the envelope and the capsid. The tegument protein pUL47 coprecipitates with two other tegument proteins, pUL48 and pUL69, as well as the major capsid protein UL86, and it is proposed that this complex is involved in release of viral DNA from the disassembling virion, as deletion of UL47 delays this process (Bechtel and Shenk, 2002, Baldick Jr and Shenk, 1996). The tegument protein ppUL99 (pp28) interacts with the virion envelope membrane via a myristylated domain (Silva et al., 2003). This

interaction is important for the accumulation of tegument proteins prior to envelopment (Sanchez et al., 2000).

The process of tegumentation occurs in two separate cellular locations during viral assembly. Initial components, including pUL83 (pp65) and UL97 kinase initiate interactions with the nucleocapsid in the nucleus (Sanchez et al., 1998, Wolf et al., 2001). The UL97 kinase is involved in DNA replication and capsid assembly. While ppUL83 (pp65) is required for the recruitment of other tegument proteins, including pUL25 and pUL69 into the virion (Zini et al., 1999, Chevillotte et al., 2009). Following nuclear egress, a second round of tegumentation occurs in the cytoplasm where ppUL99 (pp28) is added and is required for final envelopment of the virion (Sanchez et al., 2000, Silva et al., 2003).

In addition to structural roles in viral assembly, the virion tegument proteins also play important roles in the initiation of productive infection following successful cell entry, suggesting that tegument proteins may have functions unrelated to the structural role of the viral tegument. The tegument proteins are released in infected cells prior to immediate early (IE) gene expression. The UL82 gene product ppUL82 (pp71, Virus transactivator, VTA) plays a critical role in the initiation of infection by relieving hDaxx repression of virus replication by promoting hDaxx degradation via the proteasome (Cantrell and Bresnahan, 2006). ppUL82 and ppUL35 have been shown to interact and cooperatively activate the major immediate-early enhancer, enhancing the infectivity of viral DNA and accelerating the infection cycle (Baldick Jr et al., 1997, Schierling et al., 2004). pUL69 is a viral transactivator and is expressed as three differentially phosphorylated forms, only one of which is incorporated into the tegument (Winkler and Stamminger, 1996).

Other tegument proteins include the product of the UL26 open reading frame, pUL26, which contains a strong transcriptional activation domain (Stamminger et al., 2002). While, several proteins encoded by the US22 family members are also components of the tegument, including UL23, UL24, IRS1 and TRS1 (Adair et al., 2002, Romanowski et al., 1997).

1.2.1.3. Envelope glycoproteins

Once in the cytoplasm and completely tegumented, capsids bud into cytoplasmic vesicles or tubules to acquire their final envelope derived from the host cell endoplasmic reticulum

(ER)-Golgi intermediate compartment (ERGIC) membrane (Sanchez et al., 2000). This process requires pUL99; as in its absence, tegumented capsids accumulate in the cytoplasm (Silva et al., 2003).

The virion envelope is complex and many of the proteins comprising this structure remain undefined. Virion glycoproteins have been the most extensively studied virion envelope constituents. Studies have suggested the glycoproteins are present in the following order of abundance; gM/gN > gB > gH/gL/gO (gpUL100/gpUL73 > gpUL55 > gpUL75/gpUL115/gpUL74) (Britt and Boppana, 2004). There were originally believed to be three major glycoprotein complexes present on the envelope: gCI (gB), gCII (gM:gN) and gCIII (gH;gL) (Gretch et al., 1988).

gCI consists of disulphide-linked gB (gpUL55) homodimers (Kari et al., 1990). Translated as a 160kDa monomer, gB is then processed by host proteases to generate 116kDa and 55kDa polypeptides. The 55kDa species is found on the outside of the envelope and remains in complex with the 116kDa transmembrane component (Britt and Vugler, 1992, Britt and Vugler, 1989). Proteolytic cleavage of gB is dispensable for viral growth (Strive et al., 2002). However, gB is involved in the initial stages of virion-to-cell attachment. The gB glycoprotein is also a major target of human neutralising antibodies, which prevent both attachment and fusion of the virus to host cell membrane by interfering with a receptor-binding event during initial virus-host cell interaction (Marshall et al., 1992, Gicklhorn et al., 2003). Proteins thought to be involved in gB binding are cell surface heparin sulphate proteoglycans (HSPGs) as soluble gB binding is reduced by ~40% in HSPG-deficient cell lines (Boyle and Compton, 1998). After attachment the glycoprotein promotes specific interactions with epidermal growth factor receptor (EGFR) and αVβ3 integrin (Wang et al., 2005).

The gCII complex is comprised of gM (gpUL100) and gN (gpUL73) that are covalently linked by a disulphide bond, although gCII complex formation can occur in the absence of disulphide bonding (Mach et al., 2000, Mach et al., 2005). Only the initial 118aa of gM are essential for interaction with gN (Mach et al., 2005). Complex formation occurs within the ER, and is required for the transport of gM:gN to the secretory system. Both gM and gN have also been reported to interact with HSPGs on entry (Kari and Gehrz, 1992). As with gCI, gCII is also a target for neutralising antibodies in human sera. In one study most sera

failed to react to either gM or gN when expressed in isolation. However, 62% of sera were positive for the gCII (gM-gN) complex (Mach et al., 2000).

gCIII, consists of gH (gpUL75), gL (gpUL115) and gO (gpUL74) in virions purified from laboratory-adapted HCMV strains (Huber and Compton, 1998). Glycoprotein-H is a fusion receptor, while gL is a chaperone required for gH localisation; both are complexed with gO, which enhances virus entry. gH is linked to gL by disulphide bonds that are formed in the ER (Huber and Compton, 1999). gL is required for the transport of gH to the cell surface (Spaete et al 1993). The gH:gL complex binds to a gO precursor forming a 220kDa gCIII precursor before gO is post-translationally modified in a post-ER compartment to form the fully mature gCIII complex (Theiler and Compton, 2001). It has been reported that gH also binds EGFR and αVβ3 integrin (Wang et al., 2005). Both gH and gO have been implicated in viral fusion as antibodies against gH or gO inhibit this process (Paterson et al., 2002). However, although antibodies to gH block viral fusion they do not inhibit attachment (Keay and Baldwin, 1991).

In addition to gCIII, a second envelope complex involving gH and gL has been described (Wang and Shenk, 2005). This complex comprises of gH, gL and the products of UL128, UL130 and UL131A, known as the UL128locus. The co-expression of the UL128locus increases the transport of gH:gL from the ER and the gH:gL:pUL128-131A complex is required for epithelial and endothelial cell tropism (Adler et al., 2006, Ryckman et al., 2008). HCMV virions lacking this complex can be internalised but do not initiate IE gene expression in epithelial cells (Sinzger et al., 2000). *In-vitro* passage of HCMV in fibroblasts rapidly leads to mutation and loss of function of the UL128locus, leading to an inability to productively infect epithelial cells (Dolan, 2004). Strains that lack the UL128locus, such as AD169 produce virions that only contain the gH-gL-gO gCIII complex (Wang and Shenk, 2005). Thus it appears the gCIII complex allowing fibroblast tropism consists of gO:gH:gL. While the gCIII complex permitting endothelial/epithelial tropism consists of a complex of gH:gL:UL128Locus.

In addition to the major glycoprotein complexes there are also other virus-encoded glycoproteins present in the virion envelope. HCMV encodes four G coupled protein receptor homologues UL33, US27, US28 and UL74, three of which, UL33, US27 and US28, have been described as components of the virion (Varnum et al., 2004, Fraile-Ramos et al., 2001). In addition to those glycoproteins discussed above, Varnum and

colleagues (Varnum et al., 2004) identified twelve HCMV glycoproteins that are minor envelope constituents including UL79, UL33 and UL5.

1.2.1.4. Other virion constituents

In addition to viral encoded proteins, over 70 host encoded proteins have been identified in the HCMV virion including proteins involved in cell structure (α-actin, Vimentin), protein transport (clatharin, polyubiquitin B), transcription-translation (elongation factor 2) and signalling (α1-casein kinase 2) (Varnum et al., 2004). The presence of many of these host proteins within the virion is controversial. Results are dependent on the method used to purify virions and it is possible that cellular contamination may occur during the purification of infectious particles (Hudecz et al., 1985). However, support of the direct incorporation of cellular protein comes from functional studies. For example the host cell phospholipase cPLA2α was detected in virions and inhibition of cPLA2α activity in the virion correlated with a decrease of IE gene expression (Allal et al., 2004). Over 1000 annexin II molecules are present per virus particle, and HCMV infectivity could be inhibited by treating the virus with annexin-II specific mAb (Wright et al., 1995). Although a later report contradicted this finding where they found interference with annexin II had no effect on HCMV entry (Pietropaolo and Compton, 1999).

The core of HCMV virion comprises of viral DNA and 2 polyamines (spermidine and spermine). Elimination of the spermidine pool by drug treatment has been shown to interfere with HCMV replication, resulting in reduced titres and incomplete capsid formation, suggesting the spermidine and spermine are involved in DNA packaging or capsid envelopment (Gibson et al., 1984).

Although HCMV is described as a DNA virus, several viral RNA transcripts have also been detected (Bresnahan and Shenk, 2000a, Greijer et al., 2000). The viral RNA of UL21A is present in the HCMV virion. The protein product from UL21A RNA, pUL21A, has been shown to function by blocking RANTES (regulated upon activation, normal T-cell expressed and secreted), suggesting that pUL21A can modulate the host antiviral response even before the newly infecting viral genome has become transcriptionally active (Wang et al., 2004). Two viral RNAs were identified within the HCMV origin of

replication, oriLyt. One of these viral RNAs, termed vRNA-2, was shown to form a stable RNA-DNA hybrid structure in an oriLyt containing plasmid (Prichard et al., 1998).

In addition to these viral RNAs, 2 cell RNAs, those for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and U1A, were found in the HCMV virion (Greijer et al., 2000). Selective concentration of specific transcripts imply biological function, however this viewpoint is controversial as studies have also shown that RNA molecules are incorporated into the virion in proportion to cellular concentration (Terhune et al., 2004). Incorporation of cellular RNA is most likely via non-specific interactions with RNA-binding proteins such as ppUL99 (pp28) (Terhune et al., 2004).

1.2.2. HCMV genome organisation

Human Cytomegalovirus has the largest genome of any characterised human virus; consisting of ~236Kb, and is predicted to encode ~167 open reading frames, ~10miRNAs and other additional non coding transcripts (Davison, 2003, Grey and Nelson, 2008). The virus is described as having a class E isomerising genome that consists of a unique long (UL) and a unique short (US) region. Each region is flanked by inverted internal and terminal repeats, terminal repeat long (TRL), internal repeat long (IRL), terminal repeat short (TRS) and internal repeat short (IRS), Figure 1.4. A directly repeated sequence, termed *a*, is present at both termini and also in the region between the UL and US regions. The presence of the terminal and internal *a* sequences cause genome isomerisation, producing four isomers of the genome that are equally packaged into mature virions (Landolfo et al., 2003). In addition, the *a* regions contain the conserved pac1 and pac2 elements that are conserved between herpesviruses and act as a signal for the packaging of the genome into the capsid.

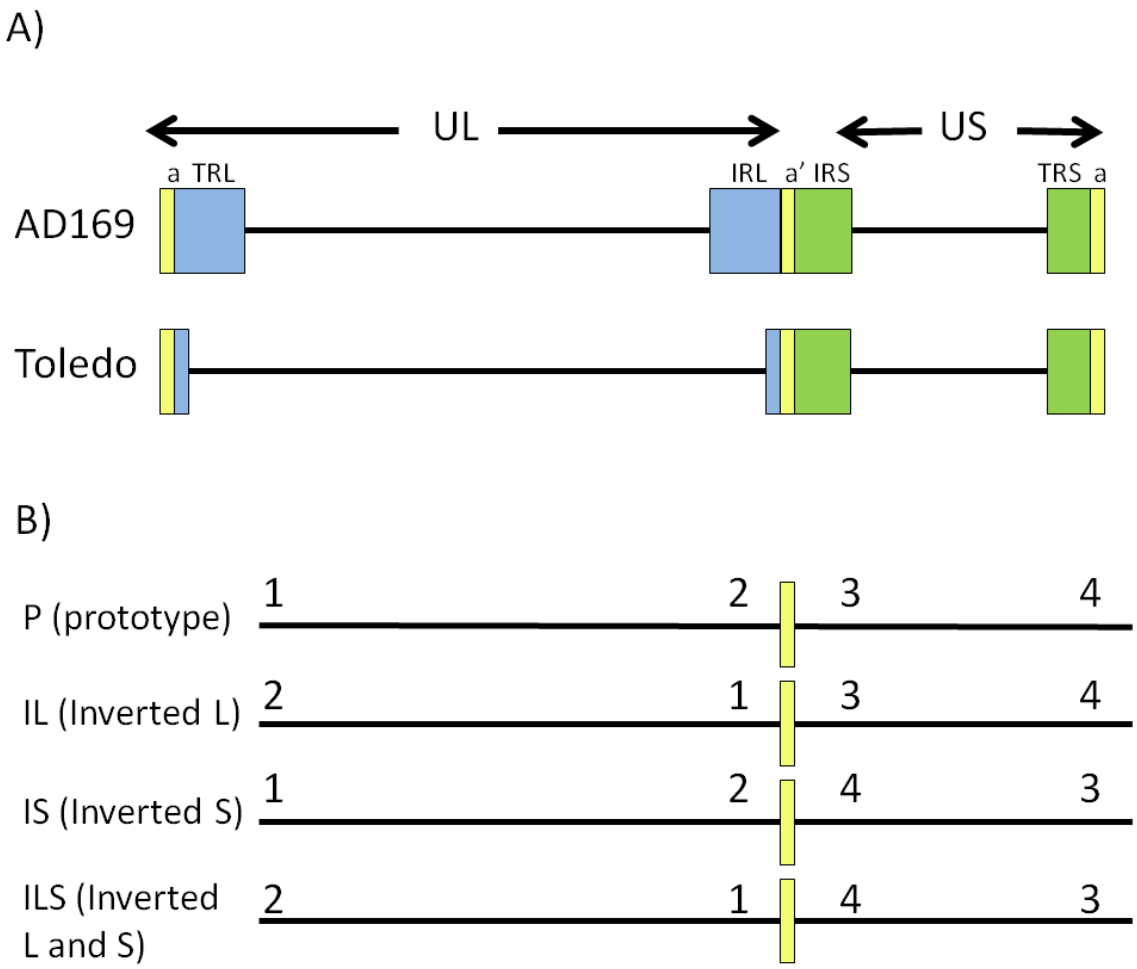


Figure 1.4. HCMV strain AD169 and Toledo genome structures
A) The HCMV genome is split into two main regions UL (unique Long) and US (unique short). These regions are flanked by terminal and inverted terminal repeats. These are termed terminal repeat long (TRL), internal repeat long (IRL), internal repeat short (IRS) and terminal repeat short (TRS). The *a* sequence is a directly repeated sequence that contains conserved packaging elements required for packaging the genome into capsids. B) The presence of terminal and internal a sequences allows for genome isomeration resulting in four genome isomers produced in viral progeny (class E genome). These include the prototype (p) inverted long (IL), inverted short (IS) and inverted long and short (ILS) genome isomers. Adapted from Landolfo et al (2003)

1.2.2.1. Genome annotation

The AD169 stock generated by Rowe et al (1956) was passaged another 56-64 times in human fibroblasts at St George's hospital, London, before being used to produce an attenuated *in-vitro* strain for use in vaccination (Elek and Stern, 1974). This strain was termed strain AD169varUK and was later cloned (Greenaway et al., 1982) and used to generate the first complete sequence of the HCMV genome (Chee, 1990). HCMV open reading frames (ORFs) were classified on their relative order within the genome and on which region they were encoded. For example UL1 is the first ORF of the UL region and US2 is the second ORF of the US region. Chee et al (1990) correctly identified that as technology and understanding of genomic structure developed then so would the annotation of the HCMV genome. This prediction was proved correct when the annotation of the HCMV genome was revisited by Davison et al (2003). Here the HCMV genome was compared to the Chimpanzee cytomegalovirus (CCMV) genome, the closest evolutionary relative of HCMV, and was predicted to comprise a total of 164-167 open frames. This number was attained by the addition of ORFs in the AD169 genome plus the number of ORFs found in the UL'b region of the Toledo strain, a region lost in strain AD169. Uncertainty remained as the existence of UL155, UL156 and UL157 found in CCMV genome could not be discounted (Davison, 2003). A later annotation to the HCMV genome came after the sequencing of the Merlin genome a clinical isolate now designated the prototype HCMV genome, Figure1.5 (Dolan, 2004, Wilkinson, 2008). A more recent analysis of the HCMV strain Merlin genome has also identified 5 non coding viral RNA transcripts (Gatherer et al., 2011).

In addition to protein coding ORFs, Grey and Nelson (2008) examined the human AD169 genome for MicroRNAs (miRNAs), an extensive class of non-coding genes that regulate gene expression through post-transcriptional repression. Using a bioinformatics approach they identified 406 potential stem-loop structures, of which 110 were conserved between chimpanzee cytomegalovirus (Grey and Nelson, 2008). Of these, 13 exhibited a significant score using the MiRscan algorithm and 5 of the 13 predicted miRNAs were found expressed during infection by examining total RNA from HCMV infected cells.

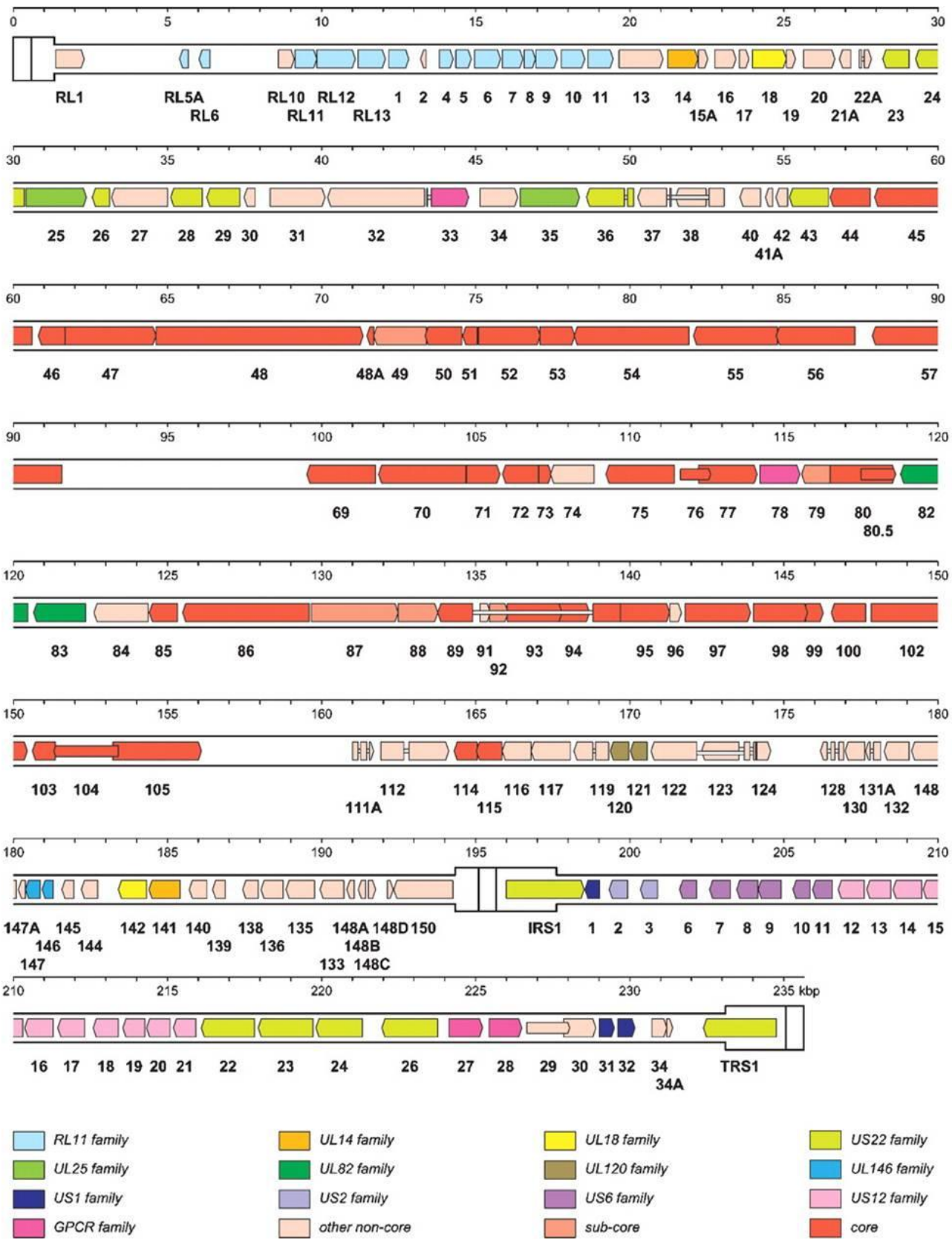


Figure 1.5. Genomic map of HCMV strain Merlin
Annotation of the wild type HCMV strain Merlin. Protein coding ORFs are indicated by arrows and have been colour coded into gene families. Reproduced from Wilkinson et al (2008), which was adapted from Dolan et al (2004)

1.2.2.2. Gene expression during infection

During productive HCMV infection, viral gene expression has been divided into three categories. These classes are based on temporal kinetics of gene expression and sensitivity to inhibition of protein or viral DNA synthesis and have been designated as immediate early (IE), early (E) and late (L). Expression of IE genes occurs immediately post viral entry and is required for the expression of both E and L genes, Figure 1.6. Transcription of the HCMV genome is performed by the host cell polymerase and its basal transcription complex.

1.2.2.3. Immediate early gene expression

Immediate early genes are defined as those that are transcribed in the absence of de novo virus-encoded protein synthesis. Four regions of IE gene expression have been mapped on the HCMV genome; the major IE (MIE) gene region encodes UL123 (IE1), UL122 (IE2) and less abundant proteins include UL36, UL37, TRS1, IRS1 and US3. By definition, IE gene expression is required to progress to productive infection. Although IE genes are primarily associated with key functions as transcriptional activators, the majority have been attributed to evading intrinsic, innate and adaptive host immune responses, immune evasion functions of these genes are discussed later in **section 1.6.**

The most abundant IE transcripts are those controlled by the MIE promoter-enhancer, one of the strongest transcriptional enhancers identified in mammalian biology (Boshart et al., 1985, Chee, 1990). The MIE promoter is located between -580 and -40 relative to the transcriptional start site and contains binding sites for an array of transcription factors, which include NF-κB and CREB (Sambucetti et al., 1989). In addition to host transcription factors, the MIE promoter is also activated by viral tegument proteins ppUL82 (pp71) and ppUL36 ensuring IE gene expression without relying on de novo viral gene expression or host transcription factors (Bresnahan and Shenk, 2000b).

UL123 (IE1/p72) and UL122 (IE2/p86) are the two major products of the MIE locus and are differentially spliced variants. These proteins contain the same 85aa N-terminus (exons 2 and 3) but following differential splicing, IE1 fuses with exon 4 and IE2 with exon 5. Three less abundant proteins are also produced; IE1p38, IE2p55 and IE2p18. With IE2p18 only produced in human infected monocyte-derived macrophages or cycloheximide-

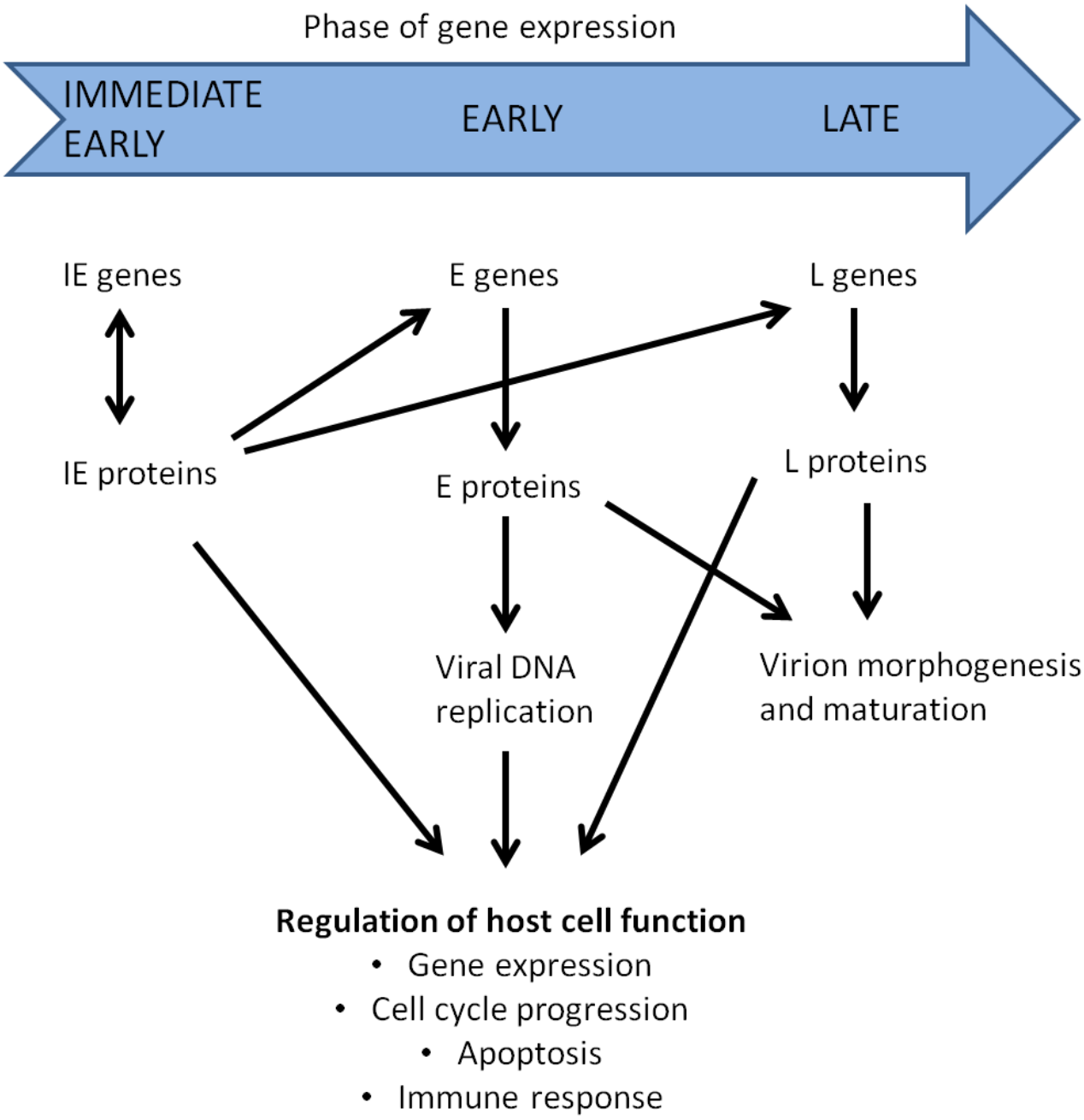


Figure 1.6. Phases of HCMV gene expression.
A simplified illustration of HCMV's temporal gene expression and the effect on viral and host gene regulation. Adapted and modified from Landolfo et al (2003).

treated HFFF cells (Kerry et al., 1995). These proteins play an important role in initiating and maintaining gene regulation during infections.

UL123 (IE1) stimulates expression from its own promoter, but is a weak activator of heterologous promoters. However, via interaction with other factors, including IE2, it can influence gene expression. The gene product initially traffics to, and induces the disruption of, Promyelocytic Leukaemia (PML) bodies (also known as ND-10 or PODs) (Kelly et al., 1995, Wilkinson et al., 1998). PML-bodies are punctuated intranuclear domains defined by their association with the tumour suppressor PML and are the site of HCMV transcription and DNA replication (Ahn and Hayward, 2000). PML-bodies are believed to constitute an intrinsic barrier to DNA-virus infection and their disruption is necessary for productive HCMV infection, as demonstrated by RNA interference knockdown of PML which significantly enhances the efficiency of infection (Tavalai et al., 2008, Tavalai et al., 2006). Post infection, PML disruption occurs within the first 6hrs. IE1 induces the dislocation of hDaxx, a PML-associated protein, which is then targeted for degradation by ppUL82. The interaction of hDaxx with histones (H2A, H2B, H3 and H4) and chromatin-associated proteins are related to its capacity to recruit histone deacetylase to sites of transcription (Woodhall et al., 2006). In preventing the recruitment of hDaxx-dependant histone deacetylase to sites of transcription, ppUL123 and ppUL82 act together to counteract an intrinsic barrier to HCMV replication (Saffert and Kalejta, 2006, Hollenbach et al., 2002).

UL122 (IE2p86) does not co-localise with PML-bodies, like UL123, but is instead located in discrete adjacent domains (Ahn and Hayward, 1997). IE2 binds a 15 base-pair cis repression signal (crs) located immediately upstream of the transcription initiation site, thereby negatively regulating expression from the MIE promoter, as well as functioning as a transactivator of both viral and host promoters (Cherrington et al., 1991, Lang and Stamminger, 1993). Through IE2's function as a transcriptional trans-activator, it mediates the switch from IE to E phase of gene expression. At early and late times of infection two additional proteins are expressed from the IE2 gene, IE2p40 and IE2p60, while IE2p40 represses expression from the MIE promoter, the other is required for efficient E and L gene expression (White et al., 2007).

UL36 and UL37 are both IE inhibitors of apoptosis (Al-Barazi and Colberg-Poley, 1996). Individually, UL36 and UL37 are dispensable, but deletion of both results in viral progeny

unable to inhibit apoptosis, resulting in programmed cell death of the infected cell. Therefore the presence of one is essential for productive HCMV infection (Patterson and Shenk, 1999, Al-Barazi and Colberg-Poley, 1996). pUL36 (vICA) blocks Fas-mediated apoptosis by binding to caspase 8 and pUL37 (vMIA) functions downstream of caspase 8 to inhibit apoptosis (Skaletskaya et al., 2001, Goldmacher et al., 1999).

TRS1 and IRS1 gene products have been shown to transactivate E promoters in co-operation with IE1 and IE2 and block interferon-induced dsRNA activated antiviral pathways (Child et al., 2004, Romanowski et al., 1997). While US3 encodes an ER-resident membrane glycoprotein that inhibits antigen presentation by retaining MHC class I heavy chains in complex in the ER (Jones et al., 1996).

1.2.2.4. Early gene expression

Expression of E genes occurs prior to viral DNA replication and requires the presence of functional IE gene products (Fortunato and Spector, 1999). Experimentally identifying E genes can be achieved by infecting and harvesting RNA in the presence of an inhibitor of HCMV DNA polymerase UL54. Inhibition of UL54 blocks L gene expression resulting in the accumulation of E gene products. However, understanding of E gene expression and regulation is incompletely understood. Many E genes continue to express into the L phase of gene expression. However, true L gene expression is dependent on viral DNA replication. Early proteins involved in viral DNA replication include the catalytic and accessory protein subunits of the viral DNA polymerase UL54 and UL44 respectively (Kerry et al., 1994, Ertl and Powell, 1992). As well as UL112-113 that encode four nuclear phosphoproteins also involved in the initiation of DNA synthesis (Penfold and Mocarski, 1997). Other E proteins include the structural proteins UL4 (gp48) and UL55 (gB) and the immune evasion proteins gpUS28 and gpUS11. Early genes are also involved in gene regulations with pUL34 repressing the expression of the IE US3 by binding transcriptional repressor elements in the promoter, comparable with the crs of the MIE promoter (LaPierre and Biegalke, 2001).

1.2.2.5. Late gene expression

True L gene expression is characterised by a dependency on Viral DNA replication and true L genes are only observed after onset of HCMV DNA synthesis and require the prior expression of IE/E gene products (Stinski, 1978). Late genes are believed to mainly encode structural proteins. The regulation of L phase gene expression is poorly understood with studies being based on a small number of genes; UL94, UL99 and UL44.

The UL94 gene product is a tegument protein that associates with ppUL99 (pp28) (Liu et al., 2009). UL94 is a true L gene controlled by a promoter with a negative response element (NRE) and a positive response element (PRE) (Wing et al., 1996, Wing et al., 1998). During the E phase of infection, IE2p86 and cellular p53 interact with the NRE to prevent expression of UL94, while during L phase the PRE is dominant driving expression of UL94 (Wing et al., 1998). Another L gene, ppUL99, also contains repressor elements that modulate gene expression (Kerry et al., 1997).

UL44 encodes a DNA polymerase accessory subunit and has three distinct transcription start sites that produce three functionally distinct proteins (Isomura et al., 2007). The proximal and distal start sites, under control of TATA elements, produce proteins with E phase kinetics and are essential for DNA replication (Iwayama et al., 1994, Isomura et al., 2008), while expression of the middle start site produces a protein with true L kinetics (Isomura et al., 2007). This gene product does not influence DNA synthesis but instead increases global viral protein synthesis and is controlled by a non-canonical TATA element (Isomura et al., 2008).

1.2.3. Non-coding RNA

Mitochondria, in addition to their role in bioenergetics, play a central role in response to apoptotic signals. Consequently, a number of viruses target mitochondria to control apoptosis and also ensure that the large energy demands of viral replication are met. The HCMV β2.7 RNA is unique in that it is the only example of a viral non-coding RNA (ncRNA) that targets the mitochondrion to maintain high levels of ATP production during infection. Transcription of β2.7 accounts for ~80% of total cellular transcription during the E stage of infection. Exactly how β2.7 RNA binds mitochondrial complex I and prevents relocalisation of essential genes thereby inhibiting proapoptotic signals remains to be

elucidated (Reeves et al., 2007). Since the description of β2.7, 4 other ncRNA's have been identified in HCMV although their function remains unknown (Gatherer et al., 2011).

1.2.4. Latency and reactivation

Classically, latency has been described as the maintenance of the viral genome without the production of cell free virus. However, in HCMV lifelong infection is associated with chronic viral re-activation and replication. This long-term low-level persistence is likely to result from a latent HCMV reservoir that constantly reactivates lytic replication. Reactivation results in productive infection, which is controlled by the cellular immune response and does not cause clinically-relevant disease in the immuno-competent population.

Studies have identified one site of latency to be $CD34^+$ haematopoietic progenitor cells. As $CD34^+$ cells undergo differentiation into cell types carrying Dendritic cell markers, viral genomes can be reactivated. However, not all lineages arising from $CD34^+$ progenitors carry viral genomes. $CD4^+$ T-cells, $CD19^+$ B-cells as well as polymorphonuclear cells do not carry HCMV genomes (Taylor-Wiedeman et al., 1991, Wang et al., 1993). The reason for this remains unclear. Sinclair et al (2008) have put forward possible theories such as HCMV infection of $CD34^+$ progenitors could alter lineage commitment, or latent infection results in lack of proliferation of certain lymphoid or myeloid cell types (Sinclair, 2008). These theories still need to be addressed .While hematopoietic mononuclear cells have received attention as one site of latency, whether other sites exist and exactly how latency is maintained and how reactivation occurs is not known (Reeves and Sinclair, 2008).

1.2.5. *In-vivo* transmission and primary infection.

Farber and Wolbach (1932) provided the first evidence for HCMV infection of individuals when cells typical of HCMV infection were detected in 12% of 183 infants who had died from other, unrelated, diseases (Farber and Wolbach, 1932). Although primary infection is generally attributed to those early on in life, HCMV is capable of infecting people of all ages. The virus can be transmitted via exposure to virus-containing bodily fluids including saliva, urine, cervical and vaginal secretions, semen, breast milk, tears, faeces and blood and is thought to be transmitted horizontally and vertically via contact with these bodily

fluids from infected individuals (Pass, 2001). HCMV is also capable of crossing the placenta and is a common cause of viral congenital malformation in humans (Stagno et al., 1987). Following primary infection, infectious virus is excreted for prolonged periods of time which enhances the spread of the virus throughout the population.

Following primary infection, HCMV spreads to the lymphoid tissues and then systematically through circulating lymphocytes and monocytes to the lymph nodes and spleen. The infection can also be localised in the duct and epithelial cells of salivary glands, kidney tubules, cervix, testes and epididymis. Pathologic changes in the immuno-competent population are rare, with the virus usually resulting in an asymptomatic infection or a mild flu-like illness and mononucleosis.

1.3. CLINICAL OUTCOMES OF HCMV INFECTION

One of the more striking observations is that severe CMV disease is found in those with weakened or compromised immune systems where infection leads to clinical manifestation with potential complications including pneumonia, hepatitis and autoantibody production (Seehofer et al., 2002, Bartholomaeus et al., 1988, Enright et al., 1993). In particular patients undergoing bone marrow transplantation or who have AIDS, where there is an impairment of T-lymphocytes, seem to be particularly susceptible (Hibberd and Snydman, 1995).

1.3.1. Congenital infection

Congenital CMV infection is one of the TORCH infections (toxoplasmosis, other infections including syphilis, rubella, CMV and herpes simplex virus), which carry a risk of significant symptomatic disease and developmental defect in newborns.

The most common complications reported in neonates associated with HCMV disease are jaundice, hepatosplenomegaly and petechiae, and these symptoms may affect up to 70% of overtly infected infants (Boppana et al., 1992). Other complications can include CNS disorders such as brain inflammation/microcepthaly and damage to the retina and cochlear which can result in blindness and deafness (Boppana et al., 1992). Primary infection

during pregnancy is thought to be one of the largest viral causes of congenital deafness and mental retardation (Stagno et al., 1977).

Congenital transmission of HCMV resulting in clinically relevant disease is more common following primary infection during pregnancy, as compared to viral reactivation. One study, by Reynolds et al (1973), reported that up to 40% of mothers who seroconvert during gestation transmitted the virus transplacentally to their child. In comparison, only 1% of children born to HCMV seropositive women who suffered a recurrent infection were born seropositive (Reynolds et al., 1973).

1.3.2. Transplantation

HCMV infection is one of the largest causes of solid organ and bone marrow transplant rejection (Hibberd and Snydman, 1995, Neiman et al., 1977, Lopez et al., 1974). Typically, rejection occurs in transplants between seropositive and seronegative patients. For HCMV seropositive recipients, the immunosuppressive treatment required prior to surgery provides an opportunity for viral reactivation and HCMV can be detected in bodily fluids in these patients (Rubin, 2001).

For seronegative recipients who receive transplanted organs from seropositive donors, HCMV present in transplanted organs can result in primary infection and subsequent complications including graft rejection (Lopez et al., 1974). The major clinical manifestations in infected solid organ recipients are fever, gastroenteritis, mononucleosis, retinitis, malaise and macular rash. While life-threatening complications such as pneumonia and hepatitis occur in fewer patients (Kotton, 2010).

In addition to solid organ transplants, patients undergoing allogenic bone marrow transplantation can also develop CMV disease. One report by Rubie et al (1993) showed the incidence of CMV infection and CMV interstitial pneumonitis (CMV-IP) in patients undergoing allogenic bone marrow transplantation were 44% and 13% respectively, with CMV-IP being the most clinically significant and life threatening (Rubie et al., 1993).

1.3.3. HIV/AIDS

In people with advanced HIV infection that have developed acquired immune deficiency syndrome (AIDS) HCMV can be reactivated as a result of an impaired immune system and consequently CMV disease is an AIDS defining condition. A HIV negative individual has a normal $CD4^+$ T-cell count varying between 600-1200 cells/mm^3. The average $CD4^+$ cell count of a HIV+ individual at the time they develop their first episode of HCMV is below 30 cells /mm^3 (Kuppermann et al., 1993). Historically, over 20 % of people with HIV have developed CMV disease within 2 years of their $CD4^+$ cell count falling below 100cells/mm^3 (Gallant et al., 1992).

In humans, $CD4^+$ helper T-lymphocytes respond to multiple structural and non-structural CMV proteins and the reduction in $CD4^+$ T-cells allows for opportunistic CMV infection (Sinclair et al., 2004). The HCMV immediate early proteins IE1 and IE2, have been shown to interact with the HIV Tat (transcriptional activator of the long terminal repeat) protein, indicating that dual infection with HCMV and HIV could play a role in HIV activation and acceleration of the onset of AIDS (Monte et al., 1997). Therefore, co-infection with HCMV and HIV has been described as an independent predictor of mortality in AIDS.

The most common manifestation of infection within this subgroup is HCMV retinitis, a progressive disease causing total retinal destruction and blindness affecting up to 5% of AIDS patients (Jacobson et al., 1988). Interestingly, CMV retinitis is almost uniquely found within this immunocompromised group, demonstrating the importance of $CD4^+$ T-cells in controlling HCMV infection. Other complications include gastro-intestinal lesions, HCMV pneumonitis and HCMV central nervous disease. However, with the onset of Highly Active Anti-Retroviral Treatment (HAART), HCMV related complications have been rapidly reduced within the HIV/AIDS population (Hammer et al., 1997, Clumeck et al., 2001, Squires et al., 2000, Eron Jr et al., 2000).

1.3.4. Treatment

HCMV is an important pathogen which is difficult to treat and currently has a significant unmet medical need. Acyclovir, which is commonly used to treat other herpesviruses, is generally ineffective (Singh et al., 1994). Two other agents are therefore more generally used; Ganciclovir and Foscarnet. These two agents have been shown to have strong *in-vivo*

activity against HCMV. However, resistance to these agents is becomingly increasingly common in the population, especially amongst AIDS patients (Jabs et al., 1998a, Jabs et al., 1998b). Cidofovir is another agent used in the treatment of CMV but is limited to use in AIDS patients due to its high toxicity. HCMV therefore provides an excellent pharmaco-economic case for drug development. Nevertheless, years of development has, thus far, failed to bring a drug to market that is both potent and safe.

Ganciclovir is a prodrug that has demonstrated efficacy against a number of the herpesviridae. Administered by intravenous (IV) injection in its inactive form, upon entering infected cells ganciclovir is phosphorylated to a monophosphate form by the enzyme encoded by the UL97 open reading frame (ORF) of HCMV (Littler et al., 1992, Sullivan et al., 1992). The monophosphate form of ganciclovir is further phosphorylated by host kinases to a triphosphate form. Ganciclovir triphosphate then competes with deoxyguanosine triphosphate as a substrate for DNA polymerase. Incorporation in the growing DNA chain slows DNA synthesis and consequently inhibits viral replication. Despite being the drug of choice for the treatment of HCMV, resistant HCMV isolates have emerged that contain mutations in UL97 that prevent the phosphorylation of ganciclovir (Chou et al., 1995).

Ganciclovir is administered by IV injection, which is inconvenient and can be complicated by infection. Valganciclovir (VGCV) is a prodrug valyl derivative of ganciclovir, which can be administered orally. After absorption through the gut, the valine moiety is rapidly cleaved off by the liver yielding ganciclovir, which is inactive until it becomes triphosphorylated and its mechanism of action is as above.

For those patients that are intolerant to ganciclovir, or have failed ganciclovir treatment, forscarnet can be used to treat HCMV disease. Forscarnet is an inorganic pyrophosphate analogue that selectively inhibits the viral DNA polymerase, encoded for by UL54. However, HCMV mutants with resistance to forscarnet have been identified (Baldanti et al., 1996).

Cidofovir (Vistide, HPMPC) is a nucleotide analogue with a prolonged intracellular half life and potent activity against a spectrum of Herpesviruses, including CMV. As a nucleotide, cidofovir does not require virus-mediated activation by phosphorylation and therefore retains activity against many ganciclovir-resistant CMV clinical isolates

(Lalezari et al., 1995). Cidofovir is also administered IV but due to its high toxicity is predominantly reserved solely for treatment of CMV retinitis.

Growing antiviral-resistance is a major issue in treating HCMV disease. In one study, Jabs et al (1998a), identified Ganciclovir-resistant isolates in 27% of CMV retinitis patients treated with ganciclovir for 9 months. Identification of a resistant isolate was associated with adverse disease outcomes, such as dissemination to the other eye or rapid progression of the retinitis (Jabs et al., 1998a). In a similar study 37% of patients treated with forscarnet for 9 months developed a forscarnet-resistant isolate, and 29% of patients treated with cidofovir for 3months a cidofovir-resistant isolate (Jabs et al., 1998b). The rapid development of resistance is of obvious concern and thus drives the need to develop more effective treatments.

1.3.5. Vaccination

Due to the lifelong persistence of viral infection, its ability to become reactivated at times of immuno suppression and the irreversible nature of some of its symptoms HCMV remains an important virus to treat. In 1999, a review of priorities for vaccine development by the Institute of Medicine (IOM) of the National Academy of Sciences (USA) ranked a vaccine to prevent CMV disease as the highest priority on the basis of life-time cost to the healthcare system and the impact of the virus on human suffering. Subsequently, this priority was also endorsed by the US vaccination programme office, which proposed a series of recommendations including support for increased funding by government agencies for HCMV vaccine research. Many attempts have been made to vaccinate against HCMV, but thus far immunisation has proven ineffective at preventing infection.

One of the earliest developments in the CMV vaccine field involved the use of a live attenuated strain of HCMV as a vaccine. The Towne strain was first described in 1975 and the Towne-virus vaccine has been used in various studies with, thus far, little success.

Trials in both CMV-positive and CMV-negative renal-transplant patients showed that vaccination with the live attenuated Towne virus was unable to prevent re-infection or re-activation of HCMV, but could dampen the pathology of infection (Plotkin et al., 1991). The vaccine has also been evaluated in healthy immunocompetent individuals and was

shown to induce virus-specific cellular immunity that was detectable 10 years post-immunisation (Plotkin et al., 1991). In a separate study, vaccinated volunteers challenged with pathogenic HCMV showed that vaccinated individuals were more resistant to viral challenge than seronegative individuals (Plotkin et al., 1990). However, natural seropositive individuals were 5-10 times less likely to develop disease than were Towne vaccinated individuals. Another trial involved administrating the Towne vaccine to women with children in daycare centres. Transmission of CMV from toddlers to their seronegative mothers occurred in ~40% of cases, but there was no difference in vaccinated versus unvaccinated women (Adler et al., 1995). Progress for the Towne vaccine diminished because of regulatory concerns and its moderate efficacy within the seropositive population.

Of those exposed to CMV nearly all appear to have antibodies in their sera raised against a variety of proteins including the envelope glycoproteins gB (gpUL55) and gH (gpUL75) . Many attempts have been made to vaccinate against HCMV glycoproteins, but until recently immunisation has proven ineffective at preventing infection. A recent paper described the successful conclusion of a phase II, placebo-controlled, randomised, double-blind clinical trial for the prevention of maternal CMV infection. The vaccine consisted of a recombinant CMV envelope glycoprotein gB (gpUL55) administered with MF59 adjuvant. Vaccine efficacy was 50% and is now being progressed to phase III clinical trials (Pass et al., 2009).

1.4. IMMUNE RESPONSE TO HCMV

Evidence from both animal experimentation and human investigation have shown that both innate and adaptive immunity play a part in the control of CMV disease. The innate immune response provides a front line defence against infection. In particular Natural Killer (NK) cells are important in controlling viral infections. Murine experiments have demonstrated that mice, genetically deficient in functional NK cells, show an increased susceptibility to MCMV (Shellam et al., 1981), while depletion of NK cells from mice leads to more serious infections after challenge (Bukowski et al., 1984). In humans, patients with defects in NK cell function also exhibit extreme sensitivity to HCMV disease as their immune systems appear unable to cope with the infection (Biron, 1989). While

indirect evidence of the importance of NK cells comes from the observation that highly conserved HCMV gene products appear to protect against NK cell cytotoxicity.

Adaptive immunity also plays an important role in controlling persistent CMV infection. CMV specific $CD4^+$ and $CD8^+$ lymphocytes play an important role in immune protection after primary infection or reactivation from latency; as demonstrated by $CD4^+$ and $CD8^+$ responses specific to HCMV antigens. In seropositive individuals CMV-specific T-cells are the most abundant subset in the T-cell repertoire comprising on average ~10% of both the $CD4^+$ and $CD8^+$ memory compartments in peripheral blood (Sylwester et al., 2005). The importance of $CD4^+$ T-cells is also evident in HIV infections, where the depletion of $CD4^+$ T-cells renders individuals susceptible to CMV infection.

B-cell function is also important in controlling infection. During primary infection, CMV specific immunoglobulin (Ig) M antibodies are produced first, later followed by IgG antibodies. IgM antibodies may be found as early as 4-7weeks and may persist as long as 16-20 weeks after initial infection, while IgG antibodies are lifelong (Middeldorp et al., 1984). Antibodies have been identified with specificity for many proteins. The majority of neutralising antibodies are directed against envelope glycoprotein B (gB). Studies have shown that more than 50% of neutralising activity in serum is directed against gB (Boppana and Britt, 1996). However, virion tegument proteins such as ppUL32 (pp150), ppUL99 (pp28) and ppUL82 (pp65) evoke strong and potent antibody responses (Britt et al., 1990, Landini et al., 1988).

1.5. MODULATION OF HOST RESPONSES BY HCMV.

As a large and complex DNA virus, HCMV encodes many ORFs that express proteins dispensable for productive replication of infectious virus in culture. These so called 'non-core' genes are thought to potentially play a role during initial infection of the host and in the modulation of the immune response. A significant number of these non-core genes have already been identified as modulators of the human immune system and of cellular defence mechanisms, Table 1.2. HCMV has co-evolved with the human immune system and this close evolutionary interplay has allowed the virus to develop an array of immuno-modulatory functions, Figure 1.7. The virus has shown a vast array of functions that

Table 1.2. HCMV gene implicated in immune modulation.

Aspect of the immune system	HCMV gene
Downregulation of MHC-1	US2, US3, US6, US11
Downregulation of MHC-II	US2, US3
NK cell evasion	UL16, UL18, UL40, UL83, UL141, UL142, miR-UL112,
Chemokine receptor	US28, UL33, UL78
TNFR homologue	UL144
IL-10 homologue	UL111A
Virokine	UL146, UL147
RANTES-binding	UL22A
Inhibitors of apoptosis	UL123 (IE1), UL122 (IE2), UL36, UL37
Interferon response	IRS1, TRS1, UL83

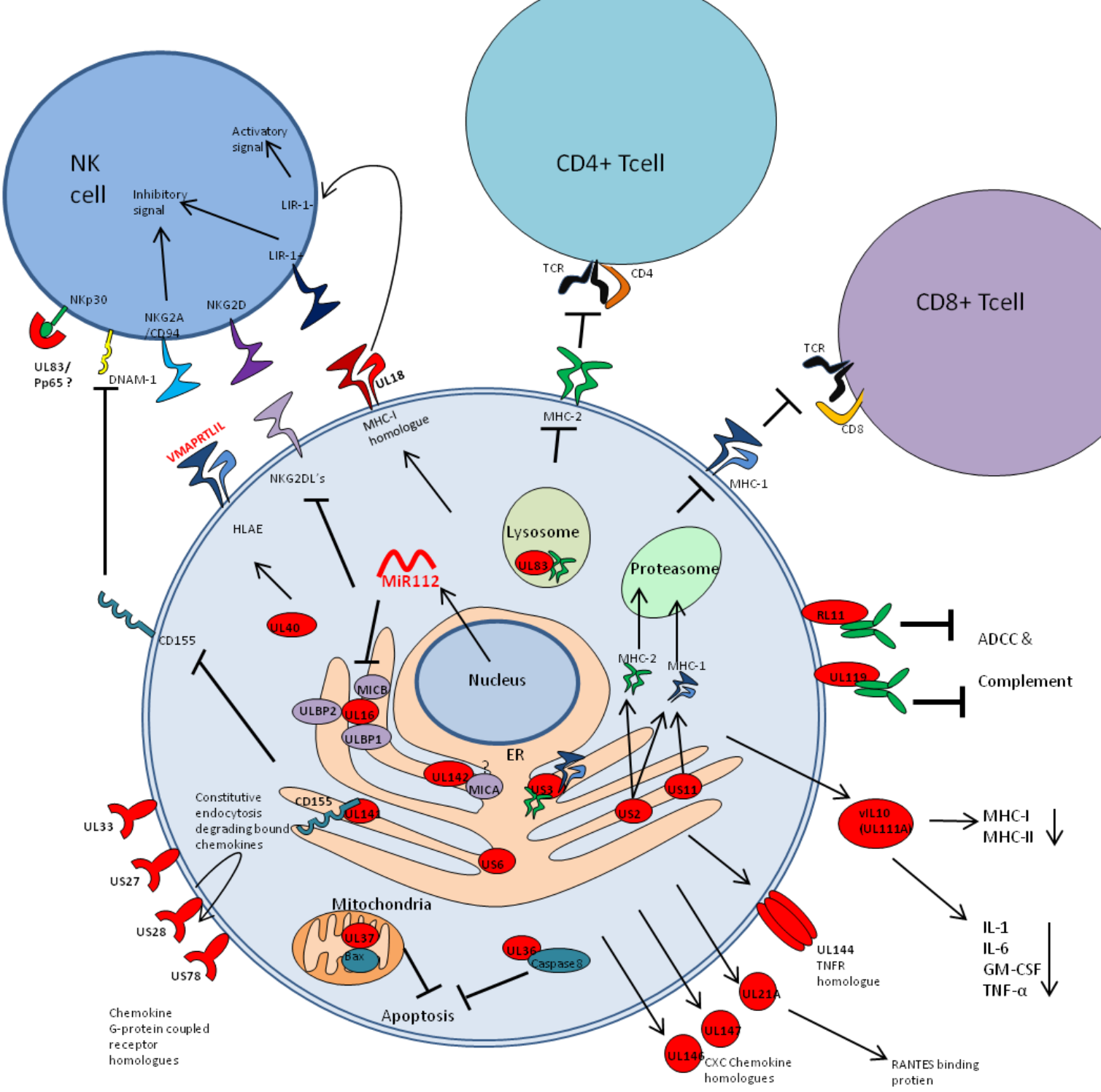


Figure 1.7. Overview of HCMV immunomodulation of infected cells.
UL18 encodes a MHC-1 homologue that inhibits LIR1+ NK cells, however activates LIR1- NK cells. UL16, UL142 and miR-Ul112 all act in concert to suppress cell surface presentation of NKG2D ligands represented here by MIICA, MICB, ULBP1 and ULBP2. UL40 upregulates the cell surface expression of HLA-E, a non-classical MHC-1 molecule. UL141 binds a sequesters CD155 in the ER preventing NK activation through DNAM-1 receptor. UL83 prevents NK activation by binding NKp30, although the as UL83 is not expressed at the infected cells surface it is unclear how this works in vivo. UL83 has also been shown to target MHC-II to the lysosome for degradation. MHC-II cell surface expression is also inhibited through ER sequestration of HLA-DR by US3 and US2 mediated degradation. MHC-I cell surface expression is inhibited byUS2 and US11 mediated proteasome degradation, US3 ER sequestration and US6 prevents TAP mediated peptide loading of MHC-I molecules. HCMV also encodes chemokine receptors US27 US28, UL33 and UL78. TNF-receptor homologue UL144, IL-10 homologue UL111A, Virokines UL46 and UL147. RANTES-binding protein UL22A. Apoptosis is also inhibited through the action of UL36 and UL37. All mechanisms are discussed in more detail in section 1.5.

interfere with many aspects of the infected cell from protein mimicry to targeted degradation; and it is these mechanisms that allow the long term survival of HCMV.

1.5.1. Modulation of the cell cycle

The phosphoproteins ppUL69 and ppUL82 are capable of imposing a G1/S-like block that deregulates the expression of many cell cycle regulated gene products (Lu and Shenk, 1999). Delivery of ppUL69, found within the virion, has been shown to impose a G1 block that is independent of synthesis of additional ppUL69 from the virion genome (Hayashi et al., 2000). Whereas, ppUL82 accelerates progression through the G1 phase and activates IE gene expression (Kalejta and Shenk, 2003). ppUL82 has also been shown to dysregulate the cell cycle by targeting members of the retinoblastoma family of proteins (p105, p107 and p130) for degradation; which are general repressors of cell proliferation (Kalejta et al., 2003).

1.5.2. Apoptosis

The IE1 (UL123) and IE2 (UL122) proteins have been shown to inhibit apoptosis by activating the PI3 kinase pathway via the cellular kinase Akt (Yu and Alwine, 2002). Additionally, IE2 appears to lead infected cells into senescence after arresting the cell cycle and inhibiting apoptosis thought to occur through the p16 (INK4a) and p53 pathways (Noris et al., 2002).

The viral non-coding RNA, β2.7, binds mitochondrial complex I and prevents the relocalisation of the essential subunit genes associated with retinoid/interferon-induced mortality-19 (GRIM-19) complex, thereby inhibiting proapoptotic signals (Reeves et al., 2007). This interaction stabilises mitochondrial membrane potential allowing the maintenance of ATP production late into infection, and is critical for the successful completion of the virus life cycle (Reeves et al., 2007).

UL36 encodes for a viral inhibitor of caspase activation (vICA), which has been shown to protect cells from extrinsic apoptosis induced via the death receptors TNFR1, Fas/CD95 or Trail (Park et al., 1996). Expression of ppUL36 inhibits Fas-mediated apoptosis via the binding to the pro-domain of caspase-8, preventing its activation (Skaletskaya et al., 2001).

HCMV also encodes a viral mitochondrial inhibitor of apoptosis (vMIA), encoded for by UL37. Two independent studies identified UL37 to bind with Bax in the mitochondria via a UL37-Bax complex, preventing Bax-mediated mitochondrial membrane permeabilisation and consequent cytochrome C leakage (Arnoult et al., 2004, Poncet et al., 2004).

UL38 also inhibits apoptosis and encodes a cell death protein (Terhune et al., 2007). Although the mechanism of UL38 action is unclear, it is thought that UL38 supports HCMV infection at least in part by blocking cellular responses to stress. TSC1/2 integrates stress signals and regulates mammalian target of rapamycin complex 1 (mTORC1), a protein complex that responds to stress by limiting protein synthesis and growth. pUL38 has been shown to interact with TSC1 and TSC2 in HCMV infected cells (Moorman et al., 2008). Additionally three pathways, collectively termed the unfolded protein response (UPR), are activated in response to ER stress and will resolve in apoptotic cell death if cell stress cannot be alleviated. In infection, pUL38 has been shown to modulate the of PKR-like ER kinase (PERK) pathway, one aspect of the UPR, and the α subunit of eukaryotic initiation factor 2 (eIF-2α), as well as induce accumulation of activating transcriptional factor 4 (ATF4) and to suppress the persistent phosphorylation of c-Jun N-terminal kinase (JNK) (Xuan et al., 2009). These activities appear crucial for protecting cells against ER stress induced by HCMV infection.

1.5.3. Cytokine and chemokine homologues

UL111a encodes a viral homologue of human IL10 (cmv-IL10) that shares only ~27% amino sequence identity with human IL10, but importantly has been shown to still signal through the IL10-receptor (Kotenko et al., 2000). Cmv-IL10 recovered from infected cell supernatants and Escherichia coli-derived recombinant –IL10 were both shown to inhibit proliferation of, and cytokine synthesis by, mitogen stimulated peripheral blood mononuclear cells (PBMC) with the potency of human IL10 (Rousset et al., 1992). Additionally, cmv-IL10 was shown to reduce cell surface expression of MHC class I and MHC class II on PBMC, analogous to human IL10 (Spencer et al., 2002). More recently cmv-IL10 has been described as down-regulating $CD4^+$ T-cell recognition of latently infected cells (Cheung et al., 2009).

UL144 is a TNF-alpha-like receptor. However, it does not bind any known TNF-related ligands (Benedict et al., 1999, Lurain et al., 1999). To date, only one ligand, the Ig superfamily member B and T lymphocyte attenuator (BTLA), has been shown to bind to the UL144 protein and inhibit T-cell proliferation *in-vitro* (Cheung et al., 2005). This may decrease the lymphocyte response to HCMV. More recently, UL144 has been shown to activate TNFR-activated receptor 6 (TRAF6) leading to NF-κB activation (Poole et al., 2006).

The HCMV gene UL146 encodes a CXC chemokine (vCXC-1), a 117 amino acid glycoprotein that, when secreted, has been shown to attract neutrophils, binds CXCR2, and induces calcium mobilisation and degranulation with similar potency to IL8 (Penfold et al., 1999). UL146 is termed a hypervariable protein and shows high degrees of variability within its coding sequence across multiple isolates (Hassan-Walker et al., 2004, Stanton et al., 2005). UL147 also encodes a CXC chemokine (vCXC-2). Unlike UL146, UL147 has only limited variability in the coding region for the mature protein, but is highly variable in the signal sequence of UL147.

The HCMV genome encodes four putative seven-transmembrane domain chemokine-like proteins that show sequence similarity with chemokine receptors belonging to the family of G-protein coupled receptors (US27, US28, UL33 and UL78). Although important in viral pathogenesis, little is known about the function of these proteins and most study has been aimed at US28.

The gpUS28 binds multiple CC chemokines, with highest affinity for fractalkine, but is also able to bind RANTES (Released upon Activation, Normal T-cell Expressed and Secreted), MCP-1 (Monocyte Chemo attractant protein-1) and MIP-1α and β (macrophage inflammatory protein) (Kledal et al 1998; (Kuhn et al., 1995, Gao and Murphy, 1994, Kledal et al., 1998, Billstrom et al., 1998, McLean et al., 2004). Located in endocytic vesicles, gpUS28 undergoes constitutive endocytosis and recycling to the cell surface, resulting in continuous internalisation of chemokines by gpUS28 and a reduction of extracellular chemokine concentration (Bodaghi et al., 1998, Fraile-Ramos et al., 2001). Following interaction with chemokines, gpUS28 couples with specific G proteins and, through these associations, activates intracellular calcium flux and the MAP kinase pathway thereby modulating the activity of transcription factors NFAT (nuclear factor of activated T-cells) and CREB (cAmp response element) (Billstrom et al., 1998, McLean et

al., 2004). gpUS28 has also been implicated in vascular smooth muscle cell migration (Streblow et al., 1999).

Additionally, HCMV also encodes a small soluble secreted RANTES binding protein, pUL21A, which acts as a decoy receptor (Wang et al., 2004). pUL21A and pUS28 may work in harness to suppress chemokine function during productive HCMV infection.

1.5.4. Modulation of the Interferon response

Interferon's (IFNs) are multifunctional cytokines with antiviral activity that are rapidly induced and released from infected cells. Released IFN then binds target cells and induces an antiviral state. HCMV is generally considered to be relatively resistant to IFN. However, in cell culture, IFN treatment can inhibit viral production (Taylor and Bresnahan, 2005). While mice lacking the IFN receptors suffer greatly increased mortality following MCMV infection (Presti et al., 1998).

Binding and cell entry of HCMV, or treatment with gB or gH, triggers a proinflammatory transcriptional profile resembling an alpha/beta interferon (IFNα/β) response, even in the absence of de-novo viral gene expression (Zhu et al., 1998, Boyle et al., 1999). The virion associated proteins are recognised by the cell as pathogen-associated molecular patterns (PAMPs) resulting in the activation of IRF3 and NF-κB. Activation of the IFN-like response is controlled by NF-κB, which may be beneficial to viral infection due to the large distribution of NF-κB binding sites on the viral genome therefore driving transcription of viral genes (Preston et al., 2001, Netterwald et al., 2005).

HCMV infection results in decreased accumulation and phosphorylation of the IFN signalling kinase STAT2 through pUL123 (Paulus et al., 2006), which inhibits transcriptional activation of IFN-regulated genes, Figure 1.8. Infection also causes a reduction in the abundance of p48/IRF9, a component of the ISGF3 transcription factor complex. During infection HCMV IE and/or E genes disable Jak/STAT signal transduction by decreasing JAK-1 expression, which inhibits IFNγ stimulated signal transduction, (Miller et al., 1998a)Figure 1.8. Inhibiting IFNγ signalling prevents the up-regulation of ISG class II transactivar (CIITA) and thereby preventing protein-protein interactions between CIITA and classII promoter-binding proteins that are believed to be required for MHC-II transcription.

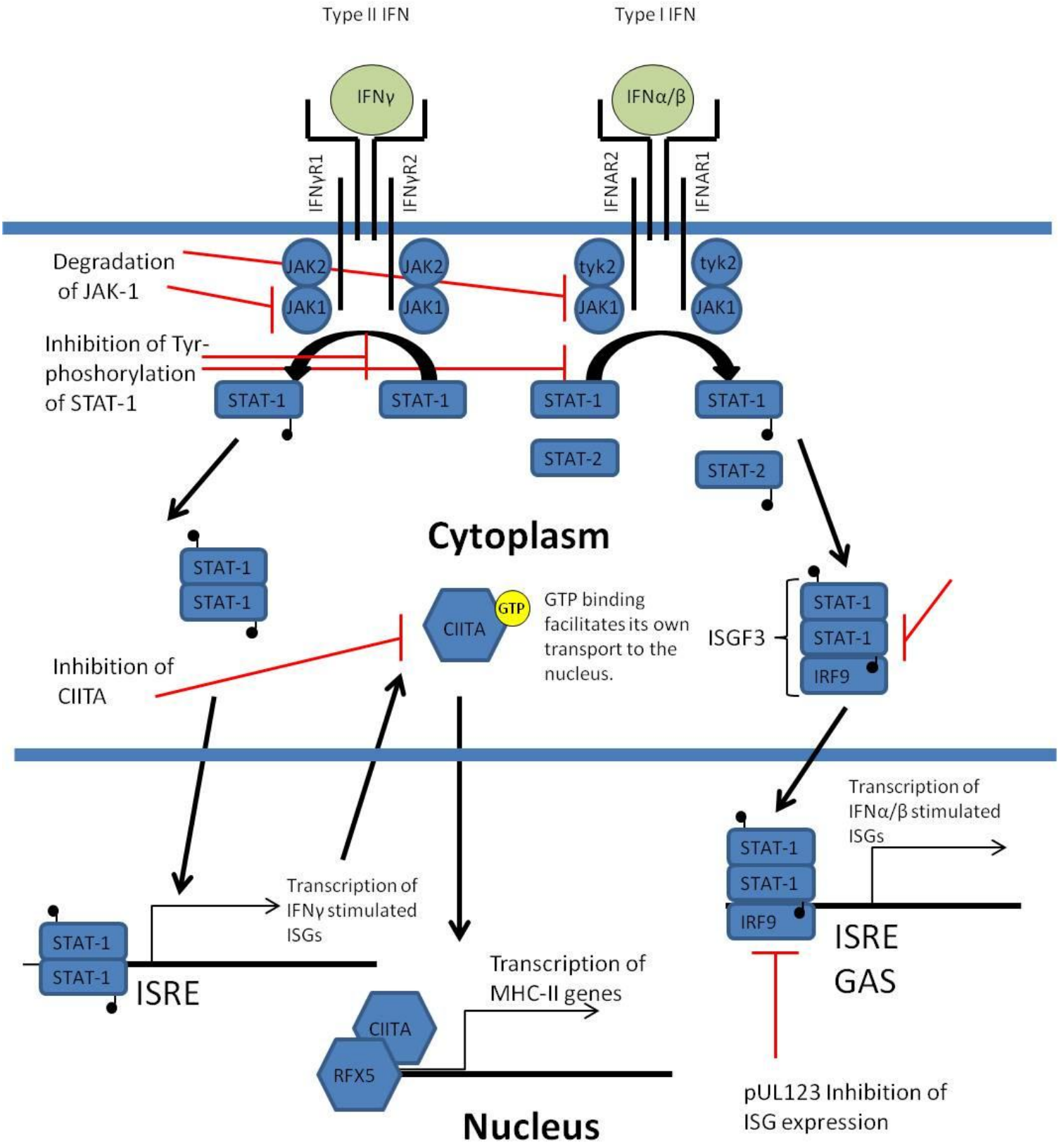


Figure 1.8. Summary of HCMV inhibition of IFN signalling through the Jak/STAT pathway.
In type II IFN signalling the activation, tyrosine phosphorylation by JAK-1 activates STAT-1 which homodimerises and translocates to the nucleus where it activates IRSEs for ISGs involved in the expression of MHC-II genes such as CIITA that promotes the expression of MHC-II. During infection HCMV IE and/or E genes disable Jak/STAT signal transduction by decreasing JAK-1 expression, which inhibits IFNγ stimulated signal transduction. HCMV has also been shown to inhibit STAT-1 and prevents the upregulation of CIITA. In type I IFN signalling JAK1 and STAT1 are also inhibited. pUL123 inhibits transcriptional activation of ISGs. HCMV infection also results in a reduction in the abundance of IRF9, a component of the ISGF3 transcription factor complex.

As HCMV progresses through the infection cycle, phosphoprotein ppUL83 (pp65) accumulates in both the nucleus and cytoplasm and has been reported to prevent IFN response. Two similarly designed experiments utilising *in-vitro* infection of HCMV with a deletion of ppUL83, or WT virus agree in concluding that ppUL83 delivered to the cell with the viral tegument functions to block IFN pathway genes very early after entry (Abate et al., 2004, Browne and Shenk, 2003). However, whether the effect of ppUL83 is to block IRF3, IRF1 and/or NFκB is not clear (Abate et al., 2004, Taylor and Bresnahan, 2005, Browne and Shenk, 2003, DeFilippis et al., 2006).

Of those ISGs transcribed as a result of IFN signalling, Protein kinase R (PKR) is an ISG that senses dsRNA and activates cellular pathways that result in the shutdown of protein synthesis and activation of the IFN response. As a result of a conformational change induced by binding to dsRNA, PKR dimerises and phosphorylates translation initiation factor eIF2α on serine 51. Phosphorylated eIF2α results in the formation of an inactive complex that involves eIF2α–$tRNA^{met}$–GTP ternary complex preventing translation initiation, and entirely eliminating viral replication. HCMV proteins pIRS1 and pTRS1 are present in the virion and expressed from immediate early times throughout infection (Romanowski et al., 1997). Both have been identified as dsRNA binding proteins required for viral evasion of Protein Kinase R (PKR). Infection with HCMV lacking both IRS1 and TRS1 results in phosphorylation of eIF2α and the shutdown of translation (Hakki and Geballe, 2005). Thus, the PKR pathway creates an intrinsic antiviral environment and IRS1 and TRS1 serve essential functions in preventing PKR binding to dsRNA preventing the shutdown on translation and induction of IFN response.

1.5.5. Protection from complement

HCMV encodes two IgG Fc receptors, RL11 and UL119, which are present on the plasma membrane of infected cells and on the envelope of the virions (Atalay et al., 2002, Lilley et al., 2001, Murayama et al., 1989). IgG-Fc receptors bind the constant Fc region of IgG molecules. A process called antibody bipolar binding and is thought to protect the virus from the complement pathway. Antibody bipolar binding is described as the Fc receptor binding the Fc region of an immunoglobulin molecule while the Fab region binds to its antigen on the virion. This process has been shown to resist complement-mediated

neutralisation and antibody-dependent cellular cytotoxicity (ADCC) in HSV (Dubin et al., 1991).

HCMV also controls the complement pathway by up-regulating the host regulators of complement activation CD55 and CD46 (Spiller et al., 1996). Host regulators of complement activation suppress the activation of C3 convertase on virus-infected cells providing a mechanism to protect HCMV-infected cells from complement mediated lysis. This technique is also utilised by virions as CD55 and another complement control protein, CD59, are incorporated into the virion envelope (Spear et al., 1995).

1.5.6. Modulation of antigen presentation

$CD8F^+$ T-cell recognition relies on the interaction of the T-cell receptor with antigen presented on Major histocompatibility complex (MHC) class I proteins and costimulatory complex on target cells. During their formation, newly synthesised MHC class I heavy chain associate with β2m in the ER. The heterodimer then associates with the transporter associated with antigen presentation (TAP) and other antigen processing machinery components to form a 'peptide loading complex'. In the cytosol, antigenic peptides processed by the proteasome are delivered to the ER lumen via the TAP complex, which is physically linked to the MHC class I molecule through the ER resident protein tapasin, which, in turn, facilitates peptide loading onto the MCH class I/β2m heterodimer. The trimeric complex is then transported through Golgi compartments to the cell surface where it can be recognised by surveying T-cells.

$CD4^+$ T-helper cell recognition relies on the interaction of the T-cell receptor with antigen presented on MHC class II proteins. The priming of HLA II-restricted $CD4^+$ T-cells by professional antigen presenting cells (APCs) is crucial for the control of HCMV infection (Gamadia et al., 2003). These cells are direct antiviral effectors and also provide help for maintenance of $CD8^+$ T-cell antiviral response. Exogenous antigen is endocytosed by an APC and destroyed by the endosomal and lysosomal proteases into peptide fragments containing 13~18 amino acid residues. The nascent assembled HLA II molecule associated with an accessory peptide, the invariant chain or Ii occupied the binding groove in the ER where it prevents MHC-II molecules from loading exogenous peptide in the ER lumen, and directs transport through the Golgi apparatus to the endosome, where Ii is destroyed by

proteases to yield a fragment called CLIP bound to HLA II molecules. CLIP is then removed by the HLA-DM, freeing all HLA-II molecules to load peptides and the HLAII/peptide complexes are transported to the cell surface where they can be recognised by $CD4^+$ T-cells.

HCMV infection results in the down-regulation of both MHC class I and class II thereby inhibiting antigen presentation to $CD8^+$ MHC class I restricted cytotoxic lymphocytes and $CD4^+$ MHC class II restricted T-helper lymphocytes. Four HCMV gene products have been implicated in the modulation of MHC class I. While two viral proteins have been shown to down-regulate both MHC- class I and class II.

1.5.6.1. Modulation of MHC class I

CD8+ T-cell recognition relies on the interaction of the T-cell receptor with antigen presented on MHC class I proteins and costimulatory complex on target cells. A cluster of genes located in the US region of HCMV (US2-11) have been identified as immune-modulatory glycoproteins (gpUS2, gpUS3, gpUS6 and gpUS11), which all act to decrease the cell surface expression of MHC class 1 proteins at various stages of the maturation process. Each of these four glycoproteins are expressed during viral infection and each is independently capable of preventing MHC class I expression at the infected cells surface. The reduction of MHC class I expression at the surface of infected cells effectively prevents the presentation of endogenous peptides to $CD8^+$ CTLs, thereby impeding cytotoxic T-cell immune surveillance, Figure 1.9. (Hengel et al 1995).

1.5.6.1.1. US2

Detected 12 hours p.i., the US2 transcript increases in abundance throughout infection (Park et al., 2002). The product of US2, gpUS2, is an ER resident, 24 kDa type I membrane glycoprotein that targets MHC class I molecules for proteasome-dependent degradation (Gewurz et al., 2002). An Ig-like ER-luminal domain of gpUS2 is essential for this process and engages the α2/α3 domain of the MHC class I heavy chain (Gewurz et al., 2001a, Gewurz et al., 2001b). Using a truncated mutant of gpUS2, Furman et al (2003) demonstrated the need for the cytoplasmic domain of US2 in catalysing the degradation of MHC class I heavy chains. The cytosolic domain was later shown to catalyse the

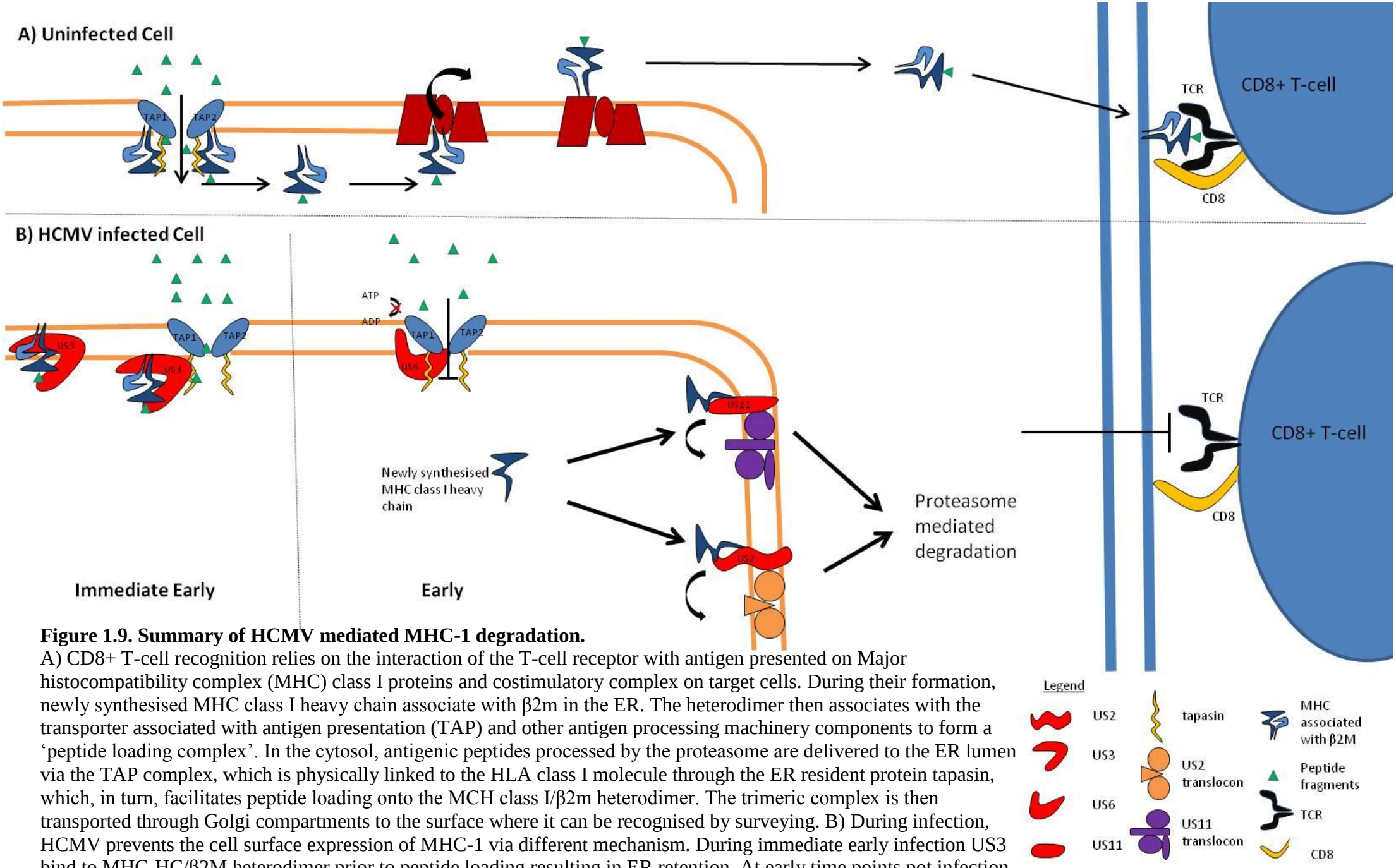


Figure 1.9. Summary of HCMV mediated MHC-1 degradation.
A) CD8+ T-cell recognition relies on the interaction of the T-cell receptor with antigen presented on Major histocompatibility complex (MHC) class I proteins and costimulatory complex on target cells. During their formation, newly synthesised MHC class I heavy chain associate with β2m in the ER. The heterodimer then associates with the transporter associated with antigen presentation (TAP) and other antigen processing machinery components to form a 'peptide loading complex'. In the cytosol, antigenic peptides processed by the proteasome are delivered to the ER lumen via the TAP complex, which is physically linked to the HLA class I molecule through the ER resident protein tapasin, which, in turn, facilitates peptide loading onto the MCH class I/β2m heterodimer. The trimeric complex is then transported through Golgi compartments to the surface where it can be recognised by surveying. B) During infection, HCMV prevents the cell surface expression of MHC-1 via different mechanism. During immediate early infection US3 bind to MHC-HC/β2M heterodimer prior to peptide loading resulting in ER retention. At early time points pot infection US6 inhibits ATP binding to TAP, preventing TAP mediated delivery of cytosolic peptides to the ER for loading. While both uS2 and US11 both target MHC-I for proteasome mediated degradation.

degradation of MHC class I by recruiting p97 ATPase into the ER associated degradation complexes (Chevalier and Johnson, 2003). P97 ATPase binds polyubiquinated proteins and functions by extracting substrates from the ER membrane before the substrate meets the proteasome (Rabinovich et al., 2002). However, whilst ubiquitination of MHC class I occurs when gpUS2 and MHC class I are bound, it was not found to be required for the initial steps of US2 mediated dislocation from the ER (Furman et al., 2003).

1.5.6.1.2. US3

gpUS3 is expressed as an 22kDa IE glycoprotein, but through alternative splicing can produce two smaller products,17kDa and 3.5kDa, US3 products are produced, which function as a dominant negative inhibitor of the full length, 22kDa, form (Liu et al., 2002, Shin et al., 2006, Ahn et al., 1996). gpUS3 forms a complex with β2m associated MHC class I heavy chains with high affinity (Ahn et al., 1996, Jones et al., 1996). This impairs the maturation and intracellular transportation of MHC heavy chains by binding to the MHC-HC/β2m heterodimer, prior to peptide loading in the ER(Lee et al., 2003). Consequently, the assembled MHC/peptide complex is retained in the ER, preventing translocation to the cell surface due to a retention signal in US3 (Lee et al., 2003). This mechanism causes a lack of MHC-1 molecules at the cell surface and means infected cells cannot be detected by T-cells that use MHC-1/foreign peptide complexes to illicit a cytotoxic response. However, US3 has also been shown to bind to tapasin, inhibiting tapasin-dependent loading of peptide to MHC class I molecules (Park et al., 2004). This mechanism only affects those HLA alleles that are dependent on tapasin for their surface expression (Park et al., 2004). Other HLA allelic products that are not tapasin dependant could express at the cell surface on US3-expressing cell lines (Park et al., 2004).

1.5.6.1.3. US6

gpUS6 contains a double-arginine motif, an ER retention signal in the C-terminal cytoplasmic domain. US6 inhibits the ATP binding to the subunit TAP1 (Hewitt et al., 2001). As a consequence, the TAP-mediated delivery of cytosolic peptides to the ER for loading to the 'peptide loading complex' is impaired. Thus TAP-dependant peptides are

unable to be loaded, resulting in retention of MHC class I in the ER and a consequent reduction of class I molecules at the surface.

1.5.6.1.4. US11

US11 encodes a type I membrane glycoprotein that resides in the ER and causes rapid dislocation of newly synthesised HLA class I heavy chains from the ER to the cytosol, where they are degraded by the proteasome. In a similar mechanism as described for US2 mediated degradation. HCMV gene products US2 and US11 differ in their ability to attack MHC class I, with US11 superior over US2 (Rehm et al., 2002). US11+ cells have been shown to reduce the half-life of newly synthesised MHC class I molecules from > 6 hours to < 2minutes (Wiertz et al., 1996). Like US2, the US11 transcript can be detected as early as 12hrs post infection (Park et al., 2002).

Heavy chain dislocation by US11 is mediated by its transmembrane domain, which contains a glutamine residue at position 192 critical for dislocation but not for interaction with MHC-1 (Lilley et al., 2003). However, unlike US2 tertiary structure has no effect on US11 mediated degradation. Screening of cellular proteins interacting with US11 but not the Gln-mutant identified Derlin-1 as an interacting protein for US11 (Lilley and Ploegh, 2004). In yeast the Derlin-1 homologue is required for the degradation of a subset of ER proteins (Lilley and Ploegh, 2004). Derlin-1 was identified as a multiple transmembrane protein responsible for recruiting to the ER the cytosolic ATPase p97, a protein required for reterotranslocation (Ye et al., 2004).

1.5.6.2. Modulation of MHC class II

Unlike MHC-I proteins, which are ubiquitously expressed, MHC-II proteins are mainly restricted to APCs, B-cells, macrophages and DCs, that take up and express exogenous antigen to $CD4^+$ T-cells. IFN signal transduction (JAK/STAT pathway) and class II transactivator (CIITA) are required for IFN stimulated MHC-II expression on non APCs. IFNγ is the most potent inducer of MHC-II expression in non APCs, such as endothelial cells and fibroblasts (Boss, 1997).

IFNγ signal transduction (JAK/STAT pathway) and class II transactivator (CIITA) are required for IFNγ stimulated MHC-II expression. CTIIA is believed to activate transcription by interacting with ubiquitous DNA binding proteins at MHCII promoters (Steimle et al., 1994, Jabrane-Ferrat et al., 1996, Moreno et al., 1995). MHC-II-α and –β chains are assembled in the ER with an invariant chain (Li) that prevents loading of peptides. The α/β/Ii complexes are then transported to the MHC-II compartment, a lysosome –like compartment where α/β dimers are loaded with peptides, normally derived from extracellular protein. HCMV infects MHC-II expressing monocyte/macrophage and endothelial cells and prevents MHC class II expression at the cell surface by varying mechanisms in both APCs and non-APCs (Lee et al., 2006, Soderberg-Naucler et al., 1997, Soderberg-Naucler et al., 1998).

Two HCMV genes target both MHC-I and MHC–II: gpUS2 binds to both HLA-DRα and HLA-DMα in the ER and promotes their degradation via the proteasome (Hegde and Johnson, 2003, Tomazin et al., 1999) . gpUS3 binding to class II α/β complexes preventing their association with the invariant chain Ii (Hegde et al., 2002). The gpUS3-MHC-II complex moves from the ER to the Golgi apparatus bypassing the MHC-II loading compartment, resulting in a reduction of peptide-loaded MHC class II complexes at the cell surface. MHC-II transcription is also suppressed by HCMV infection causing the degradation of the cellular kinase JAK, which in turn inhibits a key transcription factor (CTIIA) (Miller et al., 1998b). cmvIL10 has also been shown to down-regulate MHC class II expression on macrophages (Kotenko et al., 2000, Redpath et al., 2001). HCMV ppUL83 has also been reported to promote degradation of MHC class II by mediating the accumulation of HLA-DR in lysosomes and promoting the destruction of the HLA-DR α chain (Odeberg et al., 2003).

1.6. MODULATION OF NATURAL KILLER CELL RESPONSE BY HCMV.

Natural Killer (NK) cells comprise of ~10% of circulating lymphocytes and participate in early innate immune defence against viral infections and tumour transformation. These cytotoxic lymphocytes do not require antigen-specific recognition to lyse target cells but instead require an overriding activating signal to achieve effector function. The activation of signalling pathways results in the development of cell-mediated killing through the release of cytotoxic granules containing perforin and granzyme, as well as the expression of cytokines and chemokines that modulate subsequent steps in the adaptive immune response. NK receptors do not undergo somatic recombination like TCR genes of T-cells, or Ig genes of B-cells. Instead they are diverse and rapidly co-evolving with their ligands. Mature NK cells do not express CD34 like other lymphocytes, and are minimally defined by the $CD3^{-}CD56^{+}$ phenotype. Although they do not express CD34, NK cells are derived from CD34 lymphocytes as Cytolytic NK cells can be derived *in-vitro* from $CD34^{+}$ progenitors (Miller et al., 1992). Previous studies have demonstrated the presence of two distinct subsets of NK cells based on different levels of expression of CD56 (Cooper et al., 2001). $CD56^{Dim}$ NK cells express high levels of CD16 ($CD56^{Dim}CD16^{Bright}$) and function as potent cytolytic effector cells representing approximately 90% of NK cells in normal PBMC.

In contrast, $CD56^{bright}$ cells do not express CD16 and are not potent cytolytic effector cells. $CD56^{bright}$ cells appear to have more of a regulatory role, secreting large amounts of cytokines, including INF-γ TNF-α, lymphotoxin and IL-10(Farag and Caligiuri, 2006). NK cells therefore, have a role in inflammatory reaction(s) typically initiated as a result of the detection of foreign tissue, and are also implicated in inflammatory reaction(s) triggered by non-foreign tissue such as in autoimmunity. $CD56^{bright}$ NK cells are predominately present in lymph nodes where they appear to interact with both dendritic cells and T-cells to enhance antigen presentation (Fehniger et al., 2003).

1.6.1. NK cytotoxicity

MHC class I receptor:ligand interaction can induce inhibitory signals that represent a major failsafe mechanism preventing killing of normal MHC class I autologous cells. In contrast down-regulation or loss of MHC class I expression, during viral infection or

carcinogenesis, shifts the balance towards NK cell activation and target cell destruction by removing this inhibitory signal. Thus NK cells are tolerant towards host cells, but in disease or infection this tolerance can be readily broken. This theory where 'loss' of self antigen, namely MHC class I, leads to NK cell activation was initially described as the 'missing self hypothesis'. However, with greater appreciation of cell biology, this theory has been fine-tuned. NK cell function is determined by multiple competing activating and inhibiting receptors. The main ligands for NK these receptors are MHC class I and class-I like molecules, for example, HLA-A, -B, -C, -E, MICA, MICB and others, Figure 1.10. The inhibitory receptors counteract activating receptor signals and lead to NK cell inhibition. However, precisely how signals from competing activating and inhibitory receptors are integrated and resolved is not completely understood.

1.6.1.1. Inhibitory NK receptors

Several inhibitory receptors have been identified, but there are two main families involved in human NK regulation: the killer cell Immunoglobulin-like receptors (KIRs) and the C-type lectin-like receptors e.g. CD94/NKG2A.

In humans, killer immunoglobulin like receptors (KIRs) are primarily responsible for the recognition of allotypic determinants that are shared by different MHC class I alleles. The KIRs are type I membrane proteins encoded on chromosome 19 in a region called the leukocyte receptor complex (LRC), which spans approximately 1Mb (Wende et al., 1999). They contain either 2 or 3 extracellular domains (KIR2D or KIR3D, respectively) and are further grouped according to their function, which may be inhibitory or stimulatory. Inhibitory KIRs have a long cytoplasmic tail (L) and contain 1 or 2 Immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Dohring et al., 1996, Lanier, 1998). The best characterised members of this family are the inhibitory KIRs with specificity for HLA-C ligands. HLA-C molecules are divided into type I and type II allotypes that differ in an asparagine or a lysine residue at aa 80 respectively. KIR2DL2 and KIR2DL3 bind type I HLA-C allotypes, while KIR2DL1 binds type II HLA-C allotypes (Winter et al., 1998). Other inhibitory KIRs have specificity for determinants of HLA-B (KIR3DL1), HLA-A (KIR3DL2) and HLA-G (KIR2DL4) (Dohring et al., 1996, Rajagopalan and Long, 2005, Long and Rajagopalan, 2000).

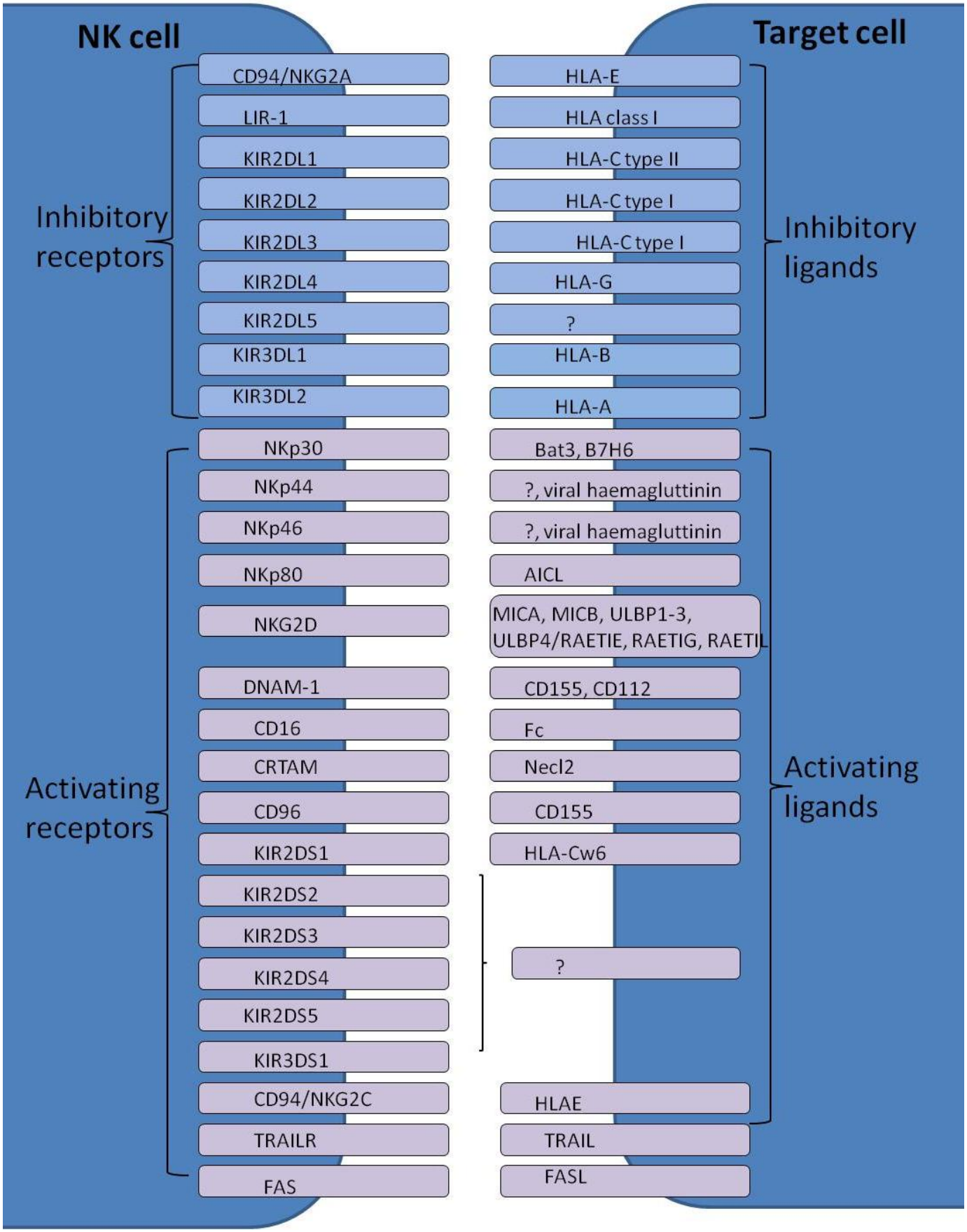


Figure 1.10. Selection of NK cell activatory and inhibitory receptors and their cognate ligands. NK activation programmes result from the integration of multiple activating and inhibitory signals that vary depending on the interacting cell. Inhibitory receptors on the NK cell are in blue and their cognate ligand expressed on the target cell are also in blue. Activatory receptors are in purple and their cognate ligand expressed on the target cell is also in purple., Selection of activatory and inhibitory ligands and their cognate ligands. Adapted from Viver et al (2008)

1.6.1.2. Activating NK receptors

Activating receptors can be split into two categories. Those that recognise MHC class I alleles, predominantly activating KIRs, and those that recognise non-MHC ligands such as the natural cytotoxicity receptors (NCRs).

Activating KIRs are primarily responsible for the recognition of allotypic determinants shared by different MHC class I alleles. The external part of the molecule interacts with ligands on the target cells, while the internal part of the molecule contains a short cytoplasmic tail and associates with adaptor molecules that provide the activating signal through Immunoreceptor tyrosine-based activatory motifs (ITAMs). Activatory KIRs include KIR2DS1 that binds HLA-Cw6, as well as KIR2DS2, KIR2DS3, KIRR2DS4, KIR2DS5 and KIR3DS1, all of which have no known activatory ligands (Stewart et al., 2005).

Activating receptors that recognise non-MHC ligands on target cells include the natural cytotoxicity receptors (NCRs), NKG2D, DNAM-1, CD16 among others, Figure1.10.

The NCRs comprise of NKp30, NKp44 and NKp46 (Pende et al., 1999, Vitale et al., 1998, Sivori et al., 1997, Pessino et al., 1998). NCRs exhibit no homology to each other but expression is restricted to NK cells. NKp30 is a polypeptide characterised by an extracellular region forming an Ig like domain connected to a transmembrane domain by a region of hydrophobic amino acids (Pende et al., 1999). The ligands for NKp30 include HLA-B associated transcript 3 (BAT3) and B7-H6 (Pogge von Strandmann et al., 2007, Brandt et al., 2009). NKp46 is characterised by 2 extracellular Ig domains (Pessino et al., 1998), while no cellular ligand has been identified for NKp46, it has been shown to bind to viral haemagluttinin (Mandelboim et al., 2001). Both NKp30 and NKp46 associate with the CD3ζ adaptor proteins that contain ITAM motifs (Pende et al., 1999, Pessino et al., 1998). The third NCR, NKp44, is a glycoprotein that associates with adaptor protein DAP12. Unlike the other NCRs, NKp44 is selectively expressed by IL2 activated NK cells (Vitale et al., 1998).

NKG2D is an activating receptor expressed on all NK cells, some T-cells and activated macrophages and associates with the adaptor protein DAP10 (Bauer et al., 1999, Wu et al., 1999) (Bauer et al 1999, Wu et al 1999, Li et al 2001). NKG2D has been shown to bind multiple MHC class I-like molecules. These include MICA, MICB, ULBP1-3,

ULBP4/RAETIE, RAETIG, RAETIL (Steinle et al., 2001, Cosman et al., 2001, Bacon et al., 2004).

DNAM-1 is a transmembrane Ig-like molecule expressed in NK-cells, monocytes, T-cells and platelets. Two DNAM-1 ligands have been identified, CD155 and CD112. Other activating receptors include CD16 that binds the Fc portion of IgG, CRTAM that recognises Necl2 and CD96 (Tactilie) that recognises CD155. Further activating ligands can be found summarised in Figure 1.10.

1.6.2. Modulation of NK cell function

The HCMV gene UL40 was the first virus gene shown to definitively protect against NK cell attack through the up-regulation of cell surface HLA-E, a non-classical MHC-1 molecule (Ulbrecht, 2000, Tomasec P, 2000, Wang, 2002). Since then more HCMV genes have been shown to modulate NK cell function. To date this includes six HCMV genes encoding proteins (UL16, UL18, UL40, UL83, UL141 and UL142) and one encoding a microRNA (miR-UL112) have been indentified in HCMV immunomodulation of NK cells, table 3 taken from Wilkinson (2008).

Table 1.3. HCMV genes implicated in NK cell modulation. Taken from Wilkinson (2008).

HCMV gene	Comment	Acts on inhibitory receptor
UL18	MHC-I homologue	LIR1 (ILT2)
UL40	Up-regulates HLA-E	CD94/NKG2A
HCMV gene	**Comment**	**Acts on activating receptor**
UL16	Sequesters MICB, ULBP1-2	NKG2D
UL83	Direct binding	NKp30
miR-UL112	Suppresses MICB	NKG2D
UL141	Sequesters CD155, acts on CD112	DNAM-1, CD96
UL142	Acts on MICA	NKG2D

1.6.2.1. Modulation of NKG2D ligands

UL16, UL142 and miR-UL112 all act in concert to suppress cell surface presentation of NKG2D ligands (NKG2DL), which act as powerful activating-ligands for NK cells via signalling through the NKG2D receptors. The prevention of the surface expression of NKG2DLs allows the modulation of NK cell effector function by reducing NK cell activation. As well as NK cells, NKG2D is also expressed on IFN-producing killer dendritic cells, αβ T-cells and γδ T-cells and is associated with the signal transducing adaptor protein DAP12 (Bauer et al., 1999, Wu et al., 1999, Li et al., 2001). In contrast to the other NKG2 proteins, the NKG2D receptor is present as a homodimer on the surface of cells and is able to recognise a wide range of target cell ligands (NKG2DL) on target cells, including MICA, MICB, ULBP1-4, RAET1E and RAET1G (Eagle and Trowsdale, 2007). Unlike MHC class I molecules, MICA and MICB are expressed in response to stress, do not associate with β_2M and do not present peptide (Groh et al., 1996). Such stress events include viral infection and HCMV IE1 and IE2 proteins have been reported to be potent activators of these ligands (Routes et al., 2005, Tomasec et al., 2007).

The cellular UL16-binding protein (ULBP) family was identified and named on the basis of the ULBP1 and ULBP2 proteins to bind gpUL16. In total gpUL16 has been shown to directly bind and sequester MICB, ULBP1 and ULBP2 in the Endoplasmic Reticulum (ER), preventing cell surface expression and consequent NK cell activation (Cosman et al., 2001, Kubin, 2001, Welte, 2003).

Together with gpUL18, UL142 constitutes the HCMV UL18 gene family. Analysis predicted that UL142 encoded intact MHC class I related α1 and α2 domains and a truncated α3 domain. When expressed from a RAd vector, gpUL142 provided efficient protection against NK cytotoxicity in autologous NK cells, while siRNA knock-down of UL142 enhanced NK cell killing (Wills, 2005). gpUL142 has also been shown to down-regulate the cell surface expression of MICA, through gpUL142 retention in the cis-Golgi (Ashiru et al., 2009). However, the truncated MICA*008 allele was not down-regulated by gpUL142 (Chalupny et al., 2006).

Micro RNAs are associated with both gene silencing and stress responses. The ORF UL112 encodes for a microRNA miR-UL112 that mediates the knockdown of MICB expression by the 3' targeting of the MICB transcript (Stern-Ginossar, 2007). Deletion of the miR-UL112 function from strains AD169 or TB40/E enabled restoration of cell surface

MICB expression and was associated with enhanced sensitivity to NK cell recognition (Sten-Ginossar et al 2007). In addition, miR-UL112 has also been shown to down-regulate IE gene expression and has been hypothesised to be involved in the induction of latency (Murphy et al., 2008).

1.6.2.2. Modulation of LIR-1

UL18 was first identified as an MHC-I homologue during the original sequencing of the HCMV laboratory strain AD169 genome (Beck and Barrell, 1988). There are two forms expressed, one a 69kDa endoglycosidase H (EndoH)-sensitive species, and the other a fully processed 110-160kDa Endo-H resistant protein (Griffin et al., 2005, Kim et al., 2004). Two conflicting reports reported that gpUL18 was both able to activate and inhibit NK cells. One report showed that gpUL18 expressed in a class I negative cell line was capable of eliciting protection against NK cell cytolysis (Reyburn et al., 1997). While a second independent study showed that gpUL18 expressing cells were more efficiently killed by NK cells (Leong et al., 1998). This apparent conflict was later resolved in a complex analysis of NK clone activity against gpUL18 expressing targets (Prod'homme et al., 2007). It was observed that expression of UL18 inhibited LIR^+ NK cells, but stimulated LIR- NK cells (Prod'homme et al., 2007). This study is consistent with a direct interaction of gpUL18 with LIR-1 resulting in a suppression of NK cell function. Whilst also implying that an additional interaction that is LIR1-independent occurs in which gpUL18 promotes NK cell recognition.

1.6.2.3. Modulation of CD94/NKG2A

The nine amino acid sequence (VMAPRTLIL) in the UL40 leader sequence was identified as an exact match to a HLA-E binding peptide (Tomasec P, 2000, Ulbrecht, 2000). HLA-E is a non-classical MHC class I molecule that binds a restricted set of peptides derived from the leader sequences of classical MHC-I molecules and HLA-G. Following peptide binding, HLA-E is transported to the cell surface where it is recognised by the NK cell inhibitory receptor complex CD94/NKG2A, suppressing NK cytotoxicity (Lee et al., 1998). gpUS6 blocks transport of peptides to the ER by inhibiting TAP, thus inhibiting cell surface expression of MHC class I including that of HLA-E. To counter this effect, UL40

–derived peptide up-regulates the cell surface expression of HLA-E, independent of TAP (Tomasec et al., 2000). Deletion of UL40 renders infected cells more susceptible to NK cytotoxicity (Wang et al., 2002).

1.6.2.4. Modulation of NK cytotoxicity receptor NKp30

As described earlier, UL83 encodes an abundant HCMV tegument protein that acts immediately post infection to suppress the induction of the IFN response (Browne and Shenk, 2003). It has also been shown to directly bind the NK cell activating receptor NKp30, in order to suppress transmission of an activating signal through adapter molecule CD3ζ, preventing NK activation (Arnon et al., 2005). However, ppUL83 has not been shown to be secreted or expressed at the cell surface. It is hypothesised therefore, that ppUL83 suppression may require its release during lysis of infected cells by an, as yet, uncharacterised mechanism(Browne and Shenk, 2003).

1.6.2.5. Modulation of DNAM-1 and CD96

CD155 (Necl-5/PVR) is a nectin-like molecule involved in NK cell activation via engagement of DNAM-1 (CD226) and CD96 (TACTILE) NK activating receptors (Bottino, 2003, Tahara-Hanaoka et al., 2004). CD112 (nectin-2) is another ligand for DNAM-1 (CD226) but not CD96 (Fuchs et al., 2004). The HCMV gpUL141 suppresses cell surface expression of CD155 via retention in the Endoplasmic reticulum (ER) and expression of gpUL141 from a RAd resulted in an inhibition of NK cytotoxicity (Tomasec et al., 2005).

While signalling through DNAM-1 directly promotes NK cytolysis, CD96 does not directly promote NK killing, rather it increases the stability of the cell-cell contact (Fuchs et al., 2004). The increased stability of the cell-cell interactions allows for more efficient killing. CD96 was first identified as a T-cell activation antigen and originally termed Tactile (T-cell activation, increased late expression (Wang et al., 1992). CD96 is an Immunoglobulin superfamily member with homology to both CD155 and 22% homology to DNAM-1 (Wang et al., 1992, Shibuya et al., 1996).

1.7. ADHESION MOLECULES: INVOLVEMENT IN THE IMMUNE SYSTEM.

Adhesion molecules are a group of cell surface proteins involved in cell-to-cell binding or binding to the extracellular matrix (ECM). They play critical roles in many biological processes. In development they play key roles in cell migration, tissue morphogenesis and axon guidance. In the immune system they allow enhanced pairing between low avidity receptors and their ligands and some adhesion molecules have also been identified as activatory ligands in their own right. Other adhesion molecules can transmit signals that direct specific effector functions during the inflammatory response and some have been identified to play a major role in the processes of metastatic tumour dissemination.

Adhesion molecules are broadly divided into 4 main groups: Integrins, lectins, cadherins and the Ig-superfamily (Ig-SF) cell adhesion molecules (CAMs). All adhesion molecules are integral membrane proteins containing cytoplasmic, transmembrane and extracellular domains. The cytoplasmic domain often interacts with cytoskeletal proteins and/or accessory proteins to carry out function. The extracellular domain extends from the cell and binds to other cells, or the ECM, by binding to other adhesion molecules of the same or different type, or an intermediary linker.

1.7.1. Nectins and nectin like molecules: Members of the Ig-superfamily

The human nectin family members are part of the $Ca2^{+}$ independent Ig-SF family of cell adhesion molecules. The family can be subdivided into nectin and nectin-like (necl) molecules that have similar domain structures but differ in their ability to bind Afadin through a PDZ binding domain found in nectin, but not necl, molecules (Takai et al., 2003).

Both nectins and necls have been shown to play major roles in cell-cell adhesion, polarisation, movement and proliferation. Generally nectins are primarily involved in the regulation of various types of cell-cell contacts including adherens junctions (AJs) and the formation of tight junctions (TJs). While nectins localise to AJs, along with Cadherins, necls do not. Instead Necls appear to have more diverse functions including mediating interactions between axons and glial cells, regulation of cell movement and proliferation (Ikeda et al., 2004, Kakunaga et al., 2005, Spiegel et al., 2007, Takai et al., 2008b).

The nectins consist of four molecules: nectin-1, -2, -3 and -4 and Necls five molecules: Necl-1, -2, -3, -4 and -5. Initially, nectin1 and nectin2 were identified as cell adhesion molecules, shortly after so were nectin3 and nectin4 (Takahashi et al., 1999, Reymond et al., 2001, Satoh-Horikawa et al., 2000, Lopez et al., 1998). All nectin family members have two or three splice variants, nectin 1α, -1β, -1γ, -2α, -2δ, -3α, -3β, and 3-γ. Most nectins, with the exception of nectin -1β, -3γ and 4, contain a conserved 4 aa motif at their C-terminus (E/A-X-Y-V, with X being any amino acid) that binds to the PDZ domain of afadin, Figure 1.11. Nectin-4 lacks this conserved motif but is still able to bind the PDZ domain of afadin at its C-terminus (Reymond et al., 2001). Afadin is an actin filament-binding protein that has multiple domains allowing binding to many proteins and is an intermediary molecule associating nectin molecules with the actin cytoskeleton. There are two isoforms of afadin, I-Afadin is ubiquitously expressed, whereas s-afadin is mainly expressed in neural tissue, although it is expressed at low levels in various other tissue types (Mandai et al., 1997, Boettner et al., 2000).

Nectins and Necls predominantly form stable interactions with other members of the nectin family. Characteristically, nectins and necls are expressed as cis-homodimers on the plasma membrane and these homodimers participate in homophilic or heterophilic trans interactions with their partners on an adjacent cell, Figure 1.12. (Tachibana et al 2000). Together with members of the cadherin family, nectins form cell-cell contacts connected to the cytoskeleton (Tachibana et al., 2000, Takahashi et al., 1999). In addition to afadin, the cytoplasmic regions of nectins can also associate with tctex-1 (Mandai et al., 1997, Mueller et al., 2002). Thus far heterophilic interactions between nectin-3/nectin-1, nectin-3/nectin-2, nectin-1/nectin-4 and nectin-3/necl-5 have been identified, reviewed in (Takai et al., 2008a).

Originally nectin-1, -2 and -3 were termed poliovirus receptor related protein -1 (PRR-1), PRR-2 and PRR-3 due to their homology to poliovirus receptor (PVR) otherwise known as necl-5 or CD155. The extracellular domains of the PRR family contain three-Ig-like domains that share 30 to 555 amino acid identities. However, none of them were reported to actually serve as poliovirus receptors. PRR-1 and PRR-2 were later shown to serve as receptors for the alphaherpesvirinae and were termed HveC and HveB respectively (Geraghty et al., 1998, Warner et al., 1998). PRR-1/HveC, PRR-2/HveB and PRR-3 were then later assigned as CD111, CD112 and CD113 respectively. From now on the CD nomenclature will be used to refer to individual members of the nectin family.

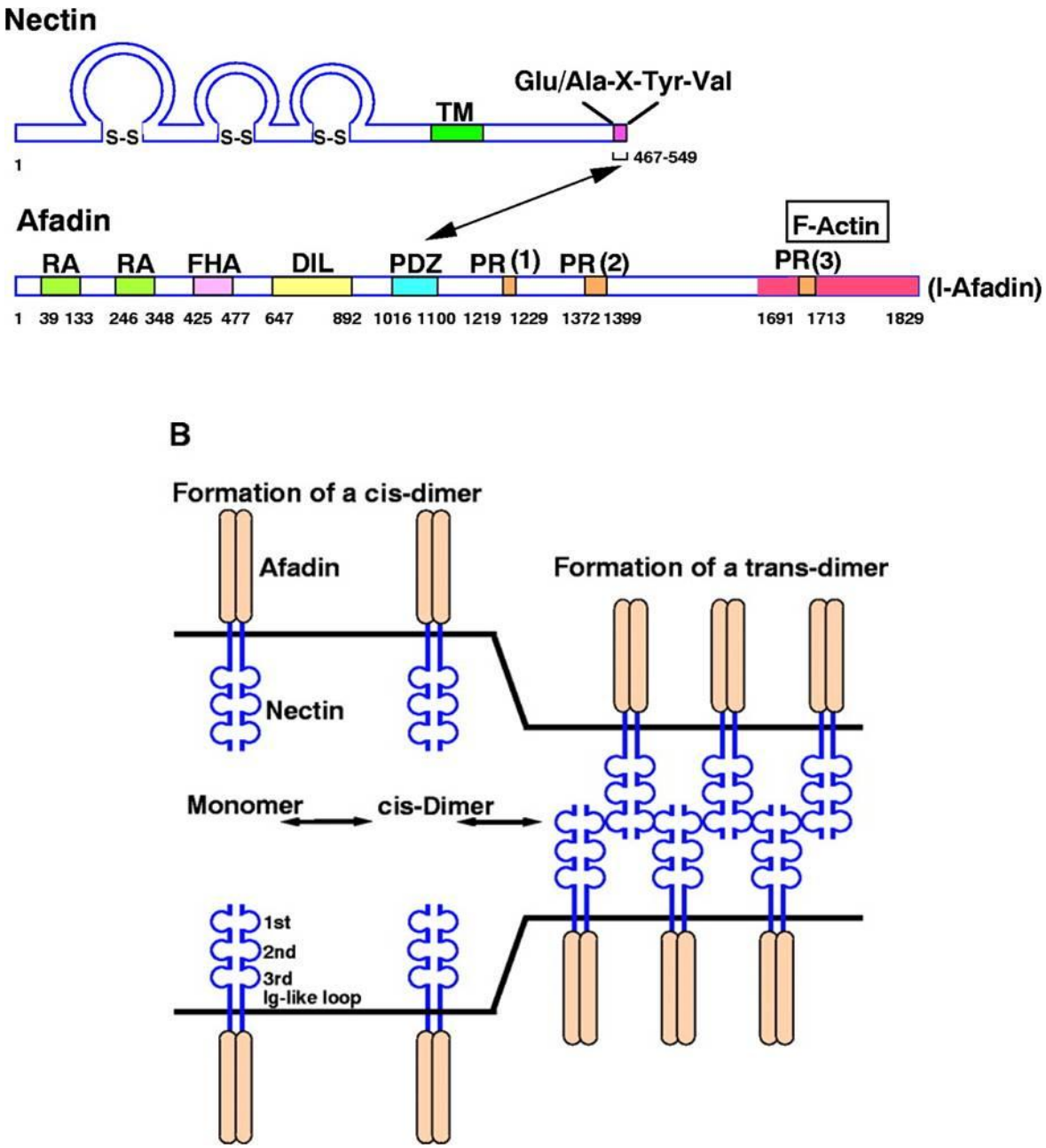


Figure 1.11. Nectin structure and proposed model for intercellular adhesion activity.
A) Nectin molecules contain 3 IgG-like loops and a C-terminal PDZ binding domain (E/A-X-Y-V) that associates nectin molecules with the afadin, an actin filament binding protein that has multiple domains allowing binding to many proteins. Therefore linking Nectins with the actin cytoskeleton. B) Characteristically nectins and necl's are expressed as cis-homodimers at the cell surface and these homodimers participate in homophilic or heterophilic interactions with their partners on an adjacent cell. Figures taken from Takai and Nakanishi (2003)

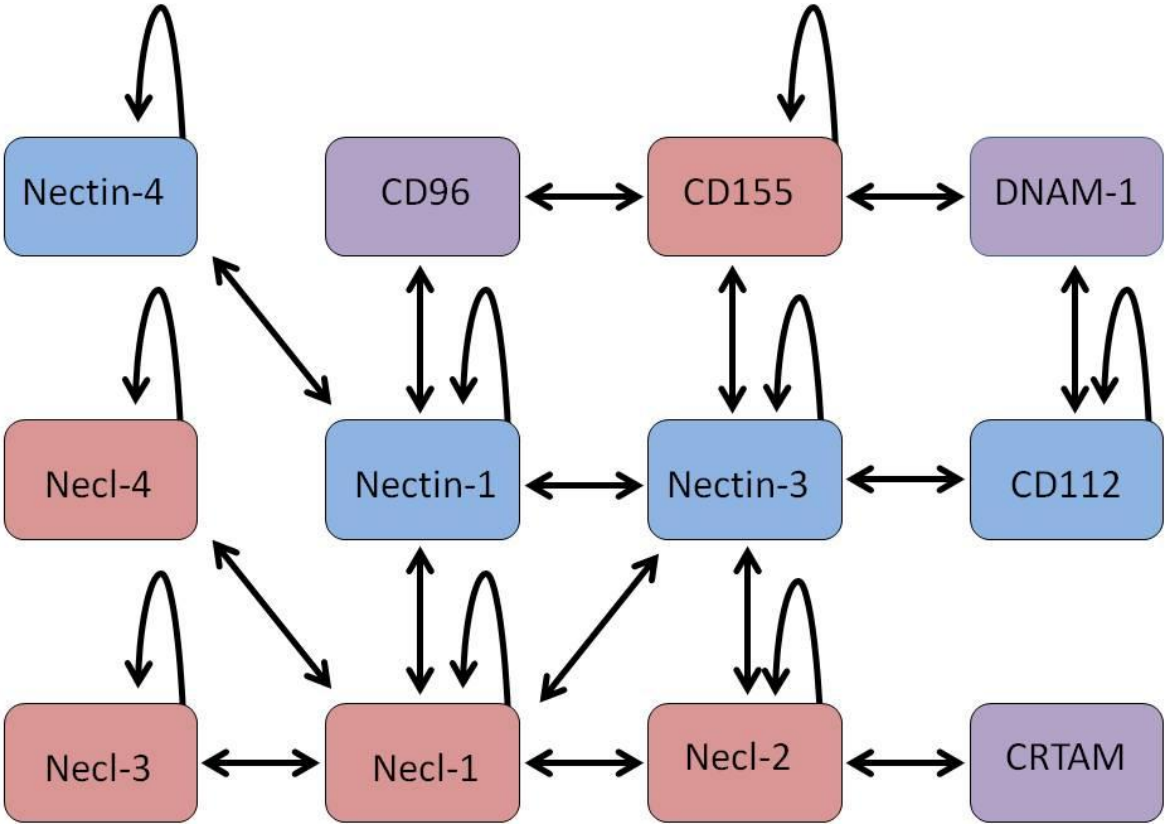


Figure 1.12. Interactions between the Nectin family and IGSF.
Nectins (**Blue**), Necl's (**Red**) and other immunoglobulin super family members (**Purple**) interact with each other by forming heterophilic *tans*-dimers (straight lines) or homophilic *cis* - or *trans*-dimers (curved arrows). Adapted from Takai et al (2008).

1.7.2. Nectins involved in cell-cell contact

CD111, CD112 and CD113 are expressed on a variety of cells including fibroblasts, epithelial cells and neurons. CD112 and CD113 are also expressed in cells that lack cadherins, such as B-cells, monocytes and spermatids, while human nectin4 is expressed mainly in the placenta (Lopez et al., 1998, Bouchard et al., 2000, Reymond et al., 2001, Ozaki-Kuroda et al., 2002).

In polarised epithelial cells, cell-cell adhesion is mediated through a junctional complex comprised of tight junctions (TJs), cell-cell adherens junctions (AJs) and desmosomes (DSs) (Farquhar and Palade, 1963). AJs are the sites of cell recognition and adhesion and are required for the later formation and maintenance of TJs and DSs (Tsukita and Furuse, 1999). Nectin-nectin trans-interactions initiate the formation of AJs and recruit cadherins to the site of cell-cell contact and cooperatively form AJs in epithelial cells, fibroblasts and synapses in neurons (Takai and Nakanishi, 2003, Takai et al., 2003, Takahashi et al., 1999). Once Cadherins are incorporated into the AJ, the cytoplasmic tail of cadherin binds the F-actin binding proteins α-catenin, α-actinin and vincilin which anchor cadherins to the actin cytoskeleton (Tachibana et al., 2000).

Nectins are also involved in the formation of TJs in epithelial cells by first recruiting junctional adhesion molecules and then claudins to the apical side of AJs in cooperation with cadherins. Junctional adhesion molecules recruit the cell polarity complex, consisting of Par3, atypical PKC and Par6 by direct binding to Par3 (Ohno, 2001). CD111 and CD113, but not CD112, also play a role in cell polarisation by directly binding Par-3 (Takekuni et al., 2003).

Following trans-interactions a series of signalling reactions occur that results in the reorganisation of the structural components of the cell including the actin cytoskeleton, filopodia formation and lamellipodia formation, Figure 1.13. Fukuyama et al (2005) described how following nectin-nectin interaction the tyrosine kinase Src is recruited and activated at cell-cell contact sites. C-Src then activates the small G protein Rap1 through the adapter proteins Crk and C3G, a Rap1-GDP/GTP exchange factor, Figure 1.13. Activated Rap1 activates phosphorylated FRG, which activates Cdc42. C-Src also activates Cdc42 through FRG, a cdc42-GDP/GTP exchange factor. Activated Cdc42 can then mediate the formation of filopodia.

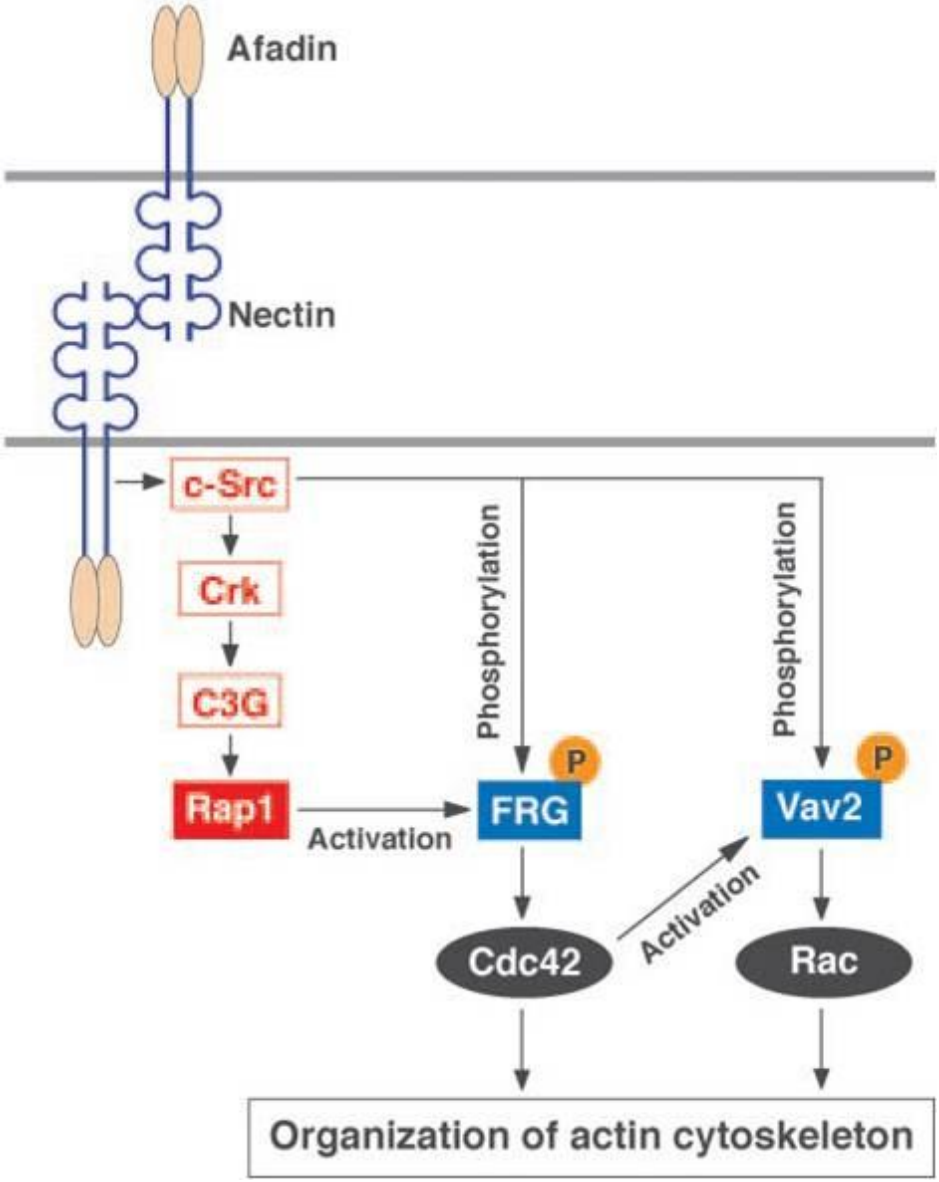


Figure 1.13 Nectin Intracellular signalling pathway.
A schematic model for the intracellular signalling pathway from trans interacting Nectin molecules to the activation of Cdc42 or Rac resulting in the reorganisation of the actin cytoskeleton. Taken form Fukuyama et al (2005).

Additionally c-Src phosphorylates Vav2, a GDP/GTP exchange factor for Rac. FRG activated Cdc42 then activates phosphorylated Vav2, which induces the activation of Rac (Fukuhara et al., 2004). The activation of Rac then induces the formation of lamellipodia (Nobes and Hall, 1995, Takahashi et al., 2008). While filopodia and lamellipodia protrusions are generally associated with cell movement they are also hypothesised to increase the number of cell-cell contacts and reduces the distance between cells enhancing cell-cell adhesion (Vasioukhin et al., 2000).

It remains unclear how c-Src and FRG are recruited and activated at the nectin-based cell-cell adhesion sites, but afadin is not likely to be necessary for this recruitment or activation because it has previously been shown that afadin is not necessary for the nectin induced, Cdc42 and Rac-mediated formation of filopodia and lamellipodia, respectively, in CD111-L cells (Kawakatsu et al., 2002). As all the work in cell signalling following trans interactions involved the use of CD111-L transfected cell lines this signalling cascade may only be relevant to CD111 and not the nectin family as a whole.

1.7.3. CD112 and CD155

Both CD112 and CD155 molecules are transmembrane glycoproteins that contain 3 Ig domains of V, C2 and C2 types in their extracellular regions and share 51% identity. Both have been described as an activatory receptor for DNAM-1 and a low avidity receptor for inhibitory receptor TIGIT (Bottino, 2003, Tahara-Hanaoka et al., 2004, Stanietsky et al., 2009). Both have shown to interact with CD113 and both are widely expressed in human tissue, including haematopoietic cells (Takai et al., 2008a).

1.7.3.1. CD112

CD112 was originally isolated as the murine homolog of human PVR but turned out to be another poliovirus-receptor-related protein and was named PRR2 (Morrison and RAcaniello 1992). The CD112 gene was mapped to 19q13.2-q13.4 by isotopic hybridisation, the same chromosomal region as CD155 (Eberle et al., 1995). Freitas et al (1998) further mapped the PRR2 gene to chromosome 19q13.2 by genome sequence analysis where it lies centromeric to the TOMM40 gene and the APOE-APC2 gene cluster. Diseases of q13.2 have mainly been associated to APOE but variations in CD112 have

been linked to T-cell non Hodgkin lymphomas and Nonsyndromic cleft lip, with or without cleft lip (nsCL/P) (Almire et al., 2007, Chengle et al., 2010, Jagomagi et al., 2010). In murine knockdown studies CD112 (-/-) males are infertile, while heterozygous males (+/-) and CD112 (-/-) females are fertile. The production of normal numbers of spermatozoa containing normal levels of DNA suggest CD112 is expressed in the testes only during the later stages of spermatogenesis (Bouchard et al., 2000). While the structural defects in spermatozoa on CD112 (-/-) mice suggest a role for CD112 for organising and reorganisation of the cytoskeleton during spermatogenesis at sertoli-cell junctions.

CD112 is a component of cell-cell AJs and interacts with I-afadin, an F-actin binding protein. The normal function of members of the nectin family is to mediate cell-cell adhesion through homotypic and heterotypic nectin-nectin interactions in cadherin based AJs. CD112-α and –δ can cis-homodimerise (α/α and δ/δ dimers) or cis-heterodimerise (α/δ dimers) (Lopez et al., 1998). An antibody against CD112 (R2.477) showed that CD112 was expressed in 96% of $CD34^+$, 88% of $CD33^+$ and 95% of $CD14^+$ haematopoietic lineages (Lopez et al., 1998). CD112 was also expressed at the intercellular junctions of adjacent cells but not at the free cellular edges (Lopez et al., 1998).

Struyf et al (2002) examined mutations in three equivalent regions of the N-terminal V-like domains of CD112 to test the effects on entry of various alphaherpesviruses, nectin-nectin interactions and with gD. Mutations in region I impaired HSV-1 entry but not PRV and did not effect nectin expression. Mutations in region II enhanced intracellular accumulation of CD111 and CD112 and had a deleterious effect on all of the activities under the study. Mutations in region III impaired entry of PRV and reduced HSV entry activity.

1.7.3.2. CD155

The first member of the nectin and necl family to be characterised, CD155 was initially identified as the cellular receptor for poliovirus (PVR). Located at 19q13.2, the CD155 gene produces four variants CD155:α, β, γ and δ through alternative splicing. CD155-α and -δ are transmembrane proteins while CD155-β and -γ are secreted (Baury et al., 2003). Little is known about the function of alternative spliced variants. However soluble CD155

is present in human serum at nanomolar concentrations and can compete with membrane bound CD155 to inhibit poliovirus entry (Baury et al., 2003).

CD155 was identified as a cognate receptor for poliovirus. Based on sequence homology to CD155, three poliovirus receptor related genes (CD111, CD112 and CD113) were indentified. However, neither CD111 nor CD112 bind poliovirus and it is assumed that their physiological functions differ from CD155. CD155 undergoes cell-matrix interactions by binding to the extracellular matrix protein vitronectin, binding with a dissociation constant (K(d)) of 72nM (Lange et al., 2001). Both CD155 and vitronectin co-localise to follicular dendritic cells and B-cells inside the germinal centres of secondary lymphoid tissues (tonsils) suggesting that the CD155/vitronectin interaction could be required for the establishment of a proper immune response at these sites (Lange et al., 2001).

In ligand binding assays, CD155 was shown to bind CD113 and the putative murine homologue of CD155, Tage4 (Mueller and Wimmer, 2003). Co-culture of CD113 and CD155 expressing Hela cells led to CD155-dependant recruitment of CD113 to cell-cell contacts. CD155 was also found to co-distribute with αV integrin microdomains on the surface of mouse transfected fibroblasts and at amniotic epithelial cell junctions (Mueller and Wimmer, 2003). These experiments show the possible trans interaction between cell-cell adherens type system (adherin/nectin) and the cell-matrix adhesion system.

The adhesion receptor CD96 (TACTILE) is a transmembrane glycoprotein and possesses 3 extracellular IG-like domains. Among peripheral blood cells, CD96 is expressed on T-cells as well as NK cells and a subpopulation of B-cells. CD96 has been reported to function as an adhesion receptor promoting NK-target cell interaction, promoting NK mediated cytolysis (Fuchs et al., 2004). CD96 has been described as a marker in acute myeloid leukaemia (AML) and acute lymphocytic leukaemia (ALL) (Hosen et al., 2007). CD96 binds to CD155 and CD111. In humans CD96 has 2 variants as a result of alternative splicing (Meyer et al., 2009). Variant 2 was shown to be predominant in AML tumour samples. Other evidence suggests that mutations in human CD96 correlate with the occurrence of a rare form of C syndrome (Opitz trigonocephaly) (Kaname et al., 2007). One such mutation causing a single amino acid change in the third domain of human CD96, which resulted in a decreased capacity of both variants to bind to CD155 suggesting that CD96 driven adhesion to CD155 may be crucial in development.

1.7.4. CD112 and CD155 in oncogenesis

While not attributed to be stress molecules CD112 and CD155 appear to be up-regulated on the cell surface of certain transformed cell lines. Both CD112 and CD155 have been shown to have involvement in NK mediated cell lysis of tumours, particularly melanoma (Pende et al., 2005a). Many cancerous cell lines and primary tumours constitutively over express CD155, which can efficiently prime cell-mediated tumour specific immunity (Tahara-Hanaoka et al., 2006). NK cells have been shown to kill ovarian carcinoma cells, neuroblastoma cells, myeloid leukaemia's and lymphoblastic leukaemia's mediated by a CD155-DNAM-1 interaction (Pende et al., 2005b, Carlsten et al., 2007, Castriconi et al., 2004, Pende et al., 2005a). The DNAM-1 ligand interaction is likely part of tumour immunosurveillance by circulating NK cells. In addition to NK cells, DNAM-1 is also expressed on a subset of T-cells and has been identified as being involved in the differentiation of Th1 T-cells, regulating their expansion and effector functions (Dardalhon et al., 2005), and may protect against Fas ligand induced apoptosis as DNAM-1 deficiency results in high sensitivity to apoptosis in NK T-cells in patients with systemic lupus (Tao et al., 2005). CD112 is expressed on $CD34^{+}$ Leukemic lymphocytes in both Acute myeloid Leukaemia (AML) and acute lymphocytic Leukaemia but not on 'normal lymphocytes', as determined by expression of CD48. Pende *et al* (2005b) showed that it was the DNAM-1 interaction and not the NKG2D interaction that was responsible for NK cell mediated lysis of these leukaemia's (Pende et al., 2005b).

CD155 expression is restricted to primates and features during the development of ventrally-derived structures within the central nervous system. Significantly it is re-expressed/overexpressed on neoplastic glia. CD155 has been proposed as playing a key role in glioma motility and invasion whereby glioma invasion was demonstrated in to be a function of CD155-mediated regulation of adhesion signalling and focal adhesion dynamics (Sloan et al., 2004). RNAi knockdown of CD155 in cultured glioma cells altered cell morphology to a larger, fusiform phenotype which resulted in reduced migration. Targeting CD155 has already been used in therapeutic targeting of glioma for regional delivery of recombinant poliovirus to achieve an oncolytic effect (Merrill et al., 2004, Sloan et al., 2005).

The CD155-DNAM-1 interaction has been described as being capable of enhancing metastasis. The interaction between CD155 on cancerous cells and DNAM-1 on platelets

resulted in the arrest of cancerous cells in pulmonary vessels and subsequent entry into tissue (Morimoto et al., 2007). Depletion of platelets in BALB/c mice resulted in a reduction of lung metastasis, suggesting that platelets mediate CD115-dependant tumour cell attachment to pulmonary vessels.

1.7.5. NK-cell and T-cell activation and inhibition

Cytotoxic lymphocytes, such as NK cells and $CD8^+$ T-cells, require adhesion molecules to migrate to sites of infection/tumour microenvironment and establish tight contact with virally infected or tumour cells. In addition, in secondary lymphoid organs, adhesion molecules facilitate $CD8^+$ T-cell and NK cell interactions with APCs, which induce their activation, expansion and differentiation. Adhesion molecules can either act directly as signalling molecules, initiating intracellular pathways, or as accessory molecules, sustaining cell contact that are necessary for T-cell receptors and NK cell receptors to engage their cognate ligands.

Both CD112 and CD155 have been identified as ligands for the activating receptor DNAM-1 and inhibitory receptor TIGIT (Bottino, 2003, Tahara-Hanaoka et al., 2004). Both DNAM-1 and TIGIT are expressed on NK cells and a subset of T-cells. In a review by Shibuya et al (2005) a hypothesis is put forward whereby a threshold is required to be exceeded in order for an activatory signal to override an inhibitory one. Constitutive CD155 expression results in an inhibitory signal overriding the activatory one. During infection/malignancy whereby CD155 cell surface expression is increased, a threshold is crossed and the activatory signal becomes dominant. During HCMV infection CD155 is down-regulated below normal cell surface expression levels and as a result is well below the threshold requirement for an activatory signal to become dominant.

1.7.5.1. Activatory receptor DNAM-1

DNAM-1 is a transmembrane immunoglobulin-like molecule that was initially identified as a T-cell specific activation marker (originally named TLiSA-1) (Burns et al., 1985). In humans, DNAM-1 is mapped to chromosome 18q22.3 and appears to be expressed on NK cells, monocytes, $CD4^+$ and $CD8^+$ T-cells, platelets and a subset of B-cells (Reymond et al., 2004, Shibuya et al., 1996, Scott et al., 1989, Shibuya et al., 1999, Shibuya et al.,

2003). Binding of DNAM-1 to its ligands triggers NK cell cytotoxicity and mediates adhesion of monocytes to endothelial cells facilitating transendothelial cell migration.

DNAM-1 signalling is dependent on physical association of DNAM-1 with co-receptor LFA-1 integrin (αLβ2) in lipid rafts and modulates its activation and capacity to bind intracellular adhesion molecules 1 (ICAM-1, CD54). LFA-1 is part of a family of leukocyte integrins that are recognised by their common β-chains (CD18) and is expressed on all T-cells, B-cells, macrophages and neutrophils. LFA-1 also has a distinct α chain (CD11a) (Kurzinger et al., 1981, Keizer et al., 1985). Mechanistically, engagement of DNAM-1 by its ligands results in phosphorylation of the cytoplasmic domain by protein kinase C. In turn this activates the tyrosine kinase Fyn recruiting actin-binding proteins 4.1G and human discs large (Ralston et al., 2004). This results in rearrangement of the actin cytoskeleton, promoting clustering of LFA-1 and formation of an adhesive complex that polarises lymphocytes towards other cells, recruiting them to sites of infection (Shibuya et al., 1999, Shibuya et al., 1996, Shibuya et al., 2003, Ralston et al., 2004).

The DNAM-1 ligands were independently indentified by two groups using two different strategies. Bottino et al (2003) generated a mAb that blocked NK cell-mediated cytotoxicity against certain tumours and purified the unknown protein expressed on the tumour target recognised by the mAb, identifying CD155 as the protein to which the mAb bound (Bottino et al., 2003). Concomitantly Tahara-Hanaoka et al (2004) generated a fusion protein of the extracellular portion of DNAM-1 with the human IgG Fc portion (DNAM-1-Fc) and used this as a probe against a retroviral cDNA library prepared from a human osteosarcoma cell line (Tahara-Hanaoka et al., 2004). Both groups identified the human poliovirus receptor (PVR) α (CD155) and its isoform PVRδ as ligands for DNAM-1. CD155 is a member of the poliovirus receptor-related (PRR) family and CD112 was the only other member of this family to be identified as a ligand for DNAM-1. Interestingly, activated T-cells and NK cells express CD96 (Tactile), which only shows ~20%homology with DNAM-1, but has also been shown to recognise CD155 as its ligand and promote NK cell adhesion and activation.

Despite the binding affinities between CD155-Fc or CD112-FC and DNAM-1-Fc being comparable (Kd = 2.3 x 10^{-7} M and 3.1 x 10^{-7} M, respectively), a BW5147 transfectant cell line expressing CD112 bound to soluble DNAM-1-Fc to a lesser extent than to a transfectant cell line expressing CD155 (Bottino, 2003). This was hypothesised to have

been caused by the homophilic interaction of CD112, but not CD155. The authors were also able to improve DNAM-1-Fc binding to the CD112 transfectant cell line with the pre-treatment of the cell line with a mAb that blocked the homophilic (cis) interaction of CD112, suggesting that DNAM-1 binding to CD112 on the cell surface may be impaired by homophilic interaction of CD112.

1.7.5.2. Inhibitory receptor TIGIT

TIGIT was identified by searching for genes that are expressed by immune cells and that might function as immunomodulatory receptors owing to a protein structure comprising an immunoglobulin domain, a transmembrane domain and an ITIM (Yu et al., 2008). Consistent with the search criteria, TIGIT was found to be expressed on the surface of $CD4^{+}CD25^{+}$ regulatory T-cells, memory T-cells and NK cells. Knockdown of TIGIT expression in primary human T-cells by RNAi had no effect on T-cell proliferation or cytokine production *in-vitro*, and exposure of T-cells to a TIGIT-specific antibody did not influence their activation (Yu et al., 2008). However, when T-cells were cultured with autologous DCs, the presence of a TIGIT-specific antibody led to a four-fold increase in interferon-γ production. Conversely the use of a TIGIT-Fc fusion protein to ligate PVR inhibited IFNγ production by T-cells in T-cell-DC co-cultures. Suggesting TIGIT might regulate T-cell responses by interacting with PVR expressed by DC's.

Exposure of monocyte-derived DCs (MDDCs) to TIGIT-Fc (or CD226-Fc) resulted in increased production of the immunosuppressive cytokine IL-10 and decreased production of IL12 following stimulation. Further analysis revealed that TIGIT-Fc engagement of CD155 on DCs induced the phosphorylation of CD155 and the downstream activation of extracellular-signal-related kinase (ERK), which is probably involved in the observed effects of CD155 ligation on DC cytokine production (Yu et al., 2008).

Yu et al (2008) then showed that TIGIT-FC treated MDDCs, but not control MDDCs, inhibited T-cell proliferation in a mixed lymphocyte response assay and that this suppression was associated with high levels of IL-10. Importantly, the suppressive effects of TIGIT-Fc were confirmed in an *in-vivo* delayed type hypersensitivity (DTH) assay; treatment of wild type, but not IL10-/- mice with TIGIT-Fc reduced DTH responsiveness. Finally DC's isolated from TIGIT-Fc treated mice contained more IL10 mRNA and less

IL12 mRNA that DCs from control mice is consistent with the idea that TIGIT-mediated modulation of DCs is responsible for the immunosuppressive effects of TIGIT *in-vivo*.

Both CD155 and CD112 were shown to be ligands for TIGIT, although TIGIT was shown to bind with lower avidity to CD112 (Stanietsky et al., 2009). However it was hypothesised that this might be due to the ability of CD112 to form homophilic interactions that might have inhibited the CD112/TIGIT interaction in a similar way that was observed by Bottino et al (2003) with the CD112/DNAM-1 interaction.

1.7.6. CD112 in other aspects of the immune response

In addition to those functions discussed thus far. CD112 has also showed functions in other aspects of the immune system

Monocytes differentiate into immature DC's (iDCs), which in turn differentiate into mature DC's (mDCs) upon appropriate stimulation. CD112 expression appears to increase with each stage of the differentiation process, unlike CD155 which is only up-regulated during the differentiation of iDC's to mDC's (Pende et al., 2006). Mature DC's are protected from NK-mediated lysis by high surface expression of HLA class 1 molecules on their cell surface which inhibit NK cell cytolysis. Immature DC's are efficiently killed by NK cells, with one of the strongest signals transmitting through CD226 (DNAM-1). Signalling through CD226 seemed to be strongest through CD112 expression (Pende et al., 2006). Therefore CD112 expression during DC maturation may play a role in NK mediated 'quality control' by selecting for those DC's that show high expression of HLA class I molecules and costimulatory ligands, optimising for an ability to prime T-cells.

Mast cells have effector functions in various immune settings. In allergic inflammation, mast cells interact with tissue-infiltrating eosinophils, forming a regulatory unit in the late and chronic phase of the allergic process. Mast cells express CD226, while eosinophils express CD112 (Bachelet et al., 2006). Engagement of CD226 by CD112 expressing eosinophils results in mast cell degranulation. When CD112 cell surface expression on eosinophils was blocked with neutralising antibodies, the hyperactivity resulting from IgE-dependant activation of mast cells was normalised during *in-vitro* co-culture of these two cell types (Bachelet et al., 2006).

1.8. CONCLUSION AND AIMS

CD112 is involved in a variety of functions including cell adhesion, contact inhibition and NK-cell activation. Specifically, CD112 signals via CD226 (DNAM-1) activating receptors to modulate NK-cell cytotoxicity. Since CD226-activating ligand, CD155, is down-regulated from cell surface during HCMV infection (Tomasec, 2005) it was important to address whether CD112 was also down-regulated from the cell surface during productive HCMV infection.

Preliminary screening of cell surface proteome of HCMV-infected cells performed in this laboratory identified that CD112 was down-regulated in HCMV-infected cells (P. Tomasec, unpublished observations). The deletion of UL141 from HCMV genome was able to restore CD112 cell surface expression in HCMV-infected cells (Prod'homme et al., 2010). Expression of UL141 on its own had no effect on CD112 cell surface expression (Tomasec, 2005). Since UL141 appeared necessary but not sufficient to modulate CD112 expression, this thesis investigated a hypothesis whereby UL141 was acting in a conjunction with another HCMV protein.

The aims of this project were

1) Perform detailed examination the effect of CMV infection on CD112 expression and analyse the effect of UL141 on CD112 in the context of CMV infection.

2) Utilise a RAd-library expressing individual HMCV ORFs to identify additional HCMV gene(s) involved in the down-regulation of CD112.

2.0. METHODS AND MATERIALS

2.1. SOLUTIONS

W/V = WEIGHT/VOLUME SOLUTION, V/V = VOLUME/VOLUME SOLUTION

Ampicillin Stock: 50 mg/mL Ampicillin (Sigma-Aldrich) was dissolved in deionised water (diH_2O) and stored at -20°C.

Ampicillin selection plates: Once LB agar autoclaved and cooled to 'hand hot', Ampicillin (1:1000 of 50 mg/mL stock), Chloramphenicol (1:1000 of 12.5mg/mL stock), X-gal (1:500 of 40mg/mL stock) and IPTG (1:500 of 100mM stock) were added before allowing to set in plates.

Caesium chloride (heavy):

5.45g/mL Caesium Chloride (Sigma-Aldrich) in 5mM Tris-HCL

Caesium Chloride (light):

1.33g/mL in 1mM EDTA, 5mM Tris, pH adjusted to pH7.8.

Chloramphenicol stock: 12.5mg/mL Choloramphenicol (Sigma-Aldrich) was dissolved in ethanol (Fisher Scientific) and stored at -20°C

DMEM-10: Dulbeco's Modified Eagle Medium with 10% Foetal calf serum (FCS) (Gibco), 10mL of Penicillin(5,000 IU/mL penicillin) and Streptomycin (5000µg/mL) (Gibco).

DOG plates: 20% (v/v) 5xM63, 1mM magnesium sulphate heptahydrate (Sigma Aldrich) and 1.5% (w/v) agar (Fisher Scientific) added before autoclaving. Once autoclaved and cooled to 'hand hot' Chloramphenicol (1:1000 of 12.5mg/mL stock), 0.2% 2-deoxy-galactose (Sigma Alrdich), 1mg/mL Biotin (Fisher Scientific), 45mg/mL Leucine (Sigma Aldrich) were added before allowing to set in plates

DNA loading buffer: 30% glycerol (Fisher Scientific) in diH2O with 0.25% (w/v) bromophenol blue (Sigma-Aldrich) in 0.25% (w/v) xylene cyanol FF (Sigma-Aldrich).

Freezing mixture: 90% Foetal calf serum (FCS) (Gibco) and 10% (v/v) dimethyl sulphoxide (DMSO) (Sigma-Aldrich).

Glycerol 10%: 10% Glycerol (Fisher Scientific) in diH_2O. Autoclaved.

IF buffer: 1% BSA (Fisher Scientific), 0.2% Saponin (Sigma) and 0.05% Sodium Azide (Sigma).

IPTG stock: 100mM stock solution generated by dissolving 23.8mg Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Melford) in 1mL diH_2O.

Kanamycin stock: 50mg/mL Kanamycin (Roche) dissolved in diH_2O and stored at -20°C.

Kanamycin selection plates (positive selection):

Once LB agar autoclaved and cooled to 'hand hot', Kanamycin (1:1000 of 25mg/L stock), Chloramphenicol (1:1000 of 12.5mg/mL stock), X-gal (1:500 of 40mg/mL stock) and IPTG (1:500 of 100mM stock) were added before allowing to set in plates.

LB broth: 1% (w/v) tryptone (Sigma Aldrich), 1% (w/v) sodium chloride (NaCl) (Sigma Aldrich), 0.5% yeast extract (Sigma Aldrich) dissolved in diH_2O then autoclaved.

Luria Bertani (LB) agar: as for LB broth but with 1.5% (w/v) agar (Fisher Scientific) added before autoclaving. Antibiotics were added before setting in plates.

LB sucrose selection plates:

1% (w/v) tryptone, 0.5% (w/v) yeast extract (Melford), 5% (w/v) sucrose (Fisher Scientific) and 1.5% (w/v) agar (Fisher Scientific) added before autoclaving. Once autoclaved and cooled to 'hand hot' Chloramphenicol (1:1000 of 12.5mg/mL stock), X-gal (1:500 of

40mg/mL stock) and IPTG (1:500 of 100mM stock) were added before allowing to set in plates.

MOPS buffer: 5% 20x MOPS buffer dissolved in diH_2O

MM9 salts: 6 g/L sodium phosphate bibasic (Sigma Aldrich), 3 g/L monopotassium sulphate (Sigma Aldrich), 1 g/L Ammonium Chloride (Sigma Aldrich), 0.5 g/L Sodium Chloride (Sigma Aldrich) dissolved in diH_2O then autoclaved.

5 x M63: 10 g/L ammonium sulphate (Sigma Aldrich), 68 g/L monopotassium phosphate, 2.5mg/L Ferrous sulphate heptgydrate (Sigma Aldrich) dissolved in diH_2O thenpH adjusted to pH7.8.

M63 minimal media plates:

20% (v/v) 5xM63, 1mM magnesium sulphate heptahydrate (Sigma Aldrich) and 1.5% (w/v) agar (Fisher Scientific) added before autoclaving. Once autoclaved and cooled to 'hand hot' Chloramphenicol (1:1000 of 12.5mg/mL stock), 0.2% Galactose (Sigma ALrdich), 1mg/mL Biotin (Fisher Scientific), 45mg/mL Leucine (Sigma Aldrich) were added before allowing to set in plates.

Paraformaldehyde (4%):

4% (w/v) Paraformaldehyde dissolved in diH_2O, pre-warmed to ~70°C. Once dissolved solution was cooled and filtered through a 0.22µm filter t to remove any particulate and stored at 4 °C

PBS: For cell culture PBS was purchased directly from Sigma Aldrich.

For all other work PBS was constituted. One phosphate buffered saline (PBS) tablet (Sigma Aldrich) was dissolved per 100mL diH_2O to generate PBS containing 0.8% (w/v) Sodium Chloride (NaCl), 0.2% (w/v) potassium chloride (KCl), 1.15% (w/v) disodium hydrogen phosphate and o.2% (w/v) potassium dihydrogen phosphate at pH7.3.

PBST: PBS with 0.1% (v/v) Tween-20 (Fisher Scientific).

Semi-dry transfer buffer:

10% (v/v) NuPAGE® Transfer buffer (Invitrogen), 10% (v/v) Methanol Fisher Scientific) dissolved in diH_2O.

Streptomycin selection plates (negative selection):

Once LB agar autoclaved and cooled to 'hand hot' Streptomycin (1:1000 of 200mg/mL stock), Chloramphenicol (1:1000 of 12.5mg/mL stock), X-gal (1:500 of 40mg/mL stock) and IPTG (1:500 of 100mM stock) were added before allowing to set in plates.

Streptomycin stock: 200mg/mL Streptomycin (Roche) dissolved in diH_2O and stored at -20°C.

TAE (50x): 242g Tris base, 57.1ml glacial acetic acid (Fisher scientific) and 100ml 0.5M ethylenediaminetetraacetic acid (EDTA), pH8.0 were mixed with water in a final volume of 1 L.

0.7% TAE Agarose gel:0.7g agarose (Invitrogen) was dissolved in 100mL TAE buffer by heating. Ethidium bromide (Sigma) was added and then poured into a mould and left to set at room temperature.

TPBST: PBS with 0.1% (v/v) Tween-20 (Fisher Scientific) and 0.1% (v.v) Triton X-100 (Fisher Scientific)

Tris buffer: 10 mL of 1M Tris-Cl pH7.4 was mixed with 2 mL 0.5M EDTA, pH 8.0 and water added to a final volume of 1L.

1M Tris-Cl 121.1g Tris-Cl (Fisher Scientific) was dissolved in 1L diH_2O and pH adjusted to pH 7.4.

X-gal stock: 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Melford) was dissolved in DMSO (Sigma) to generate a 40mg/mL stock solution. Aliquots were stored at -20°C.

2.2. CELL CULTURE:

2.2.1. Established cell lines

All cell lines used are summarised in Table 2.1. Human foetal foreskin fibroblasts (HFFF) were kindly supplied by Dr G. Farrar, Porton Down. HFFFs were immortalised with human telomerase reverse transcriptase (HFFF-hTert) and transfected with the Coxsakie Adenovirus Receptor (HFFF-hCAR) by S Llewellyn-Lacey as described in McSharry et al (2001). The 293 T-REx^{TM} cell line was obtained from Invitrogen and the human embryonic retinoblast cells, 911 were obtained from Crucell, Leiden. The human epithelial colorectal adenocarcinoma cell line Caco-2 and the human breast cancer cell line, MCF-7, were obtained from American Type Ceulture Collection (ATCC).

The human cervical epithelial adenocarcinoma cell line, Hela was obtained from ATCC. The transfectant cell lines Hela-US2 and Hela-US11 were kindly provided by Dr Emmaneual Weirtz,. The transfectant cell lines 293-GFP and 293-UL141-GFP cell lines were kindly provided by Dr P Tomasec, Cardiff, as described in Tomasec et al (2005).

2.2.2. Tissue culture media

All cell lines were grown in DMEM supplemented with 10% Foetal calf serum (FCS) (Gibco) and 20mL/L Penicillin-Streptomycin (Invitrogen), referred to here on in as DMEM-10. Most cell lines were maintained in standard 175cm^2 tissue culture flasks. 293 T-REx^{TM} and 911 cells were maintained in 175cm^2 tissue culture flasks coated with Cellbind which aids cell adherence to the flask. All cells were incubated in a static incubator (Heraeus) at 37°C and 5% CO_2. Media was warmed to 37°C before use.

2.2.3. Passage of cell lines

Confluent layers of adherent cells were passaged by first removing medium, washing the cell layer in PBS and incubating in the presence of 1 – 5 mL Trypsin/EDTA (Invitrogen) until the cells detached and fell into suspension. Once detached, cells were resuspended in 10 mL of fresh DMEM-10 and the cells were split between 1:2 and 1:5, into 175cm^2 tissue culture flasks containing fresh DMEM-10.

Table 2.1. List of cell lines.

Cell line / Abbreviation	Comment	Publication/Reference
293 T- RExTM	A cell line containing a tetracycline regulated expression system.	Invitrogen
293-GFP	Human Embryonic Kidney (HEK) cell line containing GFP.	Tomasec et al 2005
293-UL141GFP	293 cell line expressing UL141 C-terminally tagged with GFP	Tomasec et al 2005
293-UL14GFP	293 cell line expressing UL14 C-terminally tagged with GFP	Tomasec et al 2005
911	Human embryonic retinoblast cells.transfected with Ad E1 and E3 regions.	Crucell
Caco-2	Human epithelial colorectal adenocarcinoma cell line	ATCC
HFFF	Human Foetal Foreskin Fibroblasts	Obtained from Dr Graham Farrar (CAMR, Sailsbury UK)
HFFF-hCAR	HFFF immortalised by the in-house insertion of hTERT and expressing coxsakie/adenovirus receptor (CAR).	McSharry et al 2001
HFFF-hTert	HFFF immortalised but the in-house insertion of hTERT.	McSharry et al 2001
Hela	Human cervical epithelial adenocarcinoma cell line	ATCC
Hela-US2	Hela cell line expressing HCMV ORF US2	
Hela-US11	Hela cell line expressing HCMV ORF US11	
MCF-7	Human breast adenocarcinoma cell line	ATCC

2.2.4. Cell counting

A 10 µL aliquot of cell suspension was diluted 1:1 with Trypan Blue and counted on a bright line haemocytometer (Sigma) under white light. Viable cells were identified by the exclusion of the blue dye. The total number of cells present was determined using the following formula:

$$\text{Total No cells/mL} = (\text{No cells in grid}) \times 1 \times 10^4) \times 2$$

2.2.5. Cryopreservation of cell lines

Adherent cells in the log phase of growth were re-suspended and counted using a haemocytometer as described in section 2.4. The suspended cells were then recovered by placing them in a 15 mL tube and centrifuging at 1,500rpm for 3minutes at RT. The supernatant was discarded and the cell pellet re-suspended in an appropriate volume of freezing mixture (90% Foetal Calf Serum (Gibco), 10% DMSO (Gibco)) to give a final cell concentration of 1×10^6 cells/mL. 1 mL aliquots were dispensed into cryovials (Greiner) and placed in a Nalgene 5100 cryo 1°C freezing container (Merk), which contained isopropanol (Fisher Scientific) at RT. The freezing container was stored at -70°C for ~24hrs before being transferred to liquid nitrogen storage until required.

To resurrect cells from liquid nitrogen storage, cells were rapidly thawed in a 37°C waterbath (Grant). Once thawed cells were transferred to a 15 mL tube containing 10 mL pre-warmed DMEM-10. The cells were centrifuged at 1,500rpm for 5minutes at RT and the supernatant discarded. The cells were re-suspended in fresh DMEM-10 and transferred to a $25cm^2$ tissue culture flask and incubated at 37°C, 5% CO_2.

2.3. VIRUSES

2.3.1. Propagation of HCMV

HCMV strains were all propagated in HFFF-hTerts. Cells were cultured in 2 L roller bottle tissue culture flasks until cells reached ~80% confluency. Once at the correct confluence, cells were infected with the desired HCMV at a multiplicity of infection (MOI) 0.1 in ~40 mLs DMEM-10. Rollers were placed in an incubator containing a rolling platform (Heraeus) at 30 rotations a minute at 37°C, 5%CO_2. The following day medium was removed and replaced with fresh DMEM-10. Cells were monitored for cytopathic effect (cpe) and media was changed as required. When extensive cpe was observed throughout the cell monolayer (~80-90% cpe), media was removed and stored at -70°C as HCMV is secreted into the medium. Fresh 25mL media was added to the cells. During the following days media was replaced and stored at -80°C until the infection was completed.

2.3.2. HCMV purification

Frozen aliquots from respective HCMV infections were removed from storage at -80°C and defrosted in a pre-warmed 37°C waterbath. Cell debris was removed by centrifugation at 2,500rpm for 5 minutes at RT. The supernatant was pooled and decanted into pre autoclaved 250 mL Sorvall centrifuge pots (Sorvall) and centrifuged at 23,000 rpm for 120 minutes in an ultracentrifuge (Heraeus) using a SLA1500 rotor, which pelleted the virus. Following centrifugation the supernatant was discarded and the pellet was re-suspended in DMEM-10. The re-suspended pellet was gently aspirated through a 21 gauge needle to reduce the aggregation of the virus. Once resuspended the virus pellet was frozen at -80°C in 1mL aliquots until required. Infectious titres of HCMV stocks were calculated by titration. All HCMVs used in this study can be found in Table 2.2.

Table 2.2. List of HCMV's used in this study

Laboratory designation	Comment
RCMV1111	Merlin BAC derived virus
RCMV1149	Merlin BAC derived virus, ΔUL141
RCMV1278	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged
RCMV1293	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔUL2-11**
RCMV1294	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔUL13-20**
RCMV1295	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔUL22A-UL25**
RCMV1297	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔUS12-US17**
RCMV1299	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔUS27-28**
RCMV1300	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔUS29-US34A**
RCMV1318	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔUS18-22**
RCMV1332	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔRL1-RL6**
RCMV1333	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔRL10-UL1**
RCMV1528	Merlin BAC derived virus, ΔUL16 ΔUL18 UL32-GFP tagged. **ΔUS1-US11**
RCMV1579	Merlin BAC derived virus, ΔUS11
Merlin	Wild Type Merlin. Dolan et al (2004)

2.3.3. HCMV titration

HCMV viruses were titrated by limiting dilution assay in 1 x 10^6 HFFF cells that were seeded into 25cm^2 tissue culture flasks and allowed to adhere overnight. The following day serial dilutions between 10^{-2} and 10^{-8} of the virus stock were made in DMEM-10. 1mL of each HCMV dilution was added to two 25cm^2 tissue culture flasks, to give duplicate infections. Viruses were incubated on a rocking incubator for 2 hours before removing the media and replacing with 10 mL of overlay medium containing 1% Avicel (FMCBiopolymer), to prevent CMV infection from spreading throughout the flask. After 14 days medium was removed and cells were washed thoroughly in PBS to remove all avicel. Cells were fixed in Methanol and observed microscopically for plaques. The number of plaques at each dilution were counted and averaged between the duplicates to give a concentration of PFU/mL.

2.3.4. HCMV Growth curve

In order to generate a single step HCMV growth curve, 1 x 10^6 HFFF cells were seeded into 25cm^2 tissue culture flasks and allowed to adhere overnight. The following day cells were infected with HCMV at MOI 1. At day 1 and then every subsequent 2 days 1mL supernatant was taken and replaced with fresh media of equal volume. Aliquots were then titred to calculate amount of virus present in the supernatant.

2.3.5. Propagation of Recombinant Adenovirus (RAd) stocks

Recombinant Adenovirus (RAd) stocks were grown in 293-T-REx^{TM} cells in 175cm^2 tissue culture flasks. T-Rex^{TM} cells contain the tetracycline repressor protein that allows extremely low expression levels of the transgene in the repressed state, making this cell line ideal for the growth of RAd's that potentially express genes with cytotoxic effects. 293-T-Rex^{TM} cells were seeded into 175cm^2 cellbind tissue culture flask. A total of 5 flasks were used to culture a single RAd. When cells reached ~80% confluence, 10µL of unknown titred stock, or MOI 0.1 of titred stock, was added to each flask and incubated overnight at 37°C. The following day the inoculum was removed and fresh DMEM-10 was added. After 3-7days, when maximum cpe was observed, the cells and supernatant were transferred to 50mL falcon tubes and centrifuged at 1,500rpm for 5 minutes at RT. The

supernatant was discarded and the cell pellet was re-suspended in 10 mL PBS and centrifuged again at 1,500rpm for 5minutes at RT. The supernatant was discarded and the cell pellet was stored at -70°C until required.

2.3.6. RAd purification by Caesium Chloride

Firstly a Caesium Chloride density gradient was made. 1.6 mL of 3.6M Caesium Chloride (CsCl) solution (1.45 g / mL in 5mM Tris HCL, pH 7.8) was first pipetted into 14x89mm Ultra clear Beckman centrifuge tubes (Beckman) for an SW41 Ti rotor. Then 3 mL of less dense 2.5M CsCl solution (1.33 g/mL in 1mM EDTA, 5mM Tris HCL, pH 7.8) was gently layered on top.

Once the CsCl gradient was made, the cell pellet was defrosted in a 37°C waterbath and resuspended in ~10mL warm PBS. An equal volume (10 mL) of Tetrachloroethylene (Fisher) was added and mixed vigorously and centrifuged at 2,000rpm for 20minutes. The upper aqueous phase, containing the RAd was removed and gently pipetted onto the previously made CsCl gradient until the tube was filled to 2.5mm from the top. Samples were centrifuged at 23,000rpm for 120 minutes at RT using L8-M ultracentrifuge (Beckman) and SW41 Ti rotor. The virus appeared as an opalescent layer between the higher and lower density CsCl solutions. A second band higher up, resting on top of the low density CsCl solution was derived from cellular components. The virus was harvested by puncturing the tube with a 19g needle and gently pulling the lower band corresponding to the virus into a 2 mL syringe. The virus was then dialysed against 1mM MgCl2; 135mM NaCL; 10mM Tris HCL, pH 7.8; containing 10% glycerol with one change of buffer after 2hours and then left to dialyse overnight. The following day the virus diluted with autoclaved dialysis fluid to make up to 5mL volume, aliquoted as 500 µL aliquots and stored at -70°C. A list of all RAd's used in this study can be found in table2.3.

Table 2.3. List of Recombinant Adenoviruses used.

RAd	Comment	Background
RAd-Control	Empty vector	AdZ
RAd-UL141	Expresses HCMV strain Merlin UL141	AdEasy
RAd-CD112-mCherry	Expresses CD112 with a C-terminal mCherry tag	AdEasy
RAd-CD155-mCherry	Expresses CD155 with a C-terminal mCherry tag	AdEasy
RAd-GFP	Expresses EGFP	AdEasy
RAd-β.7	Expresses HCMV strain Merlin β2.7 with a C-terminal V5 tag	AdZ
RAd-RL1	Expresses HCMV strain Merlin RL1 with a C-terminal V5 tag	AdZ
RAd-RL5A	Expresses HCMV strain Merlin RL5A with a C-terminal V5 tag	AdZ
RAd-RL6	Expresses HCMV strain Merlin RL6 with a C-terminal V5 tag	AdZ
RAd-RL8A	Expresses HCMV strain Merlin RL8A with a C-terminal V5 tag	AdZ
RAd-RL9	Expresses HCMV strain Merlin RL9 with a C-terminal V5 tag	AdZ
RAd-RL10	Expresses HCMV strain Merlin RL10 with a C-terminal V5 tag	AdZ
RAd-RL11	Expresses HCMV strain Merlin RL11 with a C-terminal V5 tag	AdZ
RAd-RL12	Expresses HCMV strain Merlin RL12 with a C-terminal V5 tag	AdZ
RAd-RL13	Expresses HCMV strain Merlin RL13 with a C-terminal V5 tag	AdZ
RAd-UL1	Expresses HCMV strain Merlin UL1 with a C-terminal V5 tag	AdZ
RAd-UL2	Expresses HCMV strain Merlin UL2 with a C-terminal V5 tag	AdZ
RAd-UL4	Expresses HCMV strain Merlin UL4 with a C-terminal V5 tag	AdZ
RAd-UL5	Expresses HCMV strain Merlin UL5 with a C-terminal V5 tag	AdZ
RAd-UL6	Expresses HCMV strain Merlin UL6 with a C-terminal V5 tag	AdZ
RAd-UL7	Expresses HCMV strain Merlin UL7 with a C-terminal V5 tag	AdZ
RAd-UL7/8	Expresses HCMV strain Merlin UL7/8 with a C-terminal V5 tag	AdZ
RAd-UL8	Expresses HCMV strain Merlin UL8 with a C-terminal V5 tag	AdZ
RAd-UL9	Expresses HCMV strain Merlin UL9 with a C-terminal V5 tag	AdZ
RAd-UL10	Expresses HCMV strain Merlin UL10 with a C-terminal V5 tag	AdZ
RAd-UL11	Expresses HCMV strain Merlin UL11 with a C-terminal V5 tag	AdZ
RAd-UL13	Expresses HCMV strain Merlin UL13 with a C-terminal V5 tag	AdZ
RAd-UL14	Expresses HCMV strain Merlin UL14	AdZ
RAd-UL15A	Expresses HCMV strain Merlin UL15A with a C-terminal V5 tag	AdZ
RAd-UL16	Expresses HCMV strain Merlin UL16 with a C-terminal V5 tag	AdZ
RAd-UL17	Expresses HCMV strain Merlin UL17 with a C-terminal V5 tag	AdZ
RAd-UL18	Expresses HCMV strain Merlin UL18 with a C-terminal V5 tag	AdZ
RAd-UL19	Expresses HCMV strain Merlin UL19 with a C-terminal V5 tag	AdZ
RAd-UL20	Expresses HCMV strain Merlin UL20 with a C-terminal V5 tag	AdZ
RAd-UL21A	Expresses HCMV strain Merlin UL21A with a C-terminal V5 tag	AdZ
RAd-UL22A	Expresses HCMV strain Merlin UL22A with a C-terminal V5 tag	AdZ
RAd-UL23	Expresses HCMV strain Merlin UL23 with a C-terminal V5 tag	AdZ
RAd-UL24	Expresses HCMV strain Merlin UL24 with a C-terminal V5 tag	AdZ
RAd-UL25	Expresses HCMV strain Merlin UL25 with a C-terminal V5 tag	AdZ

RAd-UL26	Expresses HCMV strain Merlin UL26 with a C-terminal V5 tag	AdZ
RAd-UL27	Expresses HCMV strain Merlin UL27 with a C-terminal V5 tag	AdZ
RAd-UL28/29	Expresses HCMV strain Merlin UL28/29 with a C-terminal V5 tag	AdZ
RAd-UL30	Expresses HCMV strain Merlin UL30 with a C-terminal V5 tag	AdZ
RAd-UL31	Expresses HCMV strain Merlin UL31 with a C-terminal V5 tag	AdZ
RAd-UL32	Expresses HCMV strain Merlin UL32 with a C-terminal V5 tag	AdZ
RAd-UL33	Expresses HCMV strain Merlin UL33 with a C-terminal V5 tag	AdZ
RAd-UL34	Expresses HCMV strain Merlin UL34 with a C-terminal V5 tag	AdZ
RAd-UL35	Expresses HCMV strain Merlin UL35 with a C-terminal V5 tag	AdZ
RAd-UL36	Expresses HCMV strain Merlin UL36 with a C-terminal V5 tag	AdZ
RAd-UL37	Expresses HCMV strain Merlin UL37 with a C-terminal V5 tag	AdZ
RAd-UL38	Expresses HCMV strain Merlin UL38 with a C-terminal V5 tag	AdZ
RAd-UL40	Expresses HCMV strain Merlin UL40 with a C-terminal V5 tag	AdZ
RAd-UL41A	Expresses HCMV strain Merlin UL41A with a C-terminal V5 tag	AdZ
RAd-UL42	Expresses HCMV strain Merlin UL42 with a C-terminal V5 tag	AdZ
RAd-UL43	Expresses HCMV strain Merlin UL43 with a C-terminal V5 tag	AdZ
RAd-UL44	Expresses HCMV strain Merlin UL44 with a C-terminal V5 tag	AdZ
RAd-UL45	Expresses HCMV strain Merlin UL45 with a C-terminal V5 tag	AdZ
RAd-UL46	Expresses HCMV strain Merlin UL46 with a C-terminal V5 tag	AdZ
RAd-UL47	Expresses HCMV strain Merlin UL47 with a C-terminal V5 tag	AdZ
RAd-UL48	Expresses HCMV strain Merlin UL48 with a C-terminal V5 tag	AdZ
RAd-UL49	Expresses HCMV strain Merlin UL49 with a C-terminal V5 tag	AdZ
RAd-UL50	Expresses HCMV strain Merlin UL50 with a C-terminal V5 tag	AdZ
RAd-UL51	Expresses HCMV strain Merlin UL51 with a C-terminal V5 tag	AdZ
RAd-UL52	Expresses HCMV strain Merlin UL52 with a C-terminal V5 tag	AdZ
RAd-UL53	Expresses HCMV strain Merlin UL53 with a C-terminal V5 tag	AdZ
RAd-UL54	Expresses HCMV strain Merlin UL54 with a C-terminal V5 tag	AdZ
RAd-UL55	Expresses HCMV strain Merlin UL55 with a C-terminal V5 tag	AdZ
RAd-UL56	Expresses HCMV strain Merlin UL56 with a C-terminal V5 tag	AdZ
RAd-UL57	Expresses HCMV strain Merlin UL57 with a C-terminal V5 tag	AdZ
RAd-UL69	Expresses HCMV strain Merlin UL69 with a C-terminal V5 tag	AdZ
RAd-UL70	Expresses HCMV strain Merlin UL70 with a C-terminal V5 tag	AdZ
RAd-UL71	Expresses HCMV strain Merlin UL71 with a C-terminal V5 tag	AdZ
RAd-UL72	Expresses HCMV strain Merlin UL72 with a C-terminal V5 tag	AdZ
RAd-UL73	Expresses HCMV strain Merlin UL73 with a C-terminal V5 tag	AdZ
RAd-UL74	Expresses HCMV strain Merlin UL74 with a C-terminal V5 tag	AdZ
RAd-UL75	Expresses HCMV strain Merlin UL75 with a C-terminal V5 tag	AdZ
RAd-UL76	Expresses HCMV strain Merlin UL76 with a C-terminal V5 tag	AdZ
RAd-UL77	Expresses HCMV strain Merlin UL77 with a C-terminal V5 tag	AdZ
RAd-UL78	Expresses HCMV strain Merlin UL78 with a C-terminal V5 tag	AdZ
RAd-UL79	Expresses HCMV strain Merlin UL79 with a C-terminal V5 tag	AdZ
RAd-UL80	Expresses HCMV strain Merlin UL80 with a C-terminal V5 tag	AdZ
RAd-UL80.5	Expresses HCMV strain Merlin UL80.5 with a C-terminal V5 tag	AdZ
RAd-UL82	Expresses HCMV strain Merlin UL82 with a C-terminal V5 tag	AdZ

RAd-UL83	Expresses HCMV strain Merlin UL83 with a C-terminal V5 tag	AdZ
RAd-UL84	Expresses HCMV strain Merlin UL84 with a C-terminal V5 tag	AdZ
RAd-UL85	Expresses HCMV strain Merlin UL85 with a C-terminal V5 tag	AdZ
RAd-UL86	Expresses HCMV strain Merlin UL86 with a C-terminal V5 tag	AdZ
RAd-UL87	Expresses HCMV strain Merlin UL87 with a C-terminal V5 tag	AdZ
RAd-UL88	Expresses HCMV strain Merlin UL88 with a C-terminal V5 tag	AdZ
RAd-UL89	Expresses HCMV strain Merlin UL89 with a C-terminal V5 tag	AdZ
RAd-UL91	Expresses HCMV strain Merlin UL91 with a C-terminal V5 tag	AdZ
RAd-UL92	Expresses HCMV strain Merlin UL92 with a C-terminal V5 tag	AdZ
RAd-UL93	Expresses HCMV strain Merlin UL93 with a C-terminal V5 tag	AdZ
RAd-UL94	Expresses HCMV strain Merlin UL94 with a C-terminal V5 tag	AdZ
RAd-UL95	Expresses HCMV strain Merlin UL95 with a C-terminal V5 tag	AdZ
RAd-UL96	Expresses HCMV strain Merlin UL96 with a C-terminal V5 tag	AdZ
RAd-UL97	Expresses HCMV strain Merlin UL97 with a C-terminal V5 tag	AdZ
RAd-UL98	Expresses HCMV strain Merlin UL98 with a C-terminal V5 tag	AdZ
RAd-UL99	Expresses HCMV strain Merlin UL99 with a C-terminal V5 tag	AdZ
RAd-UL100	Expresses HCMV strain Merlin UL100 with a C-terminal V5 tag	AdZ
RAd-UL102	Expresses HCMV strain Merlin UL102 with a C-terminal V5 tag	AdZ
RAd-UL103	Expresses HCMV strain Merlin UL103 with a C-terminal V5 tag	AdZ
RAd-UL104	Expresses HCMV strain Merlin UL104 with a C-terminal V5 tag	AdZ
RAd-UL105	Expresses HCMV strain Merlin UL105 with a C-terminal V5 tag	AdZ
RAd-UL111A	Expresses HCMV strain Merlin UL111A with a C-terminal V5 tag	AdZ
RAd-UL112	Expresses HCMV strain Merlin UL112 with a C-terminal V5 tag	AdZ
RAd-UL114	Expresses HCMV strain Merlin UL114 with a C-terminal V5 tag	AdZ
RAd-UL115	Expresses HCMV strain Merlin UL115 with a C-terminal V5 tag	AdZ
RAd-UL116	Expresses HCMV strain Merlin UL116 with a C-terminal V5 tag	AdZ
RAd-UL117	Expresses HCMV strain Merlin UL117 with a C-terminal V5 tag	AdZ
RAd-UL119	Expresses HCMV strain Merlin UL119 with a C-terminal V5 tag	AdZ
RAd-UL120	Expresses HCMV strain Merlin UL120 with a C-terminal V5 tag	AdZ
RAd-UL121	Expresses HCMV strain Merlin UL121 with a C-terminal V5 tag	AdZ
RAd-UL122	Expresses HCMV strain Merlin UL122 with a C-terminal V5 tag	AdZ
RAd-UL123	Expresses HCMV strain Merlin UL123 with a C-terminal V5 tag	AdZ
RAd-UL124	Expresses HCMV strain Merlin UL124 with a C-terminal V5 tag	AdZ
RAd-UL128	Expresses HCMV strain Merlin UL128 with a C-terminal V5 tag	AdZ
RAd-UL130	Expresses HCMV strain Merlin UL130 with a C-terminal V5 tag	AdZ
RAd-UL131A	Expresses HCMV strain Merlin UL131A with a C-terminal V5 tag	AdZ
RAd-UL132	Expresses HCMV strain Merlin UL132 with a C-terminal V5 tag	AdZ
RAd-UL133	Expresses HCMV strain Merlin UL133 with a C-terminal V5 tag	AdZ
RAd-UL135	Expresses HCMV strain Merlin UL135 with a C-terminal V5 tag	AdZ
RAd-UL136	Expresses HCMV strain Merlin UL136 with a C-terminal V5 tag	AdZ
RAd-UL138	Expresses HCMV strain Merlin UL138 with a C-terminal V5 tag	AdZ
RAd-UL139	Expresses HCMV strain Merlin UL139 with a C-terminal V5 tag	AdZ
RAd-UL140	Expresses HCMV strain Merlin UL140 with a C-terminal V5 tag	AdZ
RAd-UL142	Expresses HCMV strain Merlin UL142 with a C-terminal V5 tag	AdZ

RAd-UL144	Expresses HCMV strain Merlin UL144 with a C-terminal V5 tag	AdZ
RAd-UL145	Expresses HCMV strain Merlin UL145 with a C-terminal V5 tag	AdZ
RAd-UL146	Expresses HCMV strain Merlin UL1446with a C-terminal V5 tag	AdZ
RAd-UL147	Expresses HCMV strain Merlin UL147 with a C-terminal V5 tag	AdZ
RAd-UL147A	Expresses HCMV strain Merlin UL147A with a C-terminal V5 tag	AdZ
RAd-UL148	Expresses HCMV strain Merlin UL148 with a C-terminal V5 tag	AdZ
RAdUL148A	Expresses HCMV strain Merlin UL148A with a C-terminal V5 tag	AdZ
RAd-UL148B	Expresses HCMV strain Merlin UL148B with a C-terminal V5 tag	AdZ
RAd-UL148C	Expresses HCMV strain Merlin UL148C with a C-terminal V5 tag	AdZ
RAd-UL148D	Expresses HCMV strain Merlin UL148D with a C-terminal V5 tag	AdZ
RAd-UL150	Expresses HCMV strain Merlin UL150 with a C-terminal V5 tag	AdZ
RAd-TRS1	Expresses HCMV strain Merlin TRS1 with a C-terminal V5 tag	AdZ
RAd-IRS1	Expresses HCMV strain Merlin IRS1 with a C-terminal V5 tag	AdZ
RAd-US1	Expresses HCMV strain Merlin US1 with a C-terminal V5 tag	AdZ
RAd-US2	Expresses HCMV strain Merlin US2 with a C-terminal V5 tag	AdZ
RAd-US3	Expresses HCMV strain Merlin US3 with a C-terminal V5 tag	AdZ
RAd-US6	Expresses HCMV strain Merlin US6 with a C-terminal V5 tag	AdZ
RAd-US7	Expresses HCMV strain Merlin US7 with a C-terminal V5 tag	AdZ
RAd-US8	Expresses HCMV strain Merlin US8 with a C-terminal V5 tag	AdZ
RAd-US9	Expresses HCMV strain Merlin US9 with a C-terminal V5 tag	AdZ
RAd-US10	Expresses HCMV strain Merlin US10 with a C-terminal V5 tag	AdZ
RAd-US11	Expresses HCMV strain Merlin US11 with a C-terminal V5 tag	AdZ
RAd-US12	Expresses HCMV strain Merlin US12 with a C-terminal V5 tag	AdZ
RAd-US13	Expresses HCMV strain Merlin US13 with a C-terminal V5 tag	AdZ
RAd-US14	Expresses HCMV strain Merlin US14 with a C-terminal V5 tag	AdZ
RAd-US15	Expresses HCMV strain Merlin US15 with a C-terminal V5 tag	AdZ
RAd-US16	Expresses HCMV strain Merlin US16 with a C-terminal V5 tag	AdZ
RAd-US17	Expresses HCMV strain Merlin US17 with a C-terminal V5 tag	AdZ
RAd-US18	Expresses HCMV strain Merlin US18 with a C-terminal V5 tag	AdZ
RAd-US19	Expresses HCMV strain Merlin US19 with a C-terminal V5 tag	AdZ
RAd-US20	Expresses HCMV strain Merlin US20 with a C-terminal V5 tag	AdZ
RAd-US21	Expresses HCMV strain Merlin US21 with a C-terminal V5 tag	AdZ
RAd-US22	Expresses HCMV strain Merlin US22 with a C-terminal V5 tag	AdZ
RAd-US23	Expresses HCMV strain Merlin US23 with a C-terminal V5 tag	AdZ
RAd-US24	Expresses HCMV strain Merlin US24 with a C-terminal V5 tag	AdZ
RAd-US26	Expresses HCMV strain Merlin US26 with a C-terminal V5 tag	AdZ
RAd-US27	Expresses HCMV strain Merlin US27 with a C-terminal V5 tag	AdZ
RAd-US28	Expresses HCMV strain Merlin US28 with a C-terminal V5 tag	AdZ
RAd-US29	Expresses HCMV strain Merlin US29 with a C-terminal V5 tag	AdZ
RAd-US30	Expresses HCMV strain Merlin US30 with a C-terminal V5 tag	AdZ
RAd-US31	Expresses HCMV strain Merlin US31 with a C-terminal V5 tag	AdZ
RAd-US32	Expresses HCMV strain Merlin US32 with a C-terminal V5 tag	AdZ
RAd-US34	Expresses HCMV strain Merlin US34 with a C-terminal V5 tag	AdZ
RAd-US34A	Expresses HCMV strain Merlin US34A with a C-terminal V5 tag	AdZ

2.3.7. RAd titration

RAd viruses were titrated by limiting dilution assay. A total of 1 x 10^6 911 cells were seeded into a flat bottomed 96 well plate (Nunc) and allowed to adhere overnight. The following day serial dilutions between 1 x 10^2 and 1 x 10^{10} of the virus stock were made in DMEM-10. 200µL of each RAd dilution was added to one row (12 wells) of a 96 well plate. After 10-14 days cells were observed microscopically for cpe and those wells that exhibited evidence of viral growth were marked. Virus titre was calculated as TCID50 units using the formula of Reed and Meunch (1938). For convenience, TCID50 were converted to pfu/mL using an accepted conversion factor (1 TCID50 unit = 0.7 infectious units).

2.3.8. Single infection of cells for assays

Primary HFFFs or HFFF-hTert's were normally infected with HCMV at MOI 10 pfu/cell or with RAds at a MOI of 100 pfu/cell for use in western blot and flow cytometry. HFFF-hCAR cells were infected with both HCMV and RAds at a MOI of 10 for use in western blot and flow cytometry. Hela, Hela-US2 and Hela-US11 were infected at MOI100 for use in flow cytometry.

Cells were seeded to the required density 24 hours prior to infection. The cell media was removed and virus inoculumn was added in minimal, yet consistent volume of media. Cells were incubated with virus inoculums for 120 minutes on a rocking platform in an incubator at 37°C, 5% CO2, after which time the inoculums was removed and replaced with fresh DMEM-10.

All viral infections include a control: An empty RAd vector for RAd infections and a mock infection (medium only) for HCMV infections.

2.3.9. Co-infection of cells for assays

For RAd-RAd co-infections of HFFF-hCARs, cells were infected at MOI10 for each virus. Each virus was added to the cell inoculums. The inoculum was then added to cells before incubation for 2hrs on a rocking platform in an incubator at 37oC, 5% CO2, after which time the inoculums was removed and replaced with fresh DMEM-10

For RAd-HCMV co-infections of HFFF, cells were infected with RAd at MOI100 and HCMV MOI 30. Each virus was added to the cell inoculum. The inoculum were then added to cells before incubation for 120 minutes on a rocking platform in an incubator at 37°C, 5% CO_2, after which time the inoculums was removed and replaced with fresh DMEM-10 .

2.3.10. Magnetofection

Magnetofection is a process of introducing nucleic acids or viruses into cells using magnetic nanoparticles coated with cationinc molecules. RAds were incubated with the magnetofection reagent, Combimag, for 20 minutes before being added to the cells pre seeded 24hours earlier in a 96 well-plate. The 96well plate was then placed on top of a magnetic plate for 20 minutes which drew the virus-bound magnetic nanoparticles onto the cell membrane thus facilitating efficient infection.

2.4. MOLECULAR BIOLOGY

2.4.1. PCR

Polymerase chain reactions (PCRs) were performed using the Expand Hi-Fi polymerase (Roche) in the manufacturers buffer (Containing Magnesium), 0.5µL dNTPs (10mM) and 0.5µL of each primer (100pmol/mL) (made to order from Invitrogen). The following thermocycling reaction was used:

1) 95°C for 2minutes
2) 95°C for 30 seconds
3) 58°C for 30 seconds
4) 72°C for 90 seconds
5) 4°C hold.

Steps 2) – 4) were repeated for 35 cycles.

2.4.2. Agarose gel electrophoresis

Separation of DNA fragments on the basis of size was performed by electrophoresis with a 0.7% (w/v) TAE agarose gel containing Ethidium Bromide. DNA samples were mixed with DNA loading buffer (6x) before adding to wells of the pre set agarose gel. DNA standards were run in parallel. Gels were run at 70-100V for 1-2hours depending on the resolution required before being visualised by UV illumination at 365nm (.

2.4.3. Isolation of DNA from an Agarose gel

Where DNA had been separated by gel electrophoresis, to be used in cloning or sequencing, bands of the expected size were excised from the gel using a clean scalpel and the DNA extracted using the illustra GFX PCR DNA and Gel band purification kit (GE healthcare). The kit is based on the principal that DNA adsorbs to silica in the presence of high salt, while contaminants will pass through. Impurities are washed away and the DNA eluted using an elution buffer.

Using a UV spectrophotometer DNA was excised from the agarose gel using a scalpel and transferred to a 1.5mL tube. Gel slices were weighed and 10µL Capture buffer 3 for every 10mg of gel slice was added (1g =1mL) and heated to 60°C for 15-30 minutes until the agarose was completely dissolved. For each purification performed one GFX microspin column was placed into one collection tube. 800µL of sample mix at a time was added to the assembled GFX microspin column and collection tube and incubated at room temperature for one minute before centrifuged at 13,000rpm for 30seconds at RT. The flow through was discarded and the sample binding steps were repeated as necessary until the entire sample was loaded. Once all the sample mix had been spun through the column, 500µL of wash buffer type 1 was added to the GFX MicroSpin column and centrifuged at 13,000rpm for 30 seconds at room temperature to remove impurities. The collection tube was discarded and the column transferred to a fresh DNAse-free 1.5mL tube. DNA was then eluted in 30µL of Elution buffer type 4 (10mM Tris-HCL, pH 8.0). Purified DNA can was stored at -20°C until required.

2.4.5. DNA extraction from solution

The basic principle involves to capturing of DNA in solution is the same for capturing DNA from agarose gel. For each purification performed, one GFX microspin column was placed into one collection tube. DNA in solution was captured in capture buffer 3 and the sample capture mixture loaded into a GFX MicroSpin column and centrifuged at 13,000rpm for 30 seconds at RT. The sample binds to the column and the supernatant is discarded. 500µL of wash buffer type 1 was then added to the GFX MicorSpin column and centrifuged at 13,000rpm for 30 seconds at RT to remove impurities. The collection tube was discarded and the column transferred to a fresh DNase-free 1.5mL tube. DNA was then eluted in 30µL of Elution buffer type 4 (10mM Tris-HCL, pH 8.0). Purified DNA was stored at -20°C until required.

2.4.6. Plasmid DNA minipreps

Small scale plasmid preparations were performed using Qiagen spin miniprep kits according to manufacturer's instructions (Qiagen). The kit is based on the alkaline lysis of bacterial cells and the adsorption of DNA to a silica gel membrane in the presence of high salt. DNA is then washed to remove impurities and eluted into Tris buffer. Bacterial cells containing the plasmid of interest were grown in an overnight culture at 32°C in 5 mL LB containing chloramphenicol (1 in 1,000 of stock solution). From the overnight culture, 4.5 mLs was pelleted by centrifugation at 4,000 rpm for 5 minutes at RT. DNA was then obtained using a spin miniprep kit (Qiagen) as described in the manufactures instructions. In brief the cell pellet is re-suspended in 250µL buffer P1 and transferred to a 1.5 mL tube. To this, 250µL of buffer P2 was added and incubated for 5 minutes at RT. Post incubation 250 µL of buffer N3 was added and mixed several times by inversion, followed by centrifugation at 13,000rpm for 10 minutes at RT. The supernatant was transferred to a new 1.5 mL tube and the pellet discarded. DNA was precipitated by adding 750µL of isopropanol, mixing by inversion and centrifuging at 13,000rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet re-suspended in 500 µL 70% ethanol followed by centrifugation at 13,000rpm for 5 minutes. The supernatant was discarded and air-dried for 15 minutes in a 37°C waterbath. Finally the DNA was re-dissolved in 30µL EB buffer (10mM Tris, pH8.5).

2.4.7. Plasmid DNA maxipreps

The BacMAX 100 kit (Machery Nagel) was used for the large scale plasmid preparation to generate transfection-quality DNA, as described in the manufacturer's instructions using the filtration method. For this procedure bacteria are subject to alkaline lysis, followed by binding to an anion-exchange resin and finally eluted after washing of the column. For large scale plasmid purification, a colony of bacteria containing plasmid of interest was used to inoculate 250mL LB media and incubated overnight in a shaking incubator at 32oC. Bacterial cells were recovered by centrifugation at 6,000 RPM for15 minutes at 4°C using 250 mL pots (Sovall) and rotor SLA1500. Supernatant was discarded and the cell pellet resuspended in 12mL manufacturers supplied buffer S1 containing RNAse. Bacteria were lysed by the addition of equal volume, 12 mL, of manufacturer lysis buffer S2 and incubated for 5minutes at RT before the addition of 12 mL manufacturers neutralisation buffer S3That contains potassium acetate that causes the formation of a precipitate containing chromosomal DNA and other cellular components. The resulting solution and precipitate was then centrifuged at 6,000 RPM for15 minutes at 4°C during which time the manufacturers BAC 100 column was equilibrated with 5mL manufactures buffer N2. Once centrifuged the lysate was poured into a funnel containing filter paper above the pre-equilibrated BAC 100 column. The flow through was allowed to empty through the column by gravity flow. Once emptied the column was washed with 2 x 18 mL manufactures wash bufferN3 to remove impurities and the DNA was eluted into pre autoclaved pots (Sorvall) with 15mL manufactures elution buffer N5 pre heated to 50°C. To precipitate the DNA, 11 mL of isopropanol was added to the elute followed by centrifugation at 15,000 RPM for 30 minutes at 4°C. The DNA precipitate was washed with 70% (v/v) ethanol followed by centrifugation at 15,000 RPM for 10minutes at RT and the pellet air dried before being resuspended in 100µL of manufacturers buffer TE.

2.4.8. Estimation of DNA concentration

To estimate DNA concentration, DNA from plasmid preparations were analysed by spectrophotometer (Biopharamacia Biotech Ultrospec 3000). Using the spectrophotometer, absorbance of a DNA solution at 260nm was compared with that of a control sample

2.4.9. Restriction enzyme digestion

Restriction enzyme digestion of DNA was undertaken according to the manufactures instructions (New England Biolabs). 10 µL reaction mixtures were set up containing: 8µL DNA, 1 µL 10x buffer and 1µL restriction enzyme. Reaction mixtures were incubated at 37°C for 2 hours or overnight. Following digestion, DNA was separated by TAE electrophoresis.

2.4.10. Glycerol stock generation

Plasmids of interest were stored as glycerol stocks, generated by mixing 500µL of an overnight bacterial culture with an equal volume of 80% (v/v) glycerol in a cryovial. Glycerol stocks were stored at -70°C.

2.4.11. DNA sequencing

DNA samples were sequenced using the 'Big Dye; terminator cycle sequencing kit (Perkin-Elmer, Boston, USA) and dideoxy terminator cycle sequencing. The sequencing reaction mixture consisted of DNA sample, 4µL reaction mix, 1µL sequencing primer (see table 2.4 for primer sequences) and distilled water to a total reaction volume of 10µL. The following thermocycling reaction condition was used:

Denaturation:	96°C x 30s	x 99 cycles
Annealing	50°C x 15s	
Elongation	60°C x 4min	

Following the thermocylcing reaction, the sequencing products were cleaned up using Performa DTR columns, according to the manufacturer's instructions. One Performa DTR column for each sequencing reaction was placed in a tube and centrifuged at 3,000rpm for 3 minutes at RT using a microfuge. The column was moved to a fresh 1.5 mL tube and the entire sequencing reaction was added to the column before being centrifuges at 3,000rpm

Table 2.4. Primer sequences used. All primer sequences are 5' --> 3' and were produced to order from Invitrogen (Paisley, UK)

Primer sequences	Direction	Comment
CCGCACCATCCCGTACTGCATGTTCCACATGTACGCGCTAGACGTGTAATCCACTCGCAGTTCGGGGACGCAACGCAGCCTGTGACG GAAGATCACTTCG	Forward	Insert selection cassette in the middle of US2
GAACGTGCGGAACTTAGATGTGATGCCGATCTTCGAGACGCTAGCCCTGCGTCTGGTACTGCAAGGGGATGTGATCTGCCTGAGGTT CTTATGGCTCTTG	Reverse	US2 Reverse for SACB
CCGCACCATCCCGTACTGCATGTTCCACATGTACGCGCTAGACGTGTAATCCACTCGCAGTTCGGGGACGCAACGCAGCCTGTTGAC AATTAATCATCGGCA	Forward	US2 Forward for GALK
GAACGTGCGGAACTTAGATGTGATGCCGATCTTCGAGACGCTAGCCCTGCGTCTGGTACTGCAAGGGGATGTGATCTGTCAGCACTG TCCTGCTCCTT	Reverse	US2 Reverse for GALK
ATGGGTGCTCGTGGCTACATTTATTGAAACAAACCGCGATCCCGGGCGTCGGTGAAACAGCGTGTGGACTGTACGCTCTTTCCCAAG TTATATCCCAGAG	oligonucleotide	Removes US2
GCACTCTACATAATAAGTTTGGCATTGATGACAGGGGGAAAAGTAGAACAACACGAGTTTTGTGCGTTGGCCTGTTGACAATTAATC ATCGGCA	Forward	US11 Forward for GALK
GATCGAGTGCCGACGCCGGTGGAGGACATCTCCGAAAGCCTCGTCGCAAAACGCTACTGGCTCCGGGACTATCGTGTTCCTCAGCAC TGTCCTGCTCCTT	Reverse	US11 Reverse for GALK
GGTGAGTCGTTTCCGAGCGACTCGAGATGCACTCCGCTTCAGTCTATATATCTGTCTTACAAGGGAAGGCTGTTCCCCTGTCTAGACT CAAAAGCTGTAAGGCTATCTTA	oligonucleotide	removes US11
ACCTCCGCGGCCGAAAAAATGTCAAACACACGCCCCTCACCACGTTCATCATTGAAAGTCTCTCCAGTCCCTATGTTGCCTGTGACGG AAGATCACTTCG	Forward	US1-11 Forward for SACB
CGTGCAAGACTACATGCTATAAGATAGCCTTACAGCTTTTGAGTCTAGACAGGGGAACAGCCTTCCCTTGTAAGACAGACTGAGGTT CTTATGGCTCTTG	Reverse	US1-11 Reverse for SACB
CACGCCCCTCACCACGTTCATCATTGAAAGTCTCTCCAGTCCCTATGTTGTCTGTCTTACAAGGGAAGGCTGTTCCCCTGTCTAGACTC AAAAGCTGTAA	oligonucleotide	Removes US1-11
CTCGTGGCTACATTTATTGA	Forward	Forward sequencing primer to confirm US2 removal
GTGCTATGGCTGCTCAATGC	Reverse	Reverse sequencing primer to confirm US2 removal
AAGTAGGTAGGTTCTCTCGT	Forward	Forward sequencing primer to confirm US11 removal
ATGTTTCTGCTCGTGGTAGT	Reverse	Reverse sequencing primer to confirm US11 removal
CATCTGGTATCCAAACTACGCC	Forward	Sequence to confirm US1-11 deletion
AATGTCGTAACAACTCCG	Forward	From CMV promoter
ACCTGATGGTGATAAGAAG	Reverse	From CMV polyA
TGCACCACCAACTGCTTAGC	Forward	GAPDH
CTGGAACGGTGAAGGTGACA	Forward	B-Actin
CCGGATCTCCTGGCTCTCA	Forward	CD112
TGGACGGCAAGAATGTGACC	Forward	CD155
GGCATGGACTGTGGTCATGAG	Reverse	GAPDH
AAGGGACTTCCTGTAACAATGCA	Reverse	B-Actin
TGAAGCGGCTGGTGACAGT	Reverse	CD112
ATCATAGCCAGAGATGGATACC	Reverse	CD155

for 3minues at RT. The flowthrough was moved to a new 0.5 mL tube and the sequencing products separated in-house on an ABI model sequencer (Applied Biosystems, Foster City, USA) by a dedicated technician at Central Biotechnology Services (Cardiff University).

2.5. RECOMBINEERING

2.5.1. The recombineering principle

Recombinant viruses were produced using a novel system that exploits DNA recombineering technology (2008). This utilises the *E.coli* bacterial strain SW102 that contains a single copy genome inserted into a single copy prokaryotic BAC (bacterial artificial chromosome) plasmid. The vector AdZ vector contains a selection cassette encoding Ampicillin resistance, *LacZ* and sucrose sensitivity (*AmpR/LacZ/SacB*) driven by the HCMV major IE promoter. The *LacZ* gene encodes a β-galactosidase that turns blue when the sugar galactose is metabolised. Colonies that contain the selection cassette are therefore resistant to Ampicillin, sensitive to sucrose and blue. The SW102 strain that maintains the vector contains a defective phage expressing the lambda red genes, which are transiently induced by heatshock and mediate homologous recombination between the vector and an oligonucleotide containing the gene of interest plus short arms of homology. A successful homologous recombination event replaces the selection cassette with the gene of interest resulting in a change in resistance, colony colour and sucrose sensitivity. Colonies that have lost the selection cassette are therefore sensitive to Ampicillin, able to grow on sucrose and white. Once sequenced to confirm correct modification, DNA is maxipreped and transfected into the relevant cell type to generate a recombinant virus. For a detailed breakdown of the recombineering protocol see Stanton et al (2008). Although a generalised protocol is described as follows:

2.5.2. Generalised recombineering protocol

Recombineering was performed as follows: 500µL of an overnight culture was diluted in 25 mL Luria-Bertani (LB) medium with chloramphenicol selection (12.5 ug/mL) in a 50 ML Duran bottle and grown at 32°C in a shaking incubator to an OD_{600} of 0.6. The culture was then transferred to a 50mL Falcon tube and heat shocked in a 42° C waterbath for

exactly 15 minutes. After 15 minutes the samples were cooled on ice, on a rocking platform for 20 minutes before pelleted at 4,000rpm for 5 mins at 0°C. The supernatant was discarded and the pellet resuspended in 1 mL ice cold ddH_2O. Once resuspended 24mL ice-cold ddH_2O was added and the samples pelleted again at 4,000rpm for 5mins at 0°C. This step was repeated once more. After the second washing and centrifugation step, the supernatant was discarded and the now competent cells were resuspended in the remaining supernatant (~200uL) and were kept on ice until electroporated with 4uL of gel-purified PCR product or 1µL oligonucleotide. A 25µL aliquot of competent cells was used for each electroporation in a 0.2cm cuvette (BioRad) at 2.5 Kv. After electroporation the bacteria were recovered in 5 mL LB for 4 hours in a 32°C shaking incubator. Recovery time depended on whether the selection cassette was inserted or removed. Post recovery 200µL bacteria were plated out onto agar selection plates and left in a 32°C incubator. After 48-72 hours correct (White) colonies were picked and grown overnight in 5 mL LB containing chloramphenicol at 32°C in a shaking incubator. DNA was minipreped, digested and sequenced to confirm correct modification. Once sequencing confirmed a correct modification, DNA was maxipreped and transfected into the relevant cell type to generate a recombinant virus.

2.5.3. Recombineering using the AdZ vector

The AdZ-5 vector contains a single copy replication deficient Ad5 ($E1^-$, $E3^-$) genome that is inserted into a single copy prokaryotic BAC (bacterial artificial chromosome) plasmid in SW102 bacteria, Figure 2.1 . The vector contains an *AmpR/LacZ/SacB* selection cassette that provides ampicillin resistance, *LacZ* and sucrose sensitivity, as described earlier. The AdZ vector also encodes a *I-sceI* cassette that allows the self excision of the RAd genome following transfection into 293 cells. The 293 cells contain the Adenovirus E1 region that allows for replicating Adenovirus to be produced and viral stocks to be grown.

Recombineering using the AdZ vector was carried out as described in the general recombineering protocol, section 2.5.2., the primers used to amplify a desired ORF were designed with regions on homology flanking the *AmpR/LacZ/SacB* selection cassette, and were designed as follows:

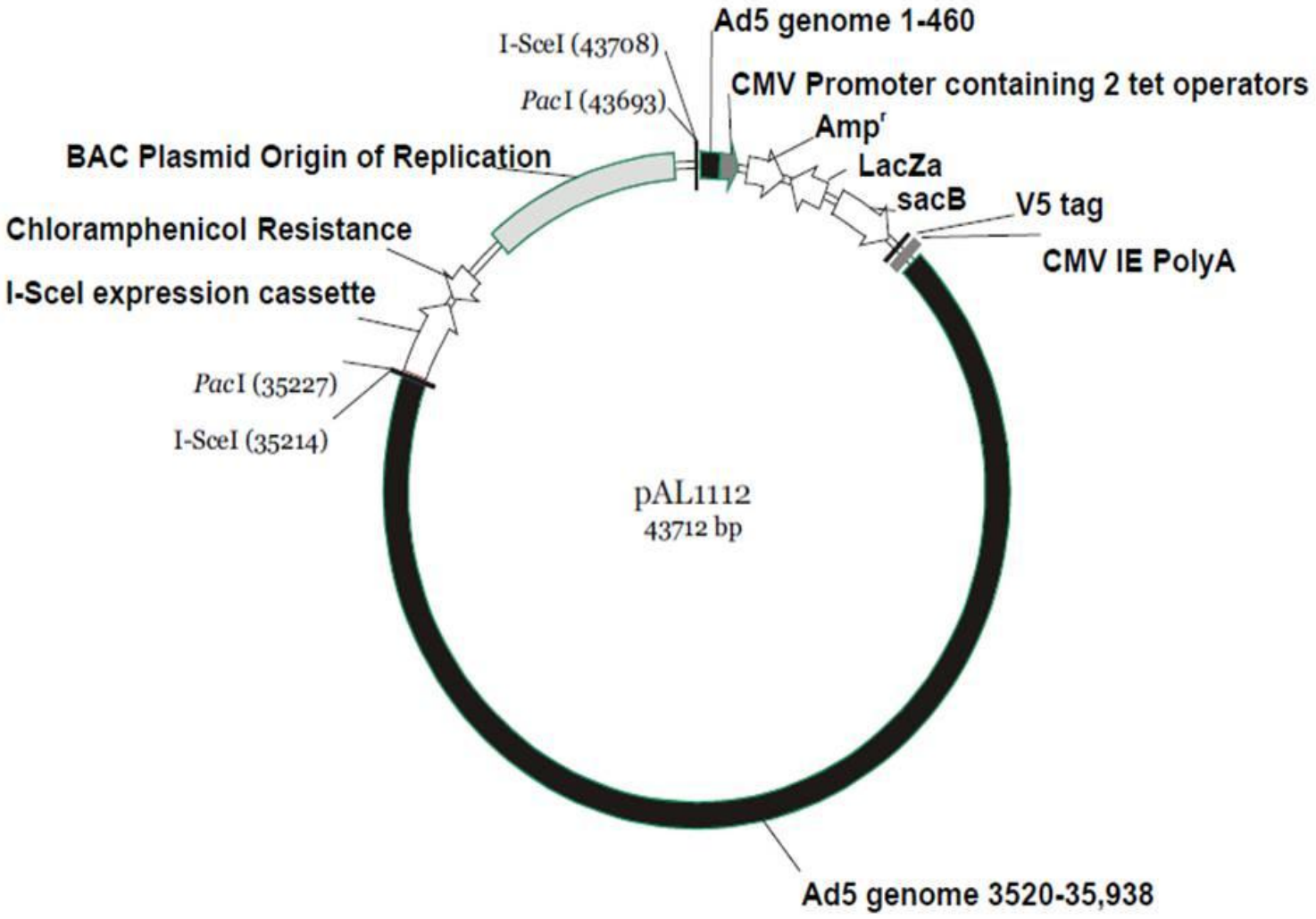


Figure 2.1. The Adz-5 vector
The AdZ-5 vector contains the Ad5 vector genome, deleted for E1 & E3 regions (rendering the vector non-replicative) in a single copy vector. It contains the HCMV major immediate early promoter & PolyA signal sequence, with a cassette in the MCS encoding ampicillin resistance, *lacZα* & *SacB*. *SacB* encodes for a gene giving sensitivity to sucrose, *lacZα* provides blue/white screening. The AdZ CMV promoter is repressible by tet repressors, thus if the vector is grown in 293TREx cells expression from the CMV promoter is prevented during growth. This allows the cloning of toxic genes, or genes which would otherwise interfere with the Ad replication. Adapted from www.Adz.cf.ac.uk Stanton et al (2008).

Forward

5’AACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAA
GACACCGGGACCGATCCAGCCTGGATCC----- required primer sequence (~20bp)-----

Reverse

TATAGAGTATACAATAGTGACGTGGGATCCCTACGTAGAATCAAGACCTAGGA
GCGGGTTAGGGATTGGCTTACCAGCGCT----- required primer sequence (~20bp)-----

Following a successful recombination event the selection cassette is lost and replaced with the desired ORF.

2.5.4. Recombineering to construct HCMV deletions

Bacteria containing the desired viral genome BAC to be modified were chosen and recombineering was carried out as described in the general recombineering protocol section 2.5.2. The following modifications were made dependant on which selection cassette was used:

2.5.4.1. Recombineering with the *AmpR/LacZ/SacB* selection cassette

Making deletions in CMV required a two-step recombineering protocol, Figure 2.2. (Stanton et al., 2010).

Bacteria containing the desired viral genome BAC to be modified were chosen and recombineering was carried out as described in the General recombineering protocol, section 2.5.2, with the following modifications:

During the first round a PCR product containing the selection cassette plus short arms of homology to an area of the genome to be deleted were used, Figure 2.2. Primers used to amplify *AmpR/LacZ/SacB* selection cassette were designed as follows:

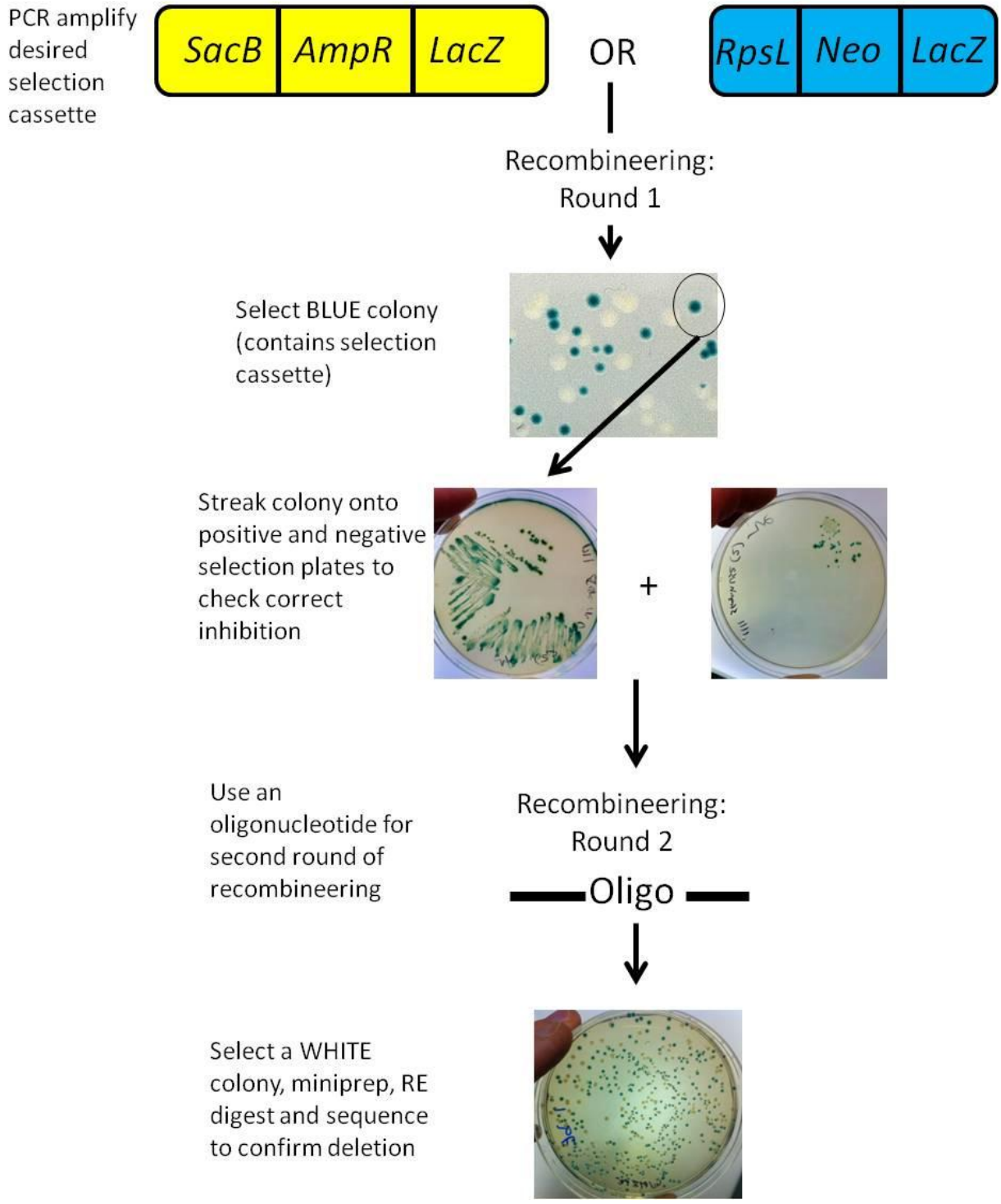


Figure 2.2. Generating HCMV deletions using AmpR/SacB/LacZ or RpsL/Neo/LacZ selection cassettes.

A two step recombineering approach is required to generate HCMV deletions. In the first round of recombineering a selection cassette is amplified using a 100bp primer. The primer is designed so that it contains ~20bp that will allow amplification of the desired selection cassette and ~ 80bp that is homologous to a region in the HCMV BAC where the selection cassette is to be inserted. Following a successful first round of recombination Blue colonies should be present. A blue colony is picked and streaked onto both positive and negative selection plates in order to check for correct inhibition. If correctly inhibited the colony is used for a second round of recombineering. During the second round a 100bp oligonucleotide is used, which is designed so that it contains50bp of homology to regions flanking either side of the genomic region to be removed. A successful recombination event will result in loss of the selection cassette and the desired genomic material. Due to the loss of the selection cassette correct colonies will be white.

Forward:

5’----------80bp homology----------CCTGTGACGGAAGATCACTTCG

Reverse:

5’----------80bp homology----------CCTGAGGTTCTTATGGCTCTTG

Following electroporation, bacteria were recovered in 1mL LB for 1hr in 32 shaking incubator. After recovery the 1mL was transferred to a tube, centrifuged at 13,000rpm for 30s, supernatant poured off and discarded. The cell pellet was resuspended in the remaining supernatant and plated onto LB-agar plates and left in an incubator at 32°C. After 48-72 hrs blue colonies were picked and sucrose sensitivity tested by spreading an individual colony onto both LB-agar and LB-sucrose selection plates.

Once sucrose sensitivity was confirmed correct colonies were picked and grown overnight in chloramphenicol and ampicillin. A second round of recombineering was then carried out as described In general recombineering protocol, section 2.5.2.

2.5.4.2. Recombineering with the *GalK* selection cassette

The *GalK* selection system was used to make deletions where the *AmpR/LacZ/SacB* selection cassette has failed. The *GalK* allows for a positive/negative selection system for manipulation of BAC’s (Warming et al., 2005). The *E.coli* galactose operon consists of four genes *GalE, GalT, GalK* and *GalM*, which are necessary for the growth and metabolism of galactose when the only carbon source. The *GalK* product galactokinase catalyses the first step in the galactose degradation pathway, phosphorylating galatcose-1-phophate. The galactokinase also catalyses the phosphorylation of a galactose analog, 2-dexygalactose-1-phosphate (DOG). The product of this reaction cannot be further metabolised, leading to a toxic build-up of 2-deoxygalactose-1-phosphate. Thus both positive and negative selection can be conferred by *GalK*, which is absent in the *E.coli* SW102 strain.

The *GalK* selection system requires two rounds of recombination, Figure 2.3: In the first the *GalK* cassette, containing homology to a specified position in the BAC is inserted by homologous recombination. The recombinant will now be able to grow on minimal media

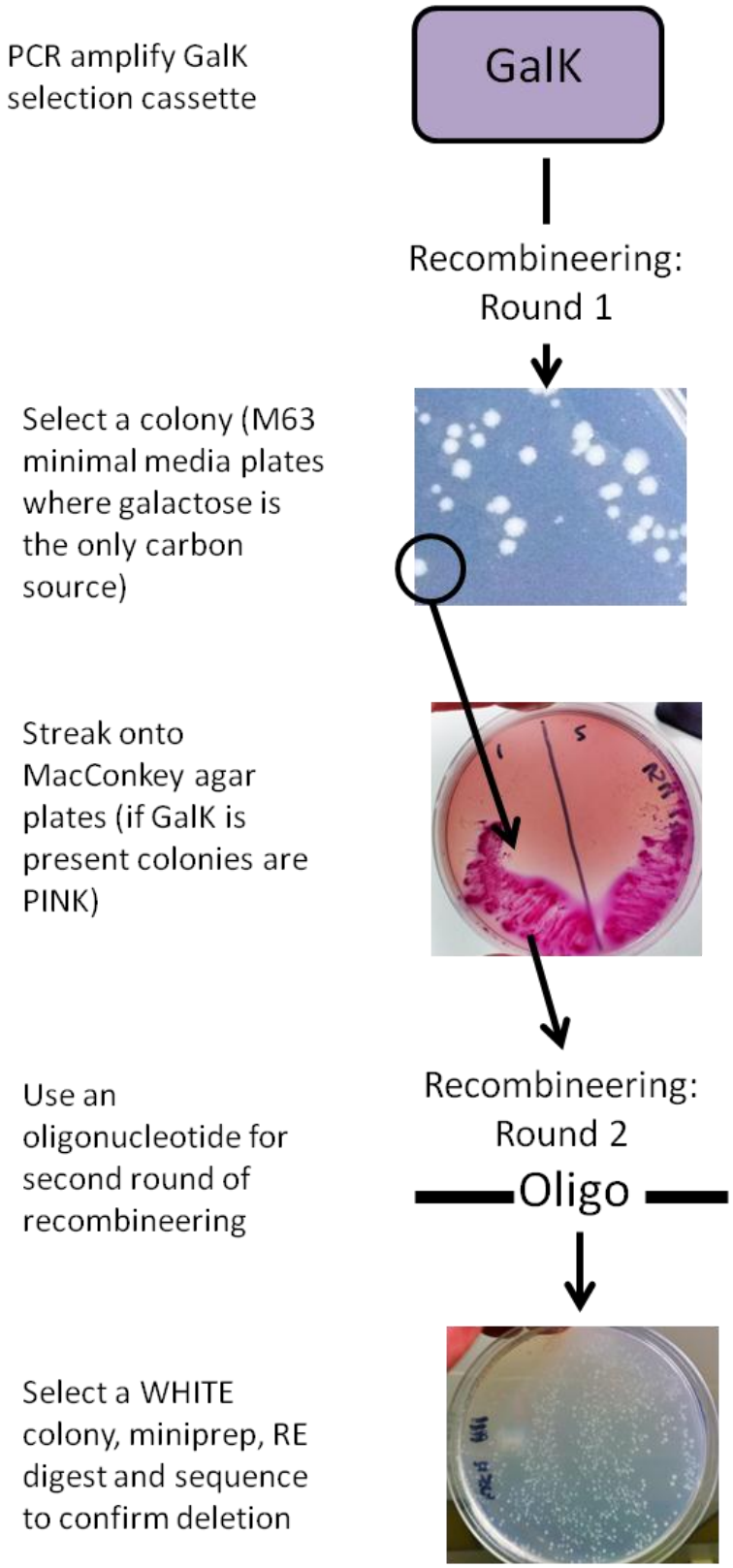


Figure 2.3. Generating HCMV deletions using *GalK* selection cassettes.
A two step recombineering approach is required to generate HCMV deletions. In the first round of recombineering the *GalK* election cassette is amplified using a 100bp primer. The primer is designed so that it contains ~20bp that will allow amplification of the *GalK* cassette and ~ 80bp that is homologous to a region in the HCMV BAC where the selection cassette is to be inserted. Following a successful first round of recombination white colonies appear on minimal media plates. A white colony is picked and streaked onto MacConkey agar plates. If *GalK* is present and functional colonies are pink. If correct a pink colony is picked and is used for a second round of recombineering. During the second round a 100bp oligonucleotide is used, which is designed so that it contains50bp of homology to regions flanking either side of the genomic region to be removed. A successful recombination event will result in loss of the selection cassette and the desired genomic material. A white colony is picked and, BAC DNA miniprepred and sequenced to confirm correct deletion. .

where galactose is the only carbon source. In the second round the *GalK* cassette is removed using a gel-purified PCR product or oligonucleotide. Correct colonies are achieved by selecting against the *GalK* cassette by resistance to 2-deoxy-galactose (DOG) on minimal plates with glycerol as the carbon source. DOG is harmless, unless phosphorylated by *GalK*.

Primers used to amplify the *GalK* selection cassette were designed as follows:

Forward:

5'----------80bp homology----------CCTGTTGACAATTAATCATCGGCA

Reverse:

5'----------80bp homology----------TCAGCACTGTCCTGCTCCTT

Round 1; is carried out as described in section 2.5.2. with the following modifications: During the first round of recombination a gel-purified PCR product encoding GalK is used. Following electroporation bacteria are recovered in 1mL LB for 1hr in a 32°C shaking incubator.

After recovery periods, the bacteria were washed twice in 1xM9 salts as follows: 1 ML culture was pelleted in a 1.5 mL tube by centrifugation at 13,000rpm for 30s at RT and the supernatant removed with a pipette. The pellet was resuspended in 1mL of 1xM9 salts before plating in serial dilutions (100ul, 100ul each of 1:10, 1:100 and 1:1000 dilutions) on M63 minimal medium plates. Washing in M9 slats is necessary to remove any rich media prior to selection on M63 minimal media plates and incubated at 32°C. Colonies appear after 5-6days on M63 media plates where galactose is the only carbon source. Individual colonies are picked and streaked onto MacConkey indicator plates. Gal- colonies will be white/colourless and the Gal+ bacteria will be bright red/pink due to a pH change resulting from fermented galactose after an overnight incubation at 32°C.

Round 2 (counter-selection); A red/pink colony was chosen from overnight incubation on MacConkey plates was inoculated in 5mL LB and choloramphenicol. Recombineering was then carried out as described in general recombineering protocol section 2.5.2, using an oligonucleotide to remove the desired part of the genome and the selection cassette. After

electroportation bacteria were recovered in 10mL LB for 4hrs at 32°C in a shaking incubator and washed in M9 salts as described in Round 1. Serial dilutions (100ul, 100ul each of 1:10, 1:100 and 1:1000 dilutions) were plated onto M63 DOG minimal medium plates and incubated for 3days at 32°. Correct colonies were picked and grown overnight at 32°C in chloramphenicol and DNA minipreped, digested and sequenced to confirm correct modification.

2.5.4.3. Recombineering with the *rpsL-Neo-LacZ* selection cassette

The *SacB* selection cassette is 4.5kB and its large size results in lower recombination efficiency. The *rpsL-Neo-LacZ* selection cassette is ~1.5kB and its smaller size allowed for higher recombination efficiency (www.genebridges.com) (Kasem et al., 2010).

The *rpsL-Neo* selection cassette (genbridges) was used to make deletions where the SacB and GalK selection cassettes had failed. The *rpsL-Neo* cassette was modified in house by Dr R Stanton to also contain LacZ. The *rpsL-Neo-LacZ* system takes advantage of the fact that the S12 ribosomal protein is the target of streptomycin. Most commonly used E.coli strains, including SW102, carry a mutation in the *rpsL* gene that confers streptomycin resistance. If the wild type *rpsL* gene is introduced then the strain will become streptomycin sensitive. The bacterial neomycin-kanamycin phosphotransferase, type II enzyme is encoded by the 'Neo' gene and confers resistance to aminoglycoside drugs such as neomycin and kanamycin. When the *rpsL-Neo-LacZ* cassette is inserted colonies will be sensitive to streptomycin and resistant to Kanamycin. When the *rpsL-Neo-LacZ* cassette is lost, colonies will be resistant to streptomycin but sensitive to kanamycin. The presence of *LacZ* in the selection cassette allowed for blue/white screening of colonies.

The *rpsL*-Neo selection system requires two rounds of recombination, Figure 2.2. In the first round the *rpsL-Neo-LacZ* cassette, containing homology to a specified position in the BAC is inserted by homologous recombination. The recombinant will now be able to grow on Kanamycin plates. In the second round the *rpsL-Neo-LacZ* is removed using an oligonucleotide. Correct colonies are achieved by plating onto Streptomycin selection plates.

Primers used to amplify *rpsL-Neo-LacZ* cassette are the same as primers used to amplify the *AmpR/SacB/LacZ* cassette, if amplified from pAL1622.

Round 1; is carried out as described in section 2.5.2. with the following modifications: During the first round of recombination a gel-purified PCR product encoding *rpsL*-Neo-LacZ is used. Following electroporation, bacteria are recovered for 1 ML LB for 1hr in a 32°C shaking incubator. After recovery, 100µL of bacteria were plated onto Kanamycin selection plates and incubated overnight in a 32°C incubator.

Round2: A blue colony is picked and inoculated in 5mL LB and grown overnight. Recombineering was carried out as described in general recombineering protocol section.... using an oligonucleotide to remove the desired part of the genome and the *rpsL-Neo-LacZ* selection cassette. Following electroporation bacteria were recovered in 1 mL LB for 1 hour at 32°C shaking incubator. After recovery, 100µL of bacteria were plated onto Streptomycin selection plates and incubated overnight in a 32°C incubator. Correct white colonies were picked and grown overnight in a 32°C incubator and DNA minipreped, digested and sequenced to confirm correct modification.

2.5.5. Generation of RAd

The AdZ5 genome does not need to be digested and can be transfected as a circular piece of maxiprepped DNA. The day before transfection 2 x 10^6 293-TRExTM cells were seeded in a 25cm^2 tissue culture flask. For transfection, 4 µg vector DNA was diluted in 100µL DMEM (no FCS or antibiotics). To the diluted DNA was added 40µL polyfect transfection reagent (Qiagen) and incubated at RT for 10minutes. During the incubation the media was changed on the cells to 3 mL fresh DMEM-10. After the 10 minute incubation 1 mL of DMEM-10 was added to the DNA complex and transferred to cells. After 24 hours the media was changed and then changed as and when required. Once sufficient cpe is observed, usually 7-10 days, cells and virus can be harvested and used to grow up further stocks.

2.5.6. Generation of HCMV

The HCMV genome is replicated using host cell machinery and virus progeny are secreted from the infected cell. For transfection 1x10^6 HFFF-hTert cells were centrifuged at 1,500rpm for 3minutes at RT and the supernatant discarded. Cells were washed in PBS and centrifuged at 1,500rpm for 3minutes at RT. Supernatant was discarded and the cell

pellet resuspended in 100 µL of nucleofector reagent (Lonza). To the suspension 2µg of BAC DNA was added and the suspension was transferred to supplied cuvettes with 2.5mm gap. Cells were electroporated using Amaxa (Amaza Biosystems) and pre-programmed setting T16. Following pulsing, cells were allowed to recover for 2 minutes before 1mL RPMI media was added to the cuvette and left for 2minutes. After recovery the contents of the cuvette were transferred to a 25cm2 T25 flask containing 5 mLs DMEM-10. Cells were incubated at 37°C/5%CO_2 and monitored for signs of cpe, with regular changes of media. Once cpe was observed throughout the monolayer cells were removed through trypsinisation and transferred to 5 x 175cm^2 tissue culture flasks containing confluent monolayer of HFFF-hTert and a stock was generated.

2.6. RT-QPCR

2.6.1. RNA extraction

At the required day post infection, media were removed from samples, cells washed in PBS and then cells lysed in 1 mL of Tri-reagent (Sigma) and stored at -80°C until required.

When required, samples were thawed at RT and 200 µL chloroform added to give a 5:1 tri:chloroform ratio. 1 mL of each sample was transferred to fresh 1mL RNase free tubes. Samples were vortexed for 10s and left at RT for 5 minutes before centrifugation at 12,500 rpm for 5 minutes at 4°C.

After centrifugation 500 µL of the aqueous phase was transferred to a fresh RNase free tube. Equal volume (500 µL) of isopropanol was added to precipitate the RNA and 1 µL (of a 5 mg/mL) glycogen was added to aid in the later visualisation of the RNA pellet. Once added the tube was inverted 10 times to ensure adequate mixing and left at -20°C for 1hour.

Post incubation samples were centrifuged at 14,000 rpm for 15 minutes at 4°C. Supernatant discarded and 500 µL ethanol added followed by centrifugation at 14,000rpm for 5minutes at 4°C. The supernatant was discarded and tubes blot dried.

RNA was obtained using the RNAeasy minelute (Qiagen) as described in the manufacturer's instructions. In brief:

The pellet was resuspended in 100µL of RNase free H_2O, followed by 350µL of RLT buffer and mixed thoroughly by inversion 10 times. This was followed by adding 250µL 100% Ethanol and mixed by pipetting up and down.

700µL of sample was passed through a minelute column into 2mL collection tubes by centrifugation at 10,000rpm for 30s. The flow through was discarded and the column transferred to a fresh collection tube.

500µL of buffer RPE was added to the column followed by centrifugation at 10,000rpm for 30s. The flow through was discarded

500µL of 80% Ethanol was added to the column. The flow through and collection tube was discarded.

The column was transferred to a new collection tube, the cap left open and centrifuged at 12,500rpm for 2minutes, any flow through was discarded along with the collection tube.

To elute the RNA, the column was transferred to a 1.5mL collection tube. 14µL of RNase free H_2O was added and centrifuged at 12,500rpm for 1minute.

Once eluted the samples were constantly kept on ice to prevent RNA degradation.

RNA concentration was assessed using a spectrophotometer (Nanodrop), and RNA integrity was assessed using an Agilent 2100 Bioanalyser (Agilent Biosystems), which was used to calculate the required amount of total RNA required for the RT reaction.

2.6.2. Reverse Transcription

A reverse transcription (RT) reaction was carried out using High cap cDNA Reverse transcriptase kit, (Applied Biosystmes), as described in the manufacturer's instructions.

RT was performed using the random hexamer method. 1 µg of RNA was added to 1µL of 100µM random hexamers (hexadeoxyribonucleotides, $pd[n]_6$, (Amersham Biosciences), 5µL of 10nM dNTPs (Amersham Biosciences), 2µL of 10x PCR buffer (Applied

Biosystems), 1 µL of reverse transcriptase, 1µl of (40 units/µL) RNase inhibitor and sterile H_2O to volume (20µL) were added to each sample and mixed.

The RT reaction was carried out using GeneAmp PCR system 9700, version 311, GeneAmp. The following reaction was used:

25°C for 10 minutes

37°C for 60minutes

37°C for 60 minutes

85°C for 5 seconds

4°C Hold

Once completed 80µL of H_2O was added and samples were stored at -20°C until required

2.6.3. QPCR

QPCR was carried out on cDNA samples obtained from the RT reaction. To each well in a 96 well plate 20µL of reaction mixture was added consisting of; 10µL syber green (applied Biosystems), 0.4µL of each primer, 1µL of diluted cDNA and to volume (20µL) DNase free water.

Samples were run on ABI 7900HT RT-PCR machine and cycling parameters were as follows

95°C for 10 minutes

95°C for 15 seconds
60°C for 1 minute
} X 40 cycles

The programme included a dissociation curve for RT-QPCR primers used, Figure 2.4.

Gene expression values were calculated based on the comparative threshold cycle (Ct) (Livak and Schmittgen, 2001), in which RNA samples for Actin and GAPDH were designated as calibrators to which other samples were compared.

The Ct data for CD112, CD155 and CD95 (Candidates) and Actin and GAPDH (Calibrators) were used to create ΔCt values ($Ct_{Candidate} - Ct_{Calibrator}$). The ΔΔCt values

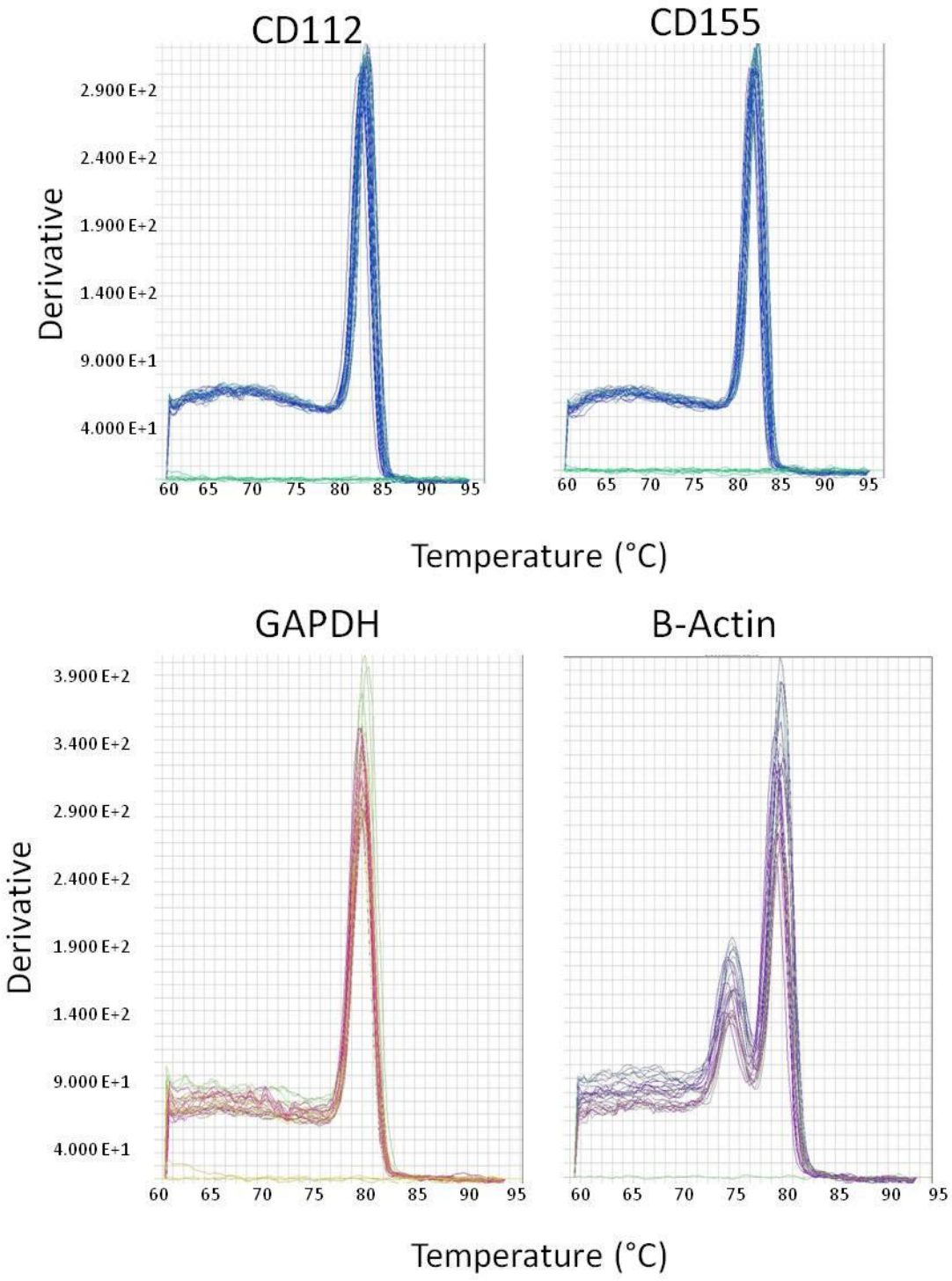


Figure 2.4. Dissociation curves for RT-QPCR primers.
The programme for QPCR included an additional reaction to determine the melting/dissociation curves for the primers to determine primer specificity.

were calculated by subtracting the ΔCt from the normaliser. In this case viral conditions were subtracted from the same day control condition for each day post infection. The Relative quantities (RQs) were calculated with the equation $RQ = 2^{-\Delta\Delta Ct}$, where RQ was normalised against the control condition for each day. The data were analysed for significance by a Turkey Post test.

2.7. WESTERN BLOTTING

2.7.1. Sample preparation of total cell lysate

Cell samples were harvested by removing the media, washing in PBS cold PBS and cells removed using a cell scraper. Detached cells were spun down at 1,500RPM for 3minutes at 4°C. The supernatant was discarded and cells washed in PBS and spun again at 1,500RPM for 3minutes at 4°C. Supernatant was discarded and the cell pellet frozen until required.

The cell pellet was resuspended in lysis buffer (25% 4X NuPAGE LDS sample buffer (Invitrogen), 1% protease inhibitor cocktail (Sigma) and 1% DTT (Sigma). Samples were sonicated (30 x 1sec pulse at 20%), vortexed for 10s and heated to 99 °C for 5minutes before being run.

2.7.2. TritonX114 enrichment of samples

Cell samples were harvested by removing the media, washing in PBS cold PBS and cells removed using a cell scraper. Detached cells were spun down at 1,500RPM for 3minutes at 4°C. The supernatant was discarded and cells washed in PBS and spun again at 1,500RPM for 3minutes at 4°C. Supernatant was discarded and the cell pellet frozen until required.

Samples were thawed and sonicated (30 x 1sec pulse at 20%) on ice in 300µL of cold lysis buffer (2% Triton X-114 in PBS, 1/100 protease inhibitors and 1/1000 1M DTT). Once sonicated the lysate was left in a clear non-stick tube on ice for 30minutes. This was followed by centrifugation at 13,000RPM at 4°C for 1 hour after which the supernatant was transferred to a fresh 1.5 mL non-stick tube. The supernatant was incubated in 37°C

waterbath for 5 minutes followed by centrifugation at 13,000rpm for 5 minutes at 37°C. The top fraction was removed and frozen for later assays, while the bottom fraction was diluted with 300µL of cold PBS and vortexed. Proteins were precipitated using the 2D MERCK clean-up kit (539180) as described in the manufacturer's instructions. 1.2mL of cold precipitation reagent was added to the 300 µL sample, which was briefly vortexed and incubated between 1hr-overnight. After precipitation samples were centrifuged at 13,000rpm for 10 minutes at 4°C. The supernatant was aspirated and discarded. The cell pellet was washed with 500µL cold wash solution, vortexed, centrifuged at 13,000rpm for 10minutes 4°C and the supernatant aspirated and discarded. The cell pellet was finally dissolved in 20-60µL of 1.5x SDS PAGE loading buffer and DTT (1in20). Samples were either frozen for later use or processed directly for western blotting by heating to 99°C for 5minutes to denature the proteins.

2.7.3. Deglycosidase treatment

To deglycosylate proteins, samples were digested with either endoglycosidase H (EndoH) (NEB) or peptide N-glycosidase F (PNGase F) (NEB). Samples were prepared using the Triton X114 method for the preparation of hydrophobic membrane proteins, as previously described, section 3.7.2. However, after precipitation, instead of re-suspending the final cell pellet in SDS loading buffer samples were instead re-suspended in denaturing buffer (NEB) to a total volume of 90µL and incubated at 99°C for 10 minutes. The sample was then split into three aliquots of 30µL for Endo H digestion, PNGase F digestion and a control undigested sample processed without any glycosidase enzyme. For Endo H digestion; 1µL (500units) enzyme, and 5µL buffer G5 (NEB) were added and made to a total of 50µL with PBS. For PNGase F digestion; 1µL (500Units) PNGase F enzyme, 5µL of supplied NP40 solution, 5µL of buffer G7 and PBS to a total volume (50µL). For the control; 5µL of buffer G5 was added and PBS to total volume, (50µL). All samples were then incubated overnight at 37°C. Post incubation samples were mixed with SDSPAGE loading buffer and run as described in sections 2.7.4.

2.7.4. Western blotting

Before use all samples were heated to 99°C for 5minutes in order to denature proteins. Samples were loaded onto a pre-set 10% Bis-Tris Midi gel Nupage Novex acrylamide gels (Invitrogen), 20µL per lane, protein standards (Invitrogen) were run in parallel. Gels were used in conjunction with Nupage electrophoresis equipment (Invitrogen) and run in MOPS buffer at 200V for 1Hour before being transferred to nitrocellulose membrane.

Following electrophoresis, proteins were transferred to nitrocellulose membrane (GE Biosciences) by semi dry transfer. Blotting paper and nitrocellulose membrane were pre-soaked in transfer buffer, then layered onto the transfer apparatus (TransferBlot SD, BioRad), with the sheet of blotting paper on the negative electrode, then the nitrocellulose on top. The polyacrylamide gel was gently removed from the electrophoresis apparatus and placed onto the nitrocellulose membrane. A final sheet of blotting paper was then layered on top of the gel. After ensuring that all air bubbles were removed, the transfer apparatus was assembled and the proteins were transferred by semi-dry transfer for 1 hour at 10V.

Following transfer, the nitrocellulose membrane was removed from the apparatus. The position of the pre-stained protein markers were marked on the membrane using an antigen-antibody (mouse) pen (Alpha Diagnostics) before the membrane was blocked with 5% milk powder in PBST for 1 hour are room temperature, or overnight at 4°C. After blocking, the blot was washed for 30minutes in PBST with three changes of buffer. An appropriate dilution of primary antibody in 5% milk powder in PBST was added to the membrane overnight at 4°C. All primary and secondary antibodies and their dilutions used can be found in table 2.5. The blot was then washed for 30minutes in PBST with three changes of PBST. Then an appropriate dilution of secondary antibody conjugated to horse-radish peroxidise (HRP), in 5% milk powder in PBST was added to the blot and incubated for 1 hour at room temperature. Blots were washed a final time in PBST before incubation with the HRP chemiluminescent substrate (Thermo Scientific) for 5 minutes. The blots were then analysed with Chemidoc chemiluminescence imaging system (UVP) or using photographic film (Kodak) and a developing machine.

To re-probe, blots were washed three times in PBST the incubated with ReStore (Pierce) for 1 hour before being washed a further three times in PBST. The stripped blots were re-

Table 2.5. Antibodies used in Immunoblot

Antibody	Dilution	Isotype	Company	Clone
Primary Antibodies				
α-V5	1:5,000	Mouse IgG2a	AbD Serotec	Sv5 Pk1
α-CD112	1:5,000	Mouse IgG	R & D	AF229
α-CD155	1:5,000	Mouse IgG	(Aoki et al., 1994)	5D1
α-Actin	1:20,000	Rabbit IgG	Sigma	A-2066
α-UL141	1:5,000	Mouse IgG1	Tomasec et al 2005	M550.2
Secondary Antibodies				
Goat α-rabbit IgG HRP	1:20,000	Goat IgG	BioRad	
Goat α-mouse IgG HRP	1:10,000	Goat IgG	BioRad	
Donkey α-goat IgG HRP	1:20,000	Donkey IgG	SantaCruz	

blocked in 5% milk powder in PBST for 1 hour at RT or overnight at 4°C then staining was carried out as described above.

2.8. MICROSCOPY

2.8.1. Phase contrast microscopy

Phase contrast images of cells were detected with a Lecia DMIBRE microscope (Lecia) and a Hamamatsu ORCA-ER camera (Hamamatsu) and analysed with Improvision Openlab 3 software (Improvision).

2.8.2. Preparation of cells for Immunofluorescence

HFFF or HFFF-hCAR cells were seeded onto a glass bottomed 96 well plate at a concentration of 5 x 105 cells/plate, and infected the following day at a MOI 10. At 72hpi, cells were washed in PBS and fixed in 2% Paraformaldehyde for 20 minutes at RT. To permeabilise cells, wells were washed twice in PBS then incubated with the relevant IF buffer for 1 hour at RT, with 2 changes of buffer.

Cells were incubated with primary antibody diluted in the relevant IF buffer for 1hour at RT. Table 2.6. lists the antibodies used in Immunofluorescence during this study. Wells were washed 5x in relevant IF buffer and incubated with secondary antibodies and/or relevant counterstains (Table 2.6.) for 1 hour at RT. After incubation wells were washed a further 5x in relevant IF to remove excess antibody before being fixed in 2% Paraformaldehyde. Fluorescence was detected with a Lecia DMIBRE microscope (Lecia) and a Hamamatsu ORCA-ER camera (Hamamatsu) and analysed with Improvision Openlab 3 software (Improvision).

2.8.3. Counterstains

4',6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds strongly to A-T rich regions in DNA and was used as a nuclear counterstain. DAPI has an absorption wavelength of 358 nm (ultraviolet) and emission at 461 nm (blue). Phalloidin-AlexaFluor-488 was used to stain actin filament and was visible on the Green channel (absorption = 495nm, emission 519nm). Both DPAI and Phalloidin were co-incubated with the relevant secondary antibody.

Table 2.6. Antibodies used in Immunofluorescence microscopy

Antibody	Dilution	Isotype	Company	Clone
Primary Antibodies				
α-CD112	1:10	Mouse IgG	Santacruz	BC12
α-CD112	Failed	Goat poly IgG	Santacruz	N20
α-CD112	Failed	Mouse IgG1	Santacruz	3H1817
α-CD112	Failed	Mouse IgG	R & D	AF229
α-CD112	Failed	Mouse IgG1	Santacruz	R2.525
α-V5	1:1000	Mouse IgG2a	AbD Serotec	Sv5 Pk1
Secondary Antibodies				
Goat α-mouse ALexaFluor 594	1:1000	F(ab')2 fragment	Molecular Probes	
Goat α-mouse ALexaFluor 488	1:1000	F(ab')2 fragment	Molecular Probes	
Other				
DAPI	1:10,000	n/a	Invitrogen	
Phalloidin-AlexaFluor 488	1:50	n/a	Molecular Probes	

2.9. FLOW CYTOMETRY

2.9.1. Cell surface staining protocol

At the required timepoint post-infection media was removed, cells were washed in PBS and incubated in Trypsin/EDTA until cells detached. Once detached, cells were resuspended in 10 mL DMEM-10 to neutralise the Trypsin and transferred to 15 mL tubes and centrifuged at 1,500rpm for 3minutes at 4°C. The supernatant was discarded and cells resupsended in 5 mL ice cold FACS buffer. Cells were then pelleted by centrifugation at 1,500rpm for 3minutes at 4°C. This washing step was repeated once more. The supernatant was again discarded and the cell pellet was resuspended in the appropriate volume of ice cold FACS buffer, 200µL for each stain to be undertaken, and 200 µL of cell suspension was added to a well of a V-bottom 96well plate.

The cell samples were incubated with the appropriate concentration of unconjugated antibodies, a list of antibodies and concentrations used can be found in table 2.7. Incubation with 1°Ab lasted for 30 minutes in a 4°C fridge, with cells being resuspended using a multi-channel pipette after 15minutes. After incubation cells were pelleted by centrifugation at 1,500rpm for 1 minute at 4°C. The supernatant was removed and cells were washed 5 times in ice cold FACS buffer. One wash step consisted of re-suspending cells in ice cold washing buffer followed by centrifugation at 1,500rpm for 1 minute at 4°C and discarding the supernatant. Cells were finally re-suspended in secondary antibody (2°Ab), diluted in ice cold FACS buffer. Incubation with the 2°Ab lasted 30 minutes, in a 4°C fridge, with re-suspension of cells after 15minutes. After incubation cells were pelleted by centrifugation at 1,500rpm for 1 minute at 4°C as above and washed 3x in ice cold FACS buffer as described above. After washing cells were fixed in 2% Paraformaldehyde and analysed by flow cytometry using a FACSCalibur (Dakocytomation, Cambridge) with CELLQuest PRO software (Becton Dickinson). FLOW JO software was also used for data analysis. Cells were selected by standard forward scatter and side scatter criteria before analysis using the appropriate laser excitation.

Table 2.7. Antibodies used in Flow cytometry

Antibody	Dilution	Isotype	Company	Clone
Primary Antibodies				
α-CD111	1:100	Mouse IgG1	SantaCruz	CK6
α-CD112	1:100	Mouse IgG2b	SantaCruz	BC12
α-CD113	1:100	Mouse	SantaCruz	N.3.82.5
α-CD155	1:100	Mouse IgG	Abcam	D171
α-MHC-1	1:100	Mouse	Serotek	W632
α-MicA/B	1:100	Mouse IgG2a	Bamomab	BAM01
α-MicB	1:100	Mouse IgG2a	Bamomab	BAM02
α-nectin4	1:100	Goat	SantaCruz	I15
α-V5	1:1000	Mouse IgG2a	AbD Serotec	Sv5 Pk1
Mouse IgG	1:1000	Mouse	Sigma	
Conjugated secondary antibodies				
Goat α-mouse AlexaFluor 647	1:1000	F(ab')2 fragment	Molecualr Probes	
Goat α-mouse AlexaFluor 488	1:1000	F(ab')2 fragment	Molecular Probes	
Donkey α-goat AlexaFluor 488	1:1000	IgG	Molecular Probes	

3.0. ANALYSIS OF CD112 EXPRESSION DURING HCMV INFECTION.

3.1. Developing assays for the detection of CD112.

Before the commencement of investigations into the expression of CD112 during HCMV infection; it was first necessary to set up appropriate assays to produce the robust, reliable and reproducible detection of CD112. Three assays were initially identified: Flow cytometry detection of cell surface CD112, detection of CD112 by immunofluorescence and detection of CD112 by immunoblot.

3.1.1. Flow cytometry detected the cell surface expression of CD112 on a range of cell lines.

Endogenous CD112 is expressed at the targets cell surface where it can engage with its activating receptor DNAM-1 on effector cells. Endogenous cell surface CD112 can be detected by two step flow cytometry, using a primary antibody against CD112 and a fluorescent conjugated secondary antibody. Of the antibodies that were commercially available upon commencement of the project, only two antibodies had validated and recommended uses for flow cytometry, sc-65333 (Santa Cruz) and MCA1937 (Serotek). Of the two antibodies tested only sc-65333 (Santa Cruz) was capable of detecting cell surface CD112 expression to a satisfactory degree, Figure 3.1. An antibody titration of sc-65333 indentified a concentration of 1 in 50 gave optimal staining (data not shown).

HCMV is capable of infecting a range of cell lines including fibroblasts, epithelial and endothelial cells (Sinzger et al., 1995). A range of cell lines were tested in order to determine base line cell surface expression of CD112 and the other nectins. These cell lines included three fibroblast cell lines, primary HFFF's, HFFF-hTert and HFFF-hCAR and two epithelial adenocarcinoma cell lines Hela and Caco-2. Fibroblast and epithelial cell lines were seeded at a concentration of $1x10^6$ and $2x10^6$ respectively in a $25cm^2$ tissue culture flask. Three days later cells were trypsinised and stained for the cell surface expression of mIgG, CD111, CD112, CD113, nectin4, CD155 and MHC-1, Figure 3.2.

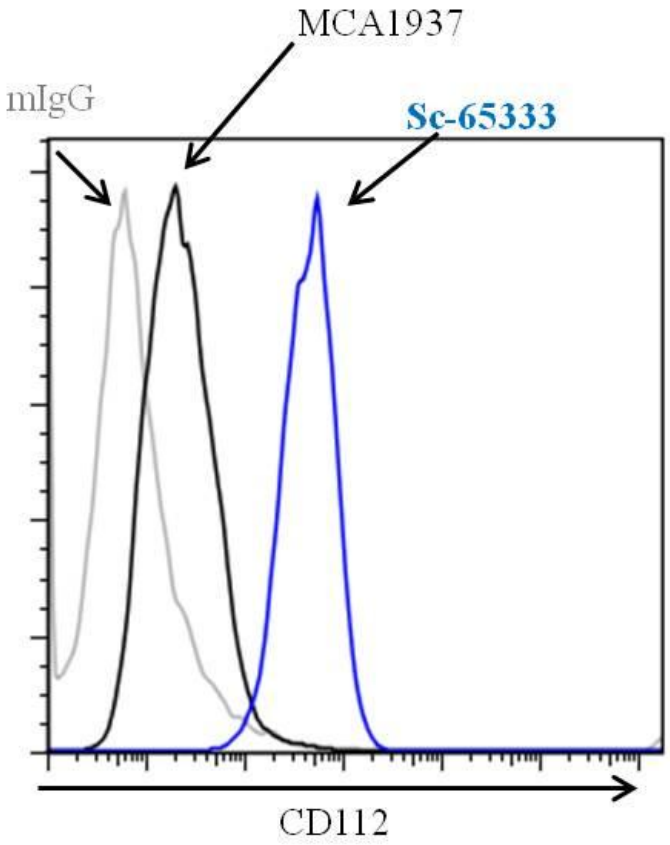


Figure 3.1. Testing antibodies for the cell surface detection of CD112 by flow cytometry.
Uninfected HFFFs were stained with two antibodies against CD112, MCA1937, Serotec **(black)** and sc-65333, Santa Cruz (**Blue**). mIgG (Grey) was used as a negative staining control.

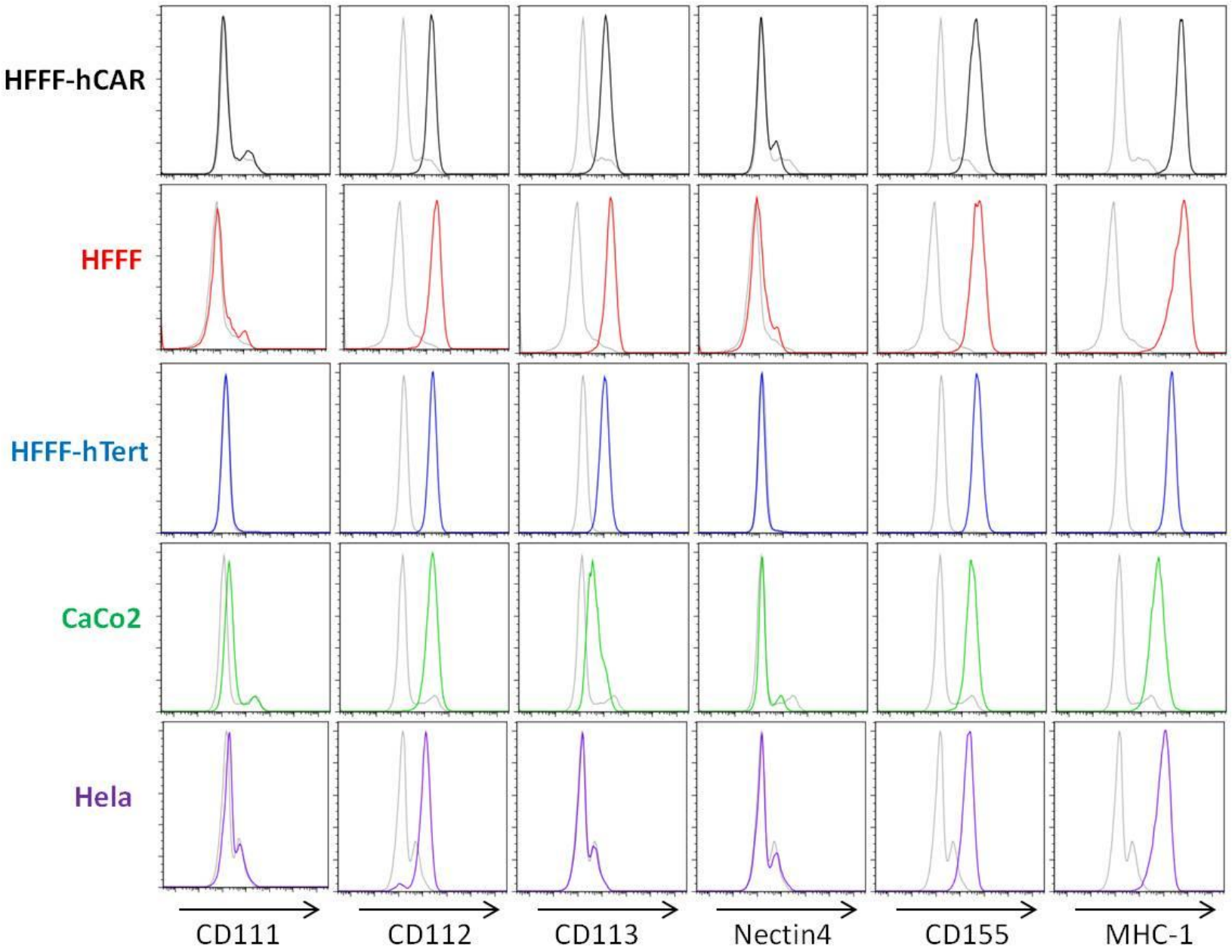


Figure 3.2. Cell surface expression of Nectin family members on several fibroblast and epithelial cell lines.
HFFF-hCAR (black), HFFF (red), HFFF-hTert (blue), CaCo2 (green) and **Hela (purple**) cells were seeded into a 25cm^2 tissue culture flask and three days later harvested and processed for flow cytometry to determine cell surface expression of the nectin family. This included CD111, CD112, CD113 and nectin4 as well as the nectin like molecule CD155 and also MHC class 1.

Mouse-IgG is not expressed on human cell lines and is therefore commonly used as a negative staining control for non specific binding of antibody. The peak achieved for mIgG was therefore used as a baseline for negative staining. In contrast, MHC-1 is commonly used as a positive staining control and was expressed on all cell lines tested. The three fibroblast cell lines are commonly used within the laboratory and cell surface expression levels of MHC-1 were as expected. Both Hela and Caco-2 cells also express MHC-1, but have lower expression levels compared to the expression level on fibroblast cell lines.

The cell surface expression of CD112 was detected on all cell lines and expression levels were similar on all cell lines irrespective of whether the cell line was epithelial or fibroblast in origin. The expression of CD155 was also detected on all cell lines, with higher cell surface expression levels on fibroblast cell lines compared with epithelial cells lines.

Nectin 4 could not be detected on any of the cell lines tested. This was as expected as nectin 4 is described in the literature as being predominantly detected in the placenta (Reymond et al., 2001). Placental tissue was not used as a positive staining control for nectin 4, so it was possible that the anti-nectin 4 antibody did not detect nectin 4.

CD111 could not be detected in any of the fibroblast cell lines tested but could be marginally detected in epithelial cell lines. It is possible that the anti-CD111 antibody was not detecting CD111 as there are reports of CD111 being detected on both fibroblasts and epithelial cell lines (Cocchi et al., 1998). Both CD111 and nectin 4 were not expected to be used further in this study so investigations into further reagents were not carried out.

CD113 was readily detected on all three fibroblast cell lines with the highest detected on HFFF's. CD113 was not detected on Hela cells and marginally detected on the Caco2 cell line. CD113 has been described as being ubiquitously expressed in the literature (Satoh-Horikawa et al., 2000).

Flow cytometry detection protocols for the cell surface expression of both DNAM-1 ligands, CD155 and CD112, were successfully set up for the detection of both proteins in fibroblast and epithelial cell lines using antibodies sc-65333 (CD112) and D171 (CD155).

3.1.2. Immunofluorescence detected CD112 at inter-cellular junctions.

Tomasec et al (2005) identified CD155 to be sequestered in the ER during HCMV infection, thus preventing its cell surface expression. Therefore, a similar effect on CD112 would not have been surprising. In order to try and visualise CD112, a panel of five monoclonal antibodies that had recommended uses for the detection of CD112 in immunofluorescence were tested to try and detect CD112 on the surface of uninfected cells. The panel of antibodies were tested in a dilution series against uninfected HFFFs in order to try and detect CD112. Of the five antibodies tested only one showed staining above background levels, Sc-6533 (Santa cruz), at a concentration of 1 in 10 (data not shown). However, HFFF's appear to have extremely low levels of CD112 expression and detection was difficult to visualise with expression as small punctate dots throughout the cell (Data not shown). Flow cytometry had identified CD112 to be expressed on epithelial cell lines and therefore CD112 expression was analysed on human breast adenocarcinoma cell line MCF7 and the human colorectal adenocarcinoma Caco2.

IF detection clearly showed CD112 at cell-to-cell contact sites, being expressed at the inter-cellular junctions of adjacent cells but not at the free cellular edges, Figure 3.3. This was true for both MCF-7 and Caco-2 cell lines and is in agreement with previous descriptions of CD112 expression in the literature (Lopez et al 1998).

3.1.3. Enrichment of CD112 for immunoblotting.

Endogenous CD112 proved difficult to detect by immunoblotting when using total cell lysate of uninfected HFFF's, Figure 3.4a. Thus a new method was investigated. When cell lysates are treated with the non-ionic detergent Triton X-114, integral membrane proteins that are amphiphilic in nature, are recovered in the detergent phase, thus allowing their concentration and partial purification (Bordier, 1981). As CD112 is a transmembrane, hydrophobic protein when fractionated with Triton X-114 CD112 was found to stably associate with the detergent fraction, Figure 3.4a. Using this method, CD112 was found almost exclusively in the detergent fraction where two distinct bands could be identified, Figure 3.4a. These two bands were of approximately 60 and 55kDA and correlated to the predicted protein sizes of 57.5 kDa (CD112-δ) and 51.4 kDa (CD112-α) respectively, which were predicted using the 'predict pI/Mw' located on the Swiss Institute of bioinformatics Expasy website (http://web.expasy.org)(Gasteiger et al., 2003), which

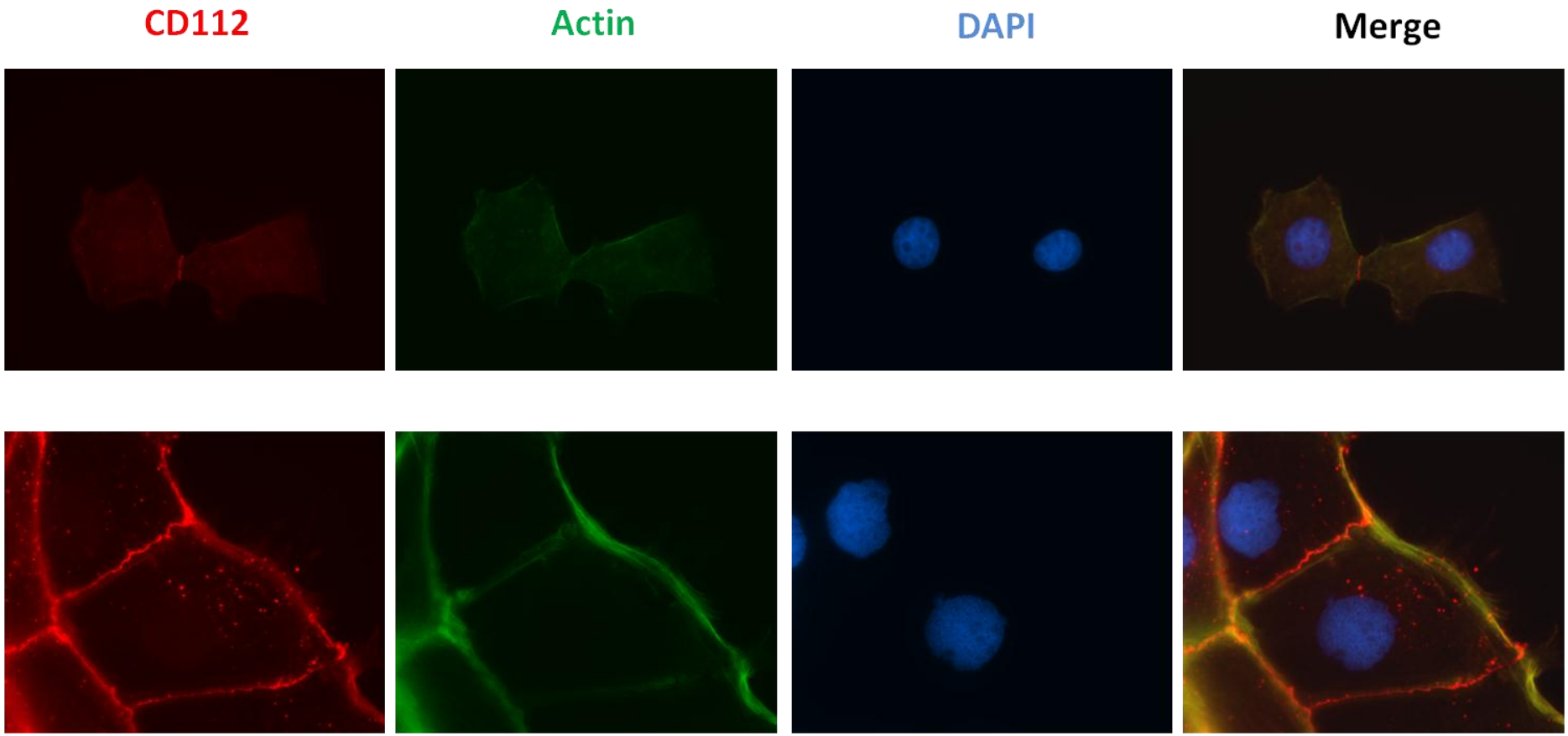
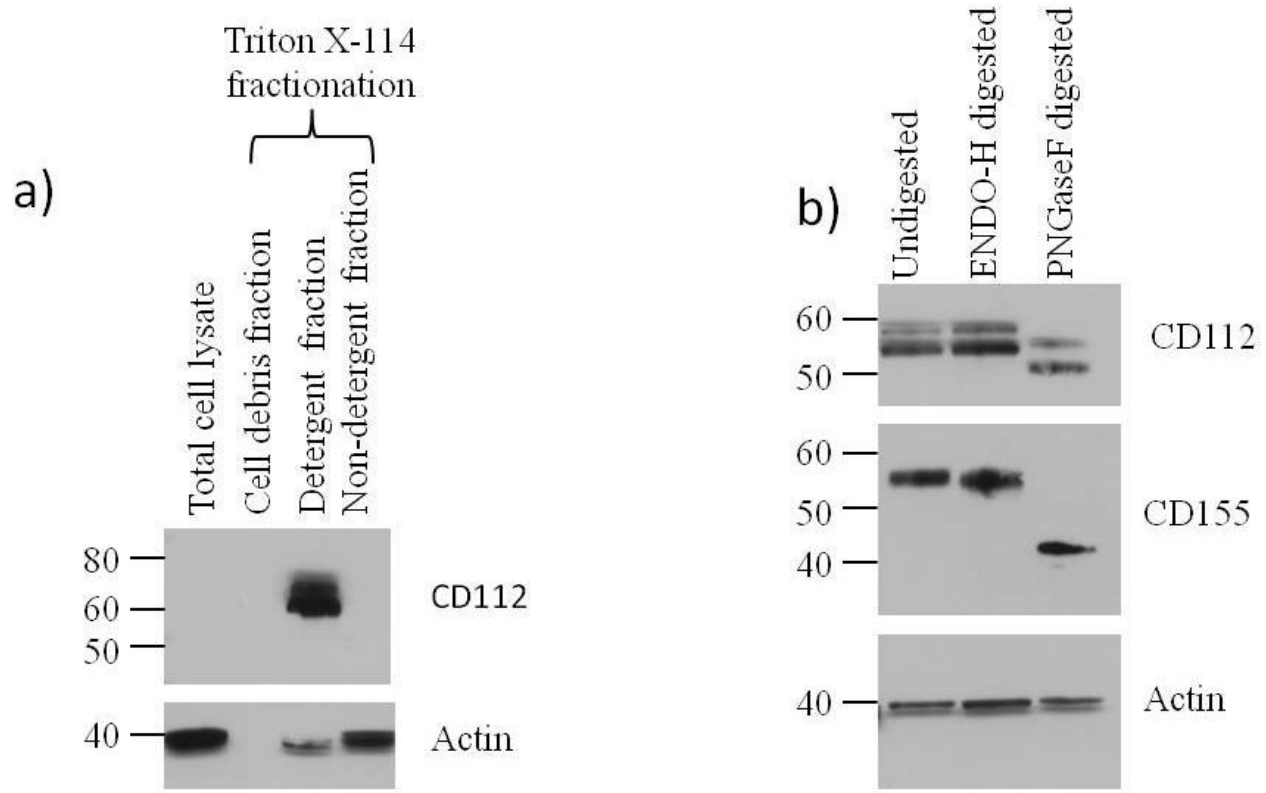


Figure 3.3. Detection of CD112 by Immunofluorescence in human epithelial cell lines MCF-7 and CaCo2.
MCF-7 or CaCo2 cells were seeded into a glass bottom 96-well plate. 2 days post seeding cells were fixed and stained for CD112 (red). Actin filaments were stained green using Phalloidin-AlexaFluor-488 and nuclei were stained blue using DAPI. Images were merged to create an overlaid image.



c)

ClustalW results

```
Q92692      MARAAALLPSRSPPTPLLWPLLLLLLLETGAQDVRVQVLPEVRGQLGGTVELPCHLLPPV  60   PVRL2_HUMAN
Q92692-2    MARAAALLPSRSPPTPLLWPLLLLLLLETGAQDVRVQVLPEVRGQLGGTVELPCHLLPPV  60   PVRL2_HUMAN
            ************************************************************

Q92692      PGLYISLVTWQRPDAPANHQNVAAFHPKMGPSFPSPKPGSERLSFVSAKQSTGQDTEAEL  120  PVRL2_HUMAN
Q92692-2    PGLYISLVTWQRPDAPANHQNVAAFHPKMGPSFPSPKPGSERLSFVSAKQSTGQDTEAEL  120  PVRL2_HUMAN
            ************************************************************

Q92692      QDATLALHGLTVEDEGNYTCEFATFPKGSVRGMTWLRVIAKPKNQAEAQKVTFSQDPTTV  180  PVRL2_HUMAN
Q92692-2    QDATLALHGLTVEDEGNYTCEFATFPKGSVRGMTWLRVIAKPKNQAEAQKVTFSQDPTTV  180  PVRL2_HUMAN
            ************************************************************

Q92692      ALCISKEGRPPARISWLSSLDWEAKETQVSGTLAGTVTVTSRFTLVPSGRADGVTVTCKV  240  PVRL2_HUMAN
Q92692-2    ALCISKEGRPPARISWLSSLDWEAKETQVSGTLAGTVTVTSRFTLVPSGRADGVTVTCKV  240  PVRL2_HUMAN
            ************************************************************

Q92692      EHESFEEPALIPVTLSVRYPPEVSISGYDDNWYLGRTDATLSCDVRSNPEPTGYDWSTTS  300  PVRL2_HUMAN
Q92692-2    EHESFEEPALIPVTLSVRYPPEVSISGYDDNWYLGRTDATLSCDVRSNPEPTGYDWSTTS  300  PVRL2_HUMAN
            ************************************************************

Q92692      GTFPTSAVAQGSQLVIHAVDSLFNTTFVCTVTNAVGMGRAEQVIFVRETPNTAG---AGA  357  PVRL2_HUMAN
Q92692-2    GTFPTSAVAQGSQLVIHAVDSLFNTTFVCTVTNAVGMGRAEQVIFVRETPRASPRDVGPL  360  PVRL2_HUMAN
            ***************************************************.::    .

Q92692      TGGIIGGIIAAIIATAVAATGILICRQQRKEQTLQGAEEDEDLEGPPSYKPPTPKAKLEA  417  PVRL2_HUMAN
Q92692-2    VWGAVGGTLLVLLLLAGGSLAFILLRVRRRRKSPGGAGG--------------------  399  PVRL2_HUMAN
            . * :** : .::  * .: .::: * :*:.::  **

Q92692      QEMPSQLFTLGASEHSPLKTPYFDAGASCTEQEMPRYHELPTLEERSGPLHPGATSLGSP  477  PVRL2_HUMAN
Q92692-2    ----------GASGDG----GFYDP-------------------KAQVLGNGDPVFWT-  424  PVRL2_HUMAN
                     *** ..     ::*.                   ::  *  * . : :

Q92692      IPVPPGPPAVEDVSLDLEDEEGEEEEEYLDKINPIYDALSYSSPSDSYQGKGFVMSRAMY  537  PVRL2_HUMAN
Q92692-2    -PVVPGP-----MEPDGKDEEEEEEEEKAEKGLMLPPPPALEDDMESQLDGSLISRRAVY  478  PVRL2_HUMAN
             ** ***     :. * :*** *****  :*   :  . : ..  :*  . .::  **:*

Q92692      V  538   PVRL2_HUMAN
Q92692-2    V  479   PVRL2_HUMAN
            *
```

Figure 3.4 Characterisation of CD112 by immunoblot.

A) Uninfected HFFF's were harvested for western blot and were prepared either using total cell lysate or enrichment using Triton-X114. Three fractions, cell debris after lysis, hydrophobic fraction that associates with TritonX114 and hydrophilic fraction which does not associate with triton X114 were compared to samples prepared by total cell lysis. B) The hydrophobic faction of uninfected HFFF's prepared by TritonX114 fractionation were treated with glycosidases, lane 1, undigested, lane 2, ENDO-H digested and lane 3, PNGaseF digested. C) a ClustalW alignment of the two CD112 isoforms performed using European Bioinformatics Institutes online ClustalW programme (www.ebi.ac.uk).

predicts Isoeletric point and molecular weight of proteins before post translational modification from an inputted AA sequence. There were no bands seen in the non-detergent fraction or in the fraction that consisted of cell debris spun down after cell lysis. The bands produced in the detergent fraction provided a cleaner band when compared to total cell lysate, where CD112 was difficult to detect and only a faint band could be seen at high exposure (high exposure data not shown).

Enrichment of CD112 in the detergent fraction of Triton X-114 provided a strong signal for CD112 and this method was used for the future detection of CD112 by immunoblot.

3.2. CD112 is a glycoprotein with two isoforms.

Immunoblot of CD112 revealed that CD112 had two distinct bands in uninfected HFFF's. This could have been due to the different glycosylation status of one protein, or due to two different isoforms. CD112 is known to have two isoforms that share approximately 70% indentify. The CD112-δ and CD112-α isoforms are identical over their extracellular and transmembrane domains but differ in their cytoplasmic domains where there is a 58aa deletion in the C-terminus spanning aa480-538, Figure 3.4c.

Endoglycosidase H (EndoH) is a recombinant glycosidase that cleaves the chitobase core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins (Maley et al., 1989). Peptide N-Glycosidase F (PNGase F) is an amidase which cleaves between the innermost GlcNAc and asparagines residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins (Maley et al., 1989). In essence, EndoH digests simple oligosacharrides whereas PNGaseF will strip all oligosaccharides leaving the naked protein.

Following digestion with EndoH the two bands of CD112 appear to be of the same molecular weight as those seen in the undigested sample at approximately 60kDa and 55kDa respectively, Figure 3.4b. Following digestion with PNGaseF, two bands are again indentified. The two bands in PNGaseF digestion are of approximately 55kDa and 50kDa respectively. As there is one band of approximately 55kDa in both EndoH and PNGaseF digested samples it is unclear whether this band is due to the same protein in both samples. It was possible the 60kDa and 55kDA proteins were both deglycosylated to 55kDa and 50kDa by PNGaseF meaning both proteins were glycosylated. A second possibility was that the 55kDa band was the same in both samples meaning that the upper 60kDA band

seen in the EndoH digested sample was deglycosylated to the 50kDa form seen in PNGaseF digested samples. As the two isoforms only differ in their intracellular domains and all glycosylation sites are predicted to be in the extracellular domain. It is assumed that the bands are due to the two CD112 isoforms that have both been deglycosylated. CD155 also appears to be glycosylated with a single band of 60kDA in undigested and EndoH digested samples and a single band of approximately 40kDa in PNGaseF digested samples. This is as expected for proteins that are expressed at the cell surface as they have been processed through the ER/Golgi so are resistant to EndoH digestion due to the complex N-glycosylation, but are sensitive to PNGaseF digestion.

3.3. The product of UL141 is essential but not sufficient for the down-regulation of CD112 during HCMV infection.

Once appropriate assays had been established, investigations began into the fate of CD112 during HCMV infection. HFFF's were seeded at $1x10^6$ cells in a $25cm^2$ tissue culture flask and infected with HCMV strain Merlin or MerlinΔUL141 and cell surface CD112 analysed 3 days p.i. UL141 had previously been shown to effect the cell surface expression of the DNAM-1 ligand CD155 and so investigations began with the MerlinΔUL141 virus to determine whether UL141 may also effect the other DNAM-1 ligand CD112.

HFFF cells infected with HCMV strain Merlin down-regulated cell surface expression of CD112, but when the UL141 ORF was deleted (Merlin ΔUL141) this effect was lost, Figure 3.5a. This demonstrated that UL141 was essential for HCMV mediated down-regulation of cell surface CD112. When UL141 was expressed from a recombinant adenoviral vector (RAd-UL141), there was no effect on cell surface CD112 expression levels, Figure 3.5c. Co-infection with both RAd-UL141 and Merlin ΔUL141 rescued the Merlin wild type phenotype of CD112 down-regulation, confirming the requirement of UL141 in this process, Figure 3.5c, d, e.

Immunoblot showed that CD112 could not be detected in HCMV strain Merlin infected cells but could be detected in MerlinΔUL141 infected cells 3 days p.i., Figure 3.6. While in HFFF-hCAR cells infected with RAd there was no decrease in CD112 seen in RAd-UL141 infected cells compared to uninfected of RAd-Ctrl (Rad-1253), Figure 3.6b. In

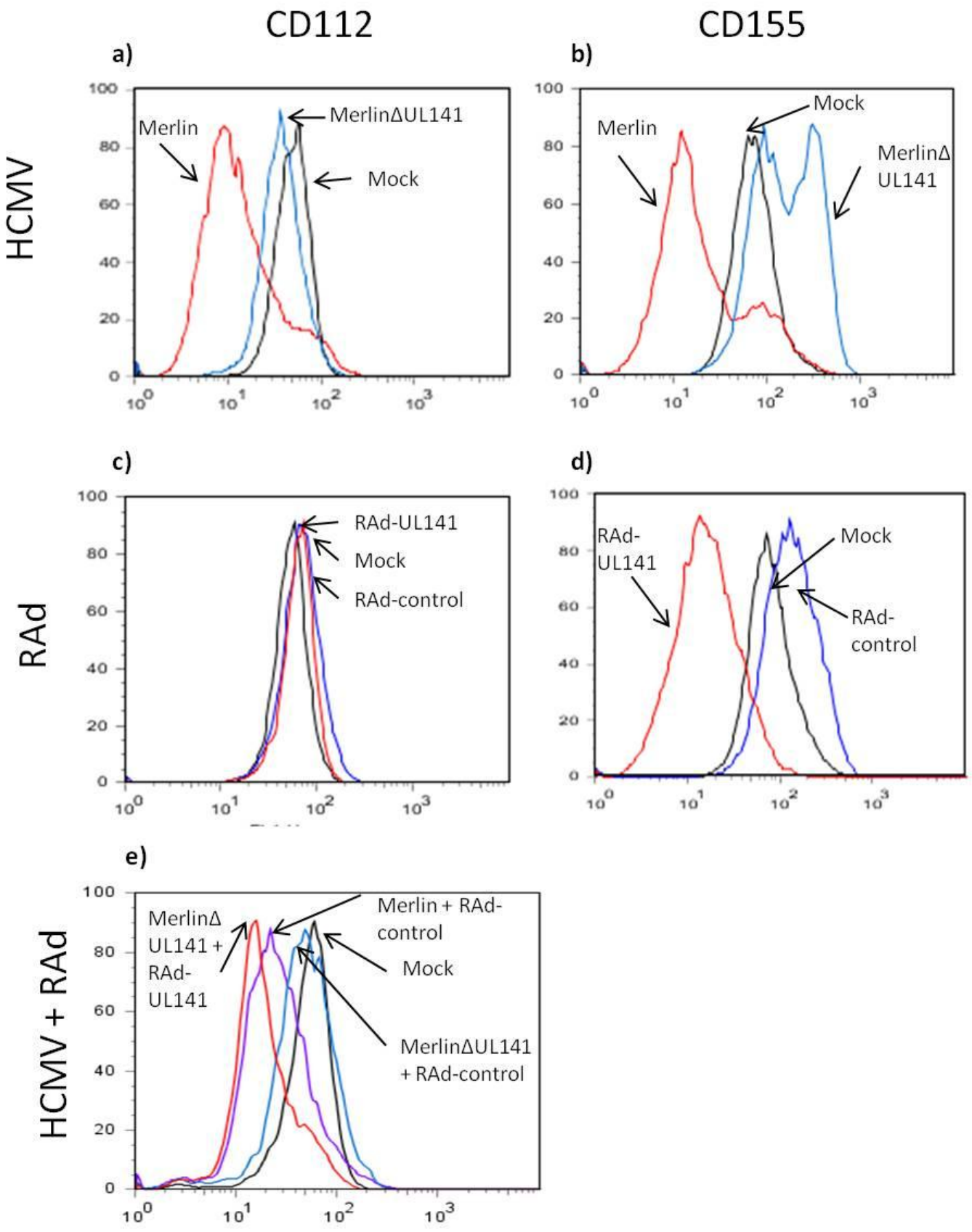


Figure 3.5. UL141- mediated downregulation of cell surface CD155 and CD112 .
HFFF cells were seeded at $1x10^6$/T25cm^2. The following day cells were infected with either HCMV, RAd or both for 2 hours. Cells were analysed by flow cytometry with mAb BC-12 (Santa Cruz) or D171 (Abcam) to detect cell surface expression of CD112 or CD155 respectively. (a) Cell surface CD112 on cells infected with strain Merlin (red), MerlinΔUL141(blue) or mock infection (black) (b) CD155 cell surface expression after infection with Merlin (red), Merlin ΔUL141 (blue) or mock infection (black) (c), CD112 surface expression after infection with RAd-UL141 (red), RAd control (blue) or mock infection (black) (d), CD155 cell surface expression after infection with RAd-UL141 (red), RAd control(blue) or mock infection (black) (e) CD112 cell surface expression after co-infection with RAd-UL141 + Merlin ΔUL141(red), RAd-Control + Merlin (Purple), RAd-Control + Merlin ΔUL141 (blue) or mock infection (Black).

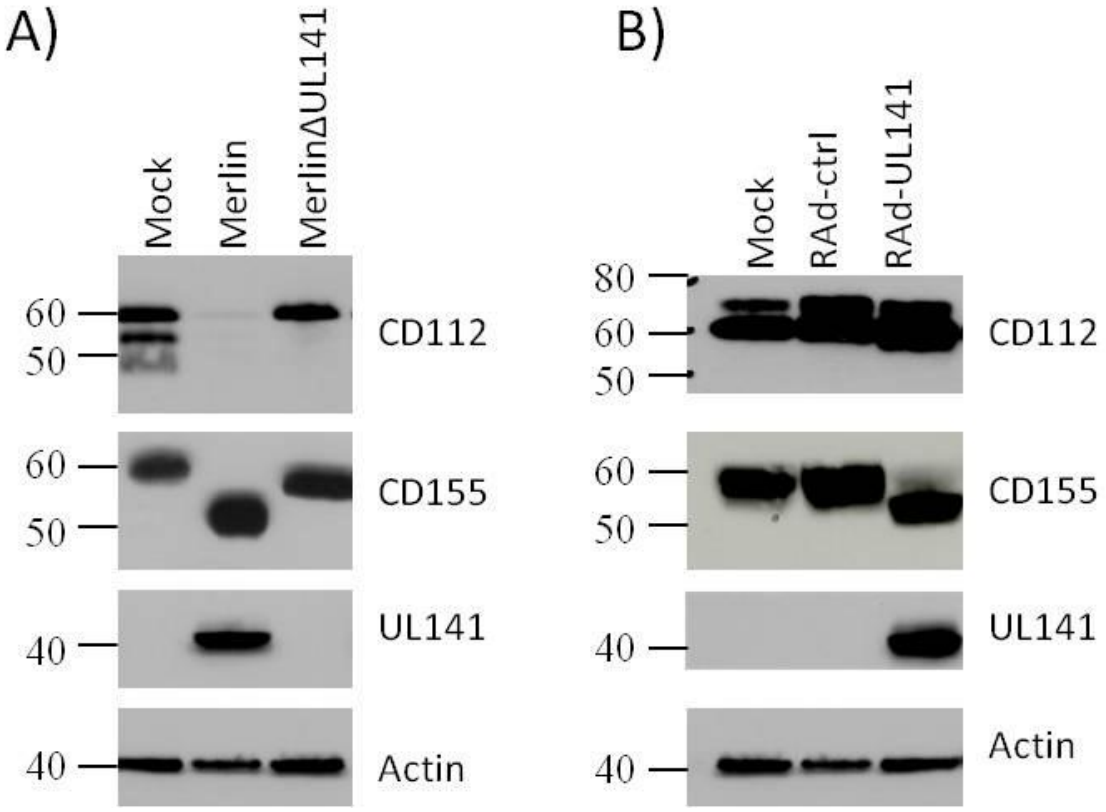


Figure 3.6: UL141- mediated downregulation of CD155 and CD112 in HCMV but not RAd infection.
HFFF cells were seeded at $1x10^6$/T25cm^2. The following day cells were infected with either HCMV or RAd for 2 hours. Cells were analysed by western blot to detect CD112. (A) detection of CD112, CD155, UL141 and Actin in cells infected with strain Merlin, MerlinΔUL141 or mock infection .(B) CD112, UL141 and actin detected in cells infected with RAd-Ctrl vector, Rad-UL141 or mock infected cells

conclusion, UL141 appeared to be required for the down-regulation of CD112 from the cell surface during Merlin infection and CD112 could no longer be detected by immunoblot indicating either that CD112 was degraded, or there was an affect before translation.

3.4. UL141-GFP cell line does not down-regulate CD112.

The cell surface expression of CD155 was down regulated in RAd-UL141 infected HFFF-hCARs, while the cell surface expression of CD112 remained unaffected. To confirm this observation the cell surface expression of the DNAM-1 ligands was analysed on a stable cell line expressing either UL141-GFP or GFP. Results mirrored those seen in RAd-UL141 infection, Figure 3.7. The UL141-GFP cell line had almost no cell surface CD155 expression compared to a GFP expressing cell line. The cell surface expression of CD112 remained unaffected and was equivalent on the two cell lines. These results confirmed that UL141 expressed in isolation had no effect on cell surface expression levels of CD112.

3.5. UL141 was not essential for viral growth/replication.

The Merlin strain provides one of the most genetically intact and stable HCMV genomes for study. However, the tendency of HCMV to accumulate genomic alterations during in vitro passage meant that wild type Merlin (wtMerlin) was unsuitable. Instead a BAC derived Merlin strain (RCMV1111) was used for this study. A BAC derived virus provided a clonal population for study, which is ideally suited for screening as a clinical sample could contain a genetically heterogeneous population. The RCMV1111 contained a premature stop codon in UL128 and a mutation in RL13 that prevents infection of endothelial cells but is otherwise genetically identical to wtMerlin.

For this thesis the recombinant virus RCMV1149 (MerlinΔUL141) was regularly utilised. It was therefore important to establish whether deletion of the UL141 ORF had any effect on viral growth/replication and plaque formation. A single step growth curve for RCMV1149 (MerlinΔUL141BAC derived virus) and RCMV1111 (MerlinBAC derived virus) were carried out and plaque size was analysed. HFFF's were infected with either

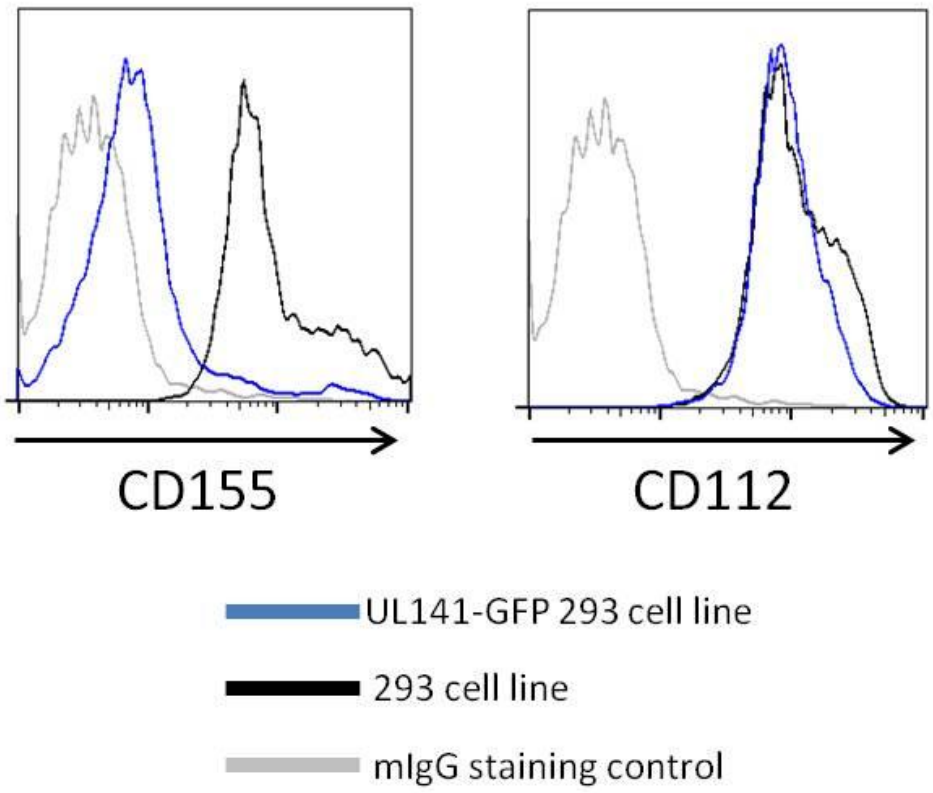


Figure 3.7. UL141-GFP 293 cells do not downregulate CD112.
2 x 10^6 293-GFP cells (**black**) and UL141-GFP stable transfected 293 cells (**Blue**) seeded into a 25cm^2 flasks and 3daye p.i. were harvested and analysed for the or cell surface expression of CD112 and CD155 by flow cytometry

virus at MOI1, supernatant was sampled at regular intervals post infection and titres for each sample were calculated. Results showed that there was no significant difference in viral growth between Merlin and MerlinΔUL141, Figure 3.8.

3.6. CD112 is notably down-regulated at 2 days p.i. during HCMV infection.

HCMV expresses different genes at different timepoints during the infection cycle defined by immediate early (IE), early (E) and Late (L) gene expression. The HCMV virion also contains a variety of proteins that act on various cellular mechanisms immediately post infection before viral transcription occurs. Identifying the timepoint for when CD112 is first down-regulated from the cell surface would narrow down gene candidates to be screened.

Infections were set up and harvested at three different days post infection with RCMV1111 (Merlin), RCMV 1149 (MerlinΔUL141) or uninfected HFFF's and cell surface mIgG, CD112, CD155 and MHC-1 was analysed by flow cytometry, Figure 3.9. Concurrent infections were also harvested and analysed by western blot, Figure 3.10. Flow cytometry allowed the detection of cell surface CD112, while western blot provided information on total CD112 and gave indications into the fate of CD112 during HCMV infection.

In RCMV1111 infected HFFF's, CD112 cell surface levels appeared to show signs of down regulation 1 day post infection. By 2 days post infection there was a clear down-regulation of cell surface CD112, which was equivalent at days 3 and days 4 p.i. The cell surface expression of CD112 remained unaffected in MerlinΔUL141 infection as expected, Figure 3.9.

The cell surface expression of CD155 was used as a control as CD155 was known to be down-regulated by Merlin, but not MerlinΔUL141 infected cells. As expected CD155 became down-regulated from the cell surface in Merlin, but not MerlinΔUL141 infections. Interestingly CD155 only began to show signs of down-regulation a day 2 post infection and was efficiently down-regulated by day 3 post infection. In Merlin ΔUL141 infection, CD155 was slightly up-regulated a day 3 post infections, Figure 3.9.

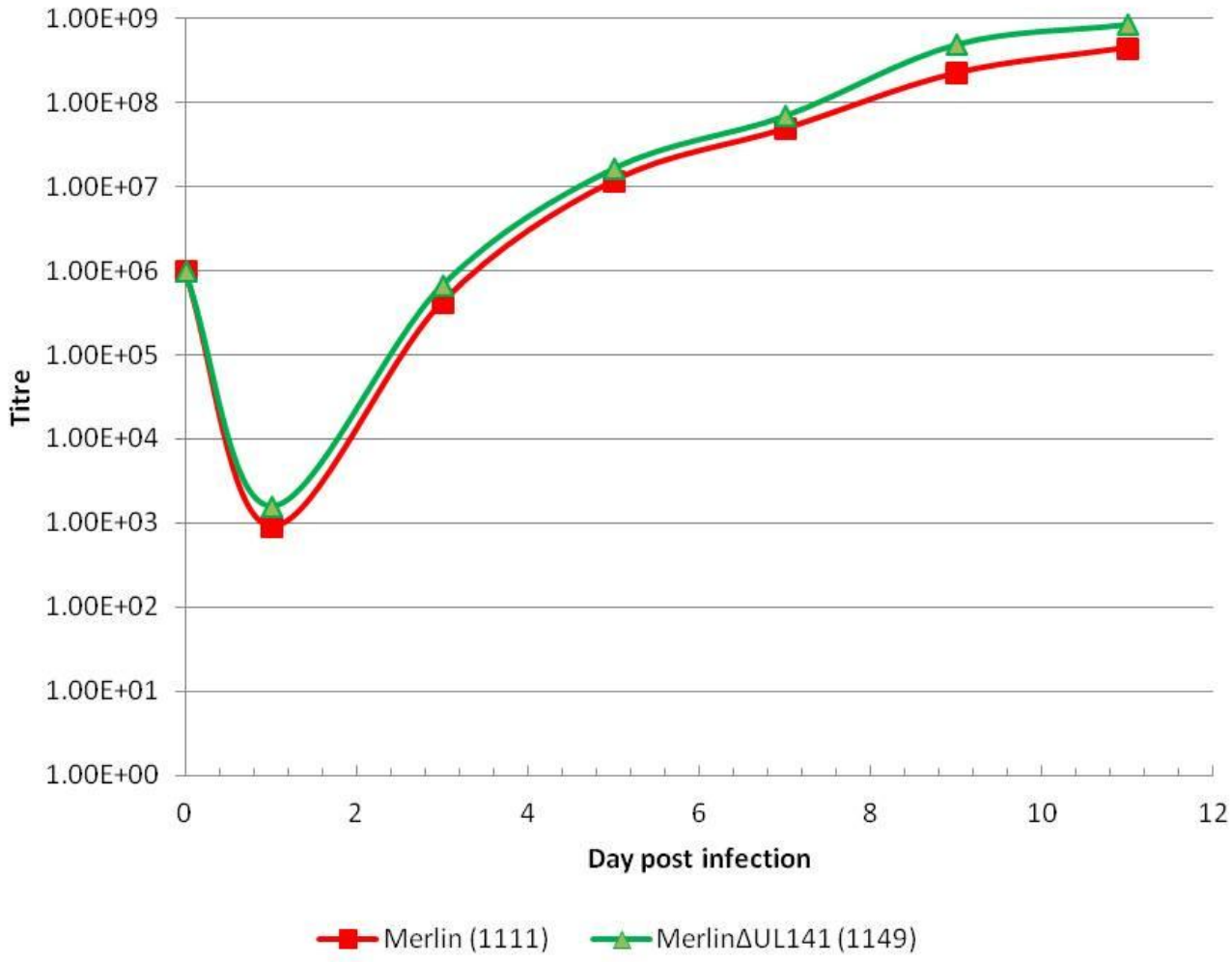


Figure 3.8. UL141 is not essential for HCMV growth.
$1x10^6$ HFFFs were infected with Merlin(1111) or MerlineΔUL141 (1149) at MOI1. Supernatant samples were taken at day 1, 3, 5, 7, 9 and 11 post infection and titred to determine PFU/mL.

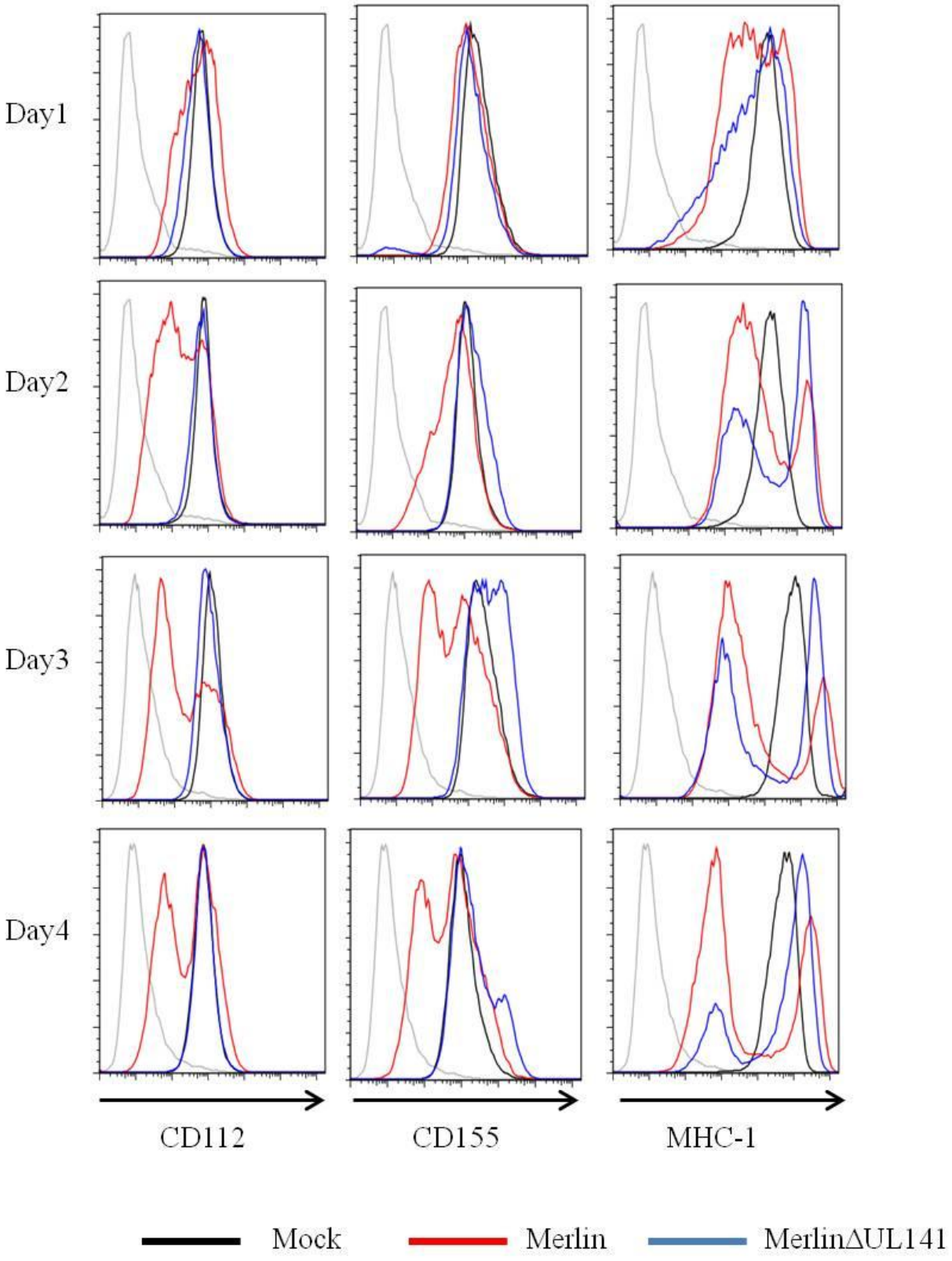


Figure 3.9 : Timecourse of cell surface expression of CDC112, CD155 and MHC-1 during Merlin infection.
1 x 10^6 HFFF's in 25cm^2 flasks were infected with Merlin (**red**), MerlinΔUL141 (**blue**) or uninfected (mock) at MOI 10. On the relevant day post infection cells were harvested and analysed for cell surface expression of CD112, CD155 and MHC-1 by flow cytometry.

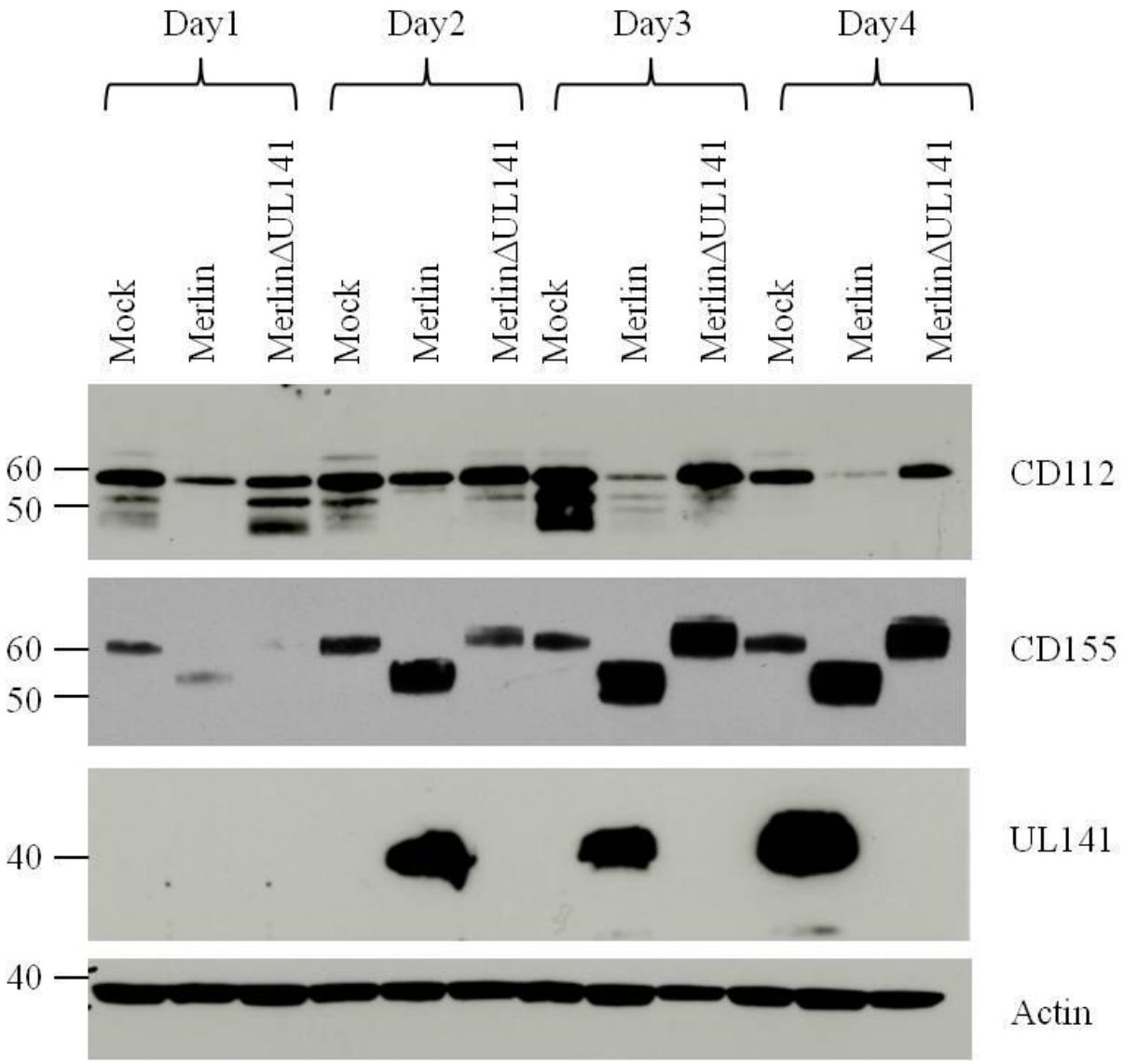


Figure 3.10 : Timecourse of cell surface expression of CD112 during Merlin and MerlinΔUL141 infection.
1 x 10^6 HFFF's in 25cm^2 flasks were infected with Merlin, MerlinΔUL141 or uninfected (mock) at MOI 10. On the relevant day post infection cells were harvested and analysed for expression of CD112, CD155, UL141 and actin by immunoblot.

It therefore appeared that CD155 and CD112 were down regulated from the cell surface with different kinetics. This could also possibly indicate a different mechanism of action against these proteins, or that different proteins are involved in the down-regulation of CD155 and CD112.

When analysed by immunoblot CD112 was detectable at day 1 and day 2 p.i. but not at day 3 and day 4 p.i. in Merlin infected cells, Figure 3.10. CD112 was detected in both mock infected and MerlinΔUL141 infected cells throughout the timecourse.

CD155 was found at a lower molecular weight form, ~50kDa, in Merlin infected cells. In uninfected and MerlinΔUL141 infected cells CD155 was found at a higher molecular weight form, ~60kDa, and CD155 appeared to increase in concentration throughout infection. UL141 could be detected in all Merlin infected HFFF's from day 2 onwards and appeared to increase in concentration as the infection progressed. UL141 was not detected in MerlinΔUL141 infected cells as expected.

It should be pointed out that 100% infection was not achieved during a timecourse of HCMV infection, figure 3.9. This is evident in the two peaks observed in during cell surface staining. The cell surface of MHC-1 clearly showed two clearly defined peaks, Figure 3.9., these peaks are representative of infected cells, which down-regulate MHC-1, and uninfected cells that up-regulate MHC-1. While the mixed population of infected and uninfected cells was not ideal, definitive conclusions could still be drawn due to the distinctive cell populations.

3.7. CD112 transcription is not negatively affected during HCMV infection.

A possible hypothesis for CD112 down-regulation was the reduction of CD112 transcription during HCMV infection. Lower transcript levels could mean that less CD112 transcript is translated into protein and could therefore account for the reduced protein levels observed at the cell surface, providing a mechanism of targeted CD112 down-regulation.

In order to determine whether the transcription of these proteins was affected during HCMV infection RT-QPCR was carried out at three different days post infection with RCMV1111, RCMV1149 or mock infection. This would provide Real time quantitative information for the transcript levels of CD155 and CD112. The relative quantity (RQ) of transcripts were determined by normalising to the concentration of candidate transcripts to both Actin and GAPDH separately, which are standard house-keeping genes used in RT-QPCR techniques.

The dataset was analysed by Peter Giles in Central Biotechnology services (CBS) for statistical analysis using a Post Turkey test and showed no statistically significant differences between expression levels in viral infection conditions and the same day mock infected cells. However, differences of expression were visually apparent when candidates were calibrated to either Actin or GAPDH. Results calibrated to GAPDH appear to show more conservative changes in expression than when calibrated to Actin levels. However the same general trends emerge:

Infection of HFFF's with either RCMV1111 or RCMV1149 appeared to show similar trends of increases in transcription for both CD112 and CD155 transcripts. This suggested that the deletion of the UL141 gene from the virus had little effect on the transcriptional profile of CD112 and CD155. This was an important observation as it demonstrated that HCMV infection does not down-regulate CD112 at the transcriptional level.

During both RCMV1111 and RCMV1149 infections CD112 mRNA appeared to be up-regulated at Day 1 post-infection with levels remaining at similar levels for the duration of the experiment. The same trend was seen when data were normalised against Actin or GAPDH. Both the FACS and immunoblot data, Figure 3.9 and Figure 3.10. respectively, showed CD112 beginning to become down-regulated at 24 hours post infection and at 48hours post-infection a solid down-regulation was observed. Therefore, when the RNA transcription data was evaluated in conjunction with the protein expression analysis it appeared that CD112 was affected at a translational or post-translational level early on in the infection cycle, thus likely to be by an early (E) or immediate early (IE) HCMV protein.

In contrast to CD112, the transcript levels of CD155 appeared to be unaffected at Day 1 post-infection but levels appeared to increase as the infection progressed, Figure 3.11. This correlated with western blot data showing the levels of CD155 increased over the course of

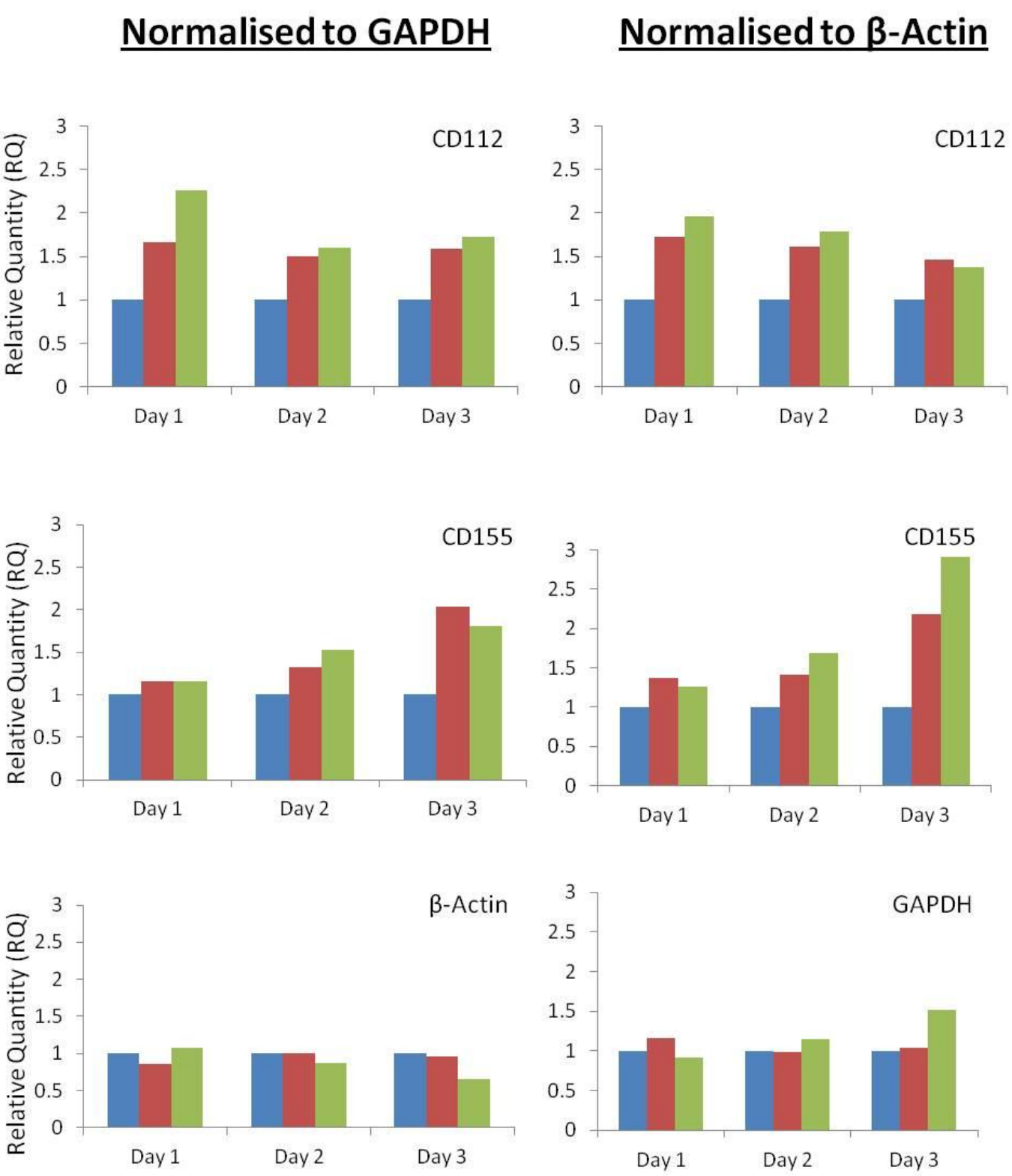


Figure 3.11. RT-QPCR analysis of CD112 and CD155 transcription during RCMV infection. 1 x 10^6 HFFF cells were infected with RCMV1111 (Red), RCMV1149 (Green) or mock infection (Blue). At day 1, day 2 or day 3 post-infection cells were lysed in 1 mL of Tri-reagent (Sigma) and stored at -80°C until required. RNA was extracted followed by Reverse transcription PCR using the random hexamer method. The cDNA was analysed by QPCR using the Sybr green technique. Relative quantity (RQ) gene expression values were calculated based on the comparative threshold cycle (Ct) as described in *Livak and Schmittgen* (2001), where both β-Actin and GAPDH were independently used as calibrators.

an infection, Figure 3.10. Results suggested that mechanisms controlling both the expression and down regulation of expression of CD112 and CD155 were controlled independently of each other.

When GAPDH and Actin were normalised to each other, Figure 3.11., expression levels gave a RQ of approximately one which was consistent with their use as reference house-keeping genes and as appropriate calibrators in the analysis and interpretation of RQ PCR data.

However, due to the small dataset it is impossible to perform reliable statistics or make any definitive conclusions. Even though in some cases there was an 'up-regulation' by two fold this is still regarded as not being particularly noticeable above background noise. For example in gene chip experiments a difference of greater than 5-fold is generally required to register a positive hit. What can be clearly concluded from this data is that the transcription of CD112 was not silenced or negatively affected by HCMV infection and that transcriptional silencing during HCMV infection is unlikely to be the cause of CD112 down-regulation. Rather CD112 down-regulation must occur at a translational or post-translational level.

3.8. Conclusion

CD112, an activating ligand for NK receptor DNAM-1, is a glycoprotein expressed as two isoforms in uninfected HFFF cells. During HCMV infection CD112 appeared to be down-regulated from the cell surface from day 2 p.i. onwards. During HCMV infection CD112 is no longer detectable by western blot from day 3 p.i. The HCMV gpUL141 appeared to be involved in this process as deletion from Merlin prevented CD112 down-regulation. However when UL141 was expressed in isolation from a RAd or in a stable cell line there appeared to be no effect on CD112 cell surface expression. RT-QPCR showed no statistically significant difference in transcript levels between Merlin and MelrinΔUL141 infected HFFF's, indicating that transcriptional silencing during HCMV infection was unlikely to be the cause of CD112 down-regulation. Instead the down-regulation CD112 cell surface expression was likely to occur at a translational or post-translational level.

4.0. SCREENING STRATEGY FOR THE IDENTIFICATION OF GENE(S) RESPONSBILE FOR CD112 DOWN-REGULATION.

4.1. Identification of screening strategy

Data presented in chapter 3.0 imply that UL141 acts in concert with at least one other HCMV gene product to down-regulate cell surface expression of CD112. The subsequent aim of experiments carried out in chapter 4.0 was to identify a second gene essential for CD112 down-regulation by the methodical screening of the HCMV genome. Two complementary approaches were projected:

1. Screen of a library of 8 HCMV block deletion mutants, covering 46 genes in the HCMV genome, for a loss of the ability to down-regulate CD112. This approach anticipated that the second HCMV gene acting with UL141 played an essential role in CD112 down-regulation and its deletion would restore CD112 cell surface expression.

2. Screen of a library of 166 RAd vectors encoding individual HCMV genes co-infected with RAd-UL141 for a gain of the ability to down-regulate CD112, anticipating that a combination of two specific HCMV genes would be sufficient for this effect.

Both approaches were predicted to independently identify gene(s) involved in CD112 down-regulation.

4.2. Identification of appropriate screening assays.

Before commencing screening an appropriate assay was sought that would provide both high throughput potential and a robust and reliable readout in order to efficiently screen through the large number of potential targets. Four approaches were identified. Approaches 1-3 were based on the finding that CD112 appeared to be degraded during HCMV infection. Approach 4 was based on the finding that CD112 cell surface expression was down-regulated during HCMV infection.

1. Co-infection of HCMV with RAd-CD112-mCherry; the RAd vector construct would provide expression of fluorescently tagged CD112-mCherry, thus enabling sensitive and live monitoring of expression using mCherry fluorescence.

2. Co-infection of RAd-CD112-mCherry with RAd-UL141 and RAd vectors encoding individual HCMV genes. This would allow the live monitoring of expression intensity and cellular localisation to be monitored. Spectrophotometry would allow the measurement of florescence intensity.

3. Analysis of the expression of endogenous CD112 by immunoblotting. Method was optimised in chapter 3.0. to enhance sensitivity.

4. Analysis of CD112 cell surface expression by Flow cytometry. Method was optimised in chapter 3.0. to provide reliable and robust readout.

A timecourse of CD112 expression in chapter 3.0., figure 3.9 and Figure 3.10., identified both CD112 and CD155 to be down-regulated at 3 days p.i. This timepoint was therefore chosen for the development of screening assays.

4.2. Developing screening system to detect CD112 down-regulation by CD112-mCherry over-expression

A 96 well plate assay format was sought as a primary screening method in order to exploit its high throughput potential. A CD112-mCherry reporter was used as it simultaneously allowed the assessment of changes in fluorescence intensity and intracellular localisation of CD112-mCherry. Since HCMV UL141 re-localised CD155 from the cell surface to the ER, a similar fate befalling CD112 would not have been surprising. Two approaches utilising RAd-CD112-mCherry were identified: Co-infection of RAd-CD112-mCherry with either (1) HCMV or (2) RAd expressing individual HCMV ORFs.

4.2.1 Co-infection of HCMV and RAd-CD112-mCherry

During initial assay development, HFFF's, were co-infected with HCMV strain Toledo encoding GFP (HCMV-GFP) and either RAd-CD112-mCherry or RAd-CD155-mCherry. RAd-CD155-mCherry was to be used as a control as CD155 is sequestered in the ER during HCMV infection (Tomasec et al., 2005). The tracking of productive HCMV infection was monitored as GFP fluorescence, expression of GFP being driven by HCMV

β2.7 early promoter, while the expression of mCherry allowed changes in CD112 localisation or intensity to be monitored.

Cells were seeded at $5x10^5$ across a glass bottomed 96-well plate and 3days p.i. infections were fixed and fluorescence monitored using fluorescent microscopy. Interestingly, at 3days p.i., cells appeared either green (HCMV infection) or red (RAd infection) and the proportion of HCMV infected cells (green) decreased with increasing RAd-CD112-mCherry MOI (red) (figure not shown). This implied:

a) HCMV infection induced efficient degradation of CD112-mCherry.

b) Exclusive infections with either HCMV-GFP or RAd-CD112-mCherry.

c) HCMV infection had a negative effect on the promoter driving CD112-mCherry expression.

d) CD112-mCherry expression had a negative effect on HCMV replication or GFP expression.

In an attempt to address point b), RAd magnetofection (Combimag, OzBiosciences) was used to force HCMV and RAd-CD112-mCherry co-infection. Magnetofection is a process of enhancing receptor-independent RAd infection using magnetic nanoparticles.

Cells were infected with Toledo-GFP and 2 hours later magnetofected with RAd-CD112-mCherry or control RAd-CD155-mCherry. HCMV infection was monitored as green fluorescence and CD112-mCherry or CD155-mCherry as red fluorescence. Results clearly indicated that successful Toledo-GFP and RAd co-infection occurred using this approach as the same cell expressed both GFP and mCherry, although GFP expression was weak in cells expressing CD112-mCherry compared to infected cells expressing CD155-mCherry (data not shown). This was in agreement with the hypothesis that the over expression of CD112-mCherry either a) inhibited the progression of HCMV infection or b) inhibited expression of GFP from β2.7 early promoter.

Following the verification the co-infection of HCMV with RAd using magnetofection technique, the assay setup was tested in a final format imitating the projected screen of library of HCMV mutants. Cells infected with HCMV Merlin (down-regulates CD112) or HCMV MerlinΔUL141 (does not down-regulate CD112) were magnetofected with either RAd-CD155-mCherry or RAd-CD112-mCherry. As expected, when HCMV Merlin was

co-infected with RAd-CD155-mCherry, the CD155-mCherry formed intracellular foci, Figure 4.1. However, in cells infected with RAd-CD112-mCherry no obvious difference in CD112-mCherry localisation could be observed between Merlin and MerlinΔUL141 infections, Figure 4.1. It was therefore concluded that this method was not suitable for high throughput screening.

4.2.2. Co-infection of RAd-CD112 mCherry, RAd-UL141 and a RAd from the HCMV-RAd-library

A 96-well plate assay format was sought in order to exploit the potential for a high throughput assay with robust readout. The basis of the assay was co-infection of RAd-CD112-mCherry with RAd-UL141 plus RAd vectors encoding individual HCMV genes. The readout for the assay was primarily a visual one with wells monitored for changes in both CD112-mCherry florescent intensity (due to degradation or inhibited transcription) and for changes in CD112-mCherry subcellular location (e.g. ER retention). As UL141 retains CD155 in the ER, a similar effect on CD112 would not have been surprising as UL141 is also involved in the down-regulation of CD112 from the cell surface (Figure 3.5.) Changes in florescence intensity were to be later reconfirmed by spectrophotometry.

However, it became evident that expression of CD112-mCherry from RAd was unsuitable for use with the HFFF-hCAR cell line. As early as 1 day after infection of cells with RAd-CD112-cherry, the fluorescence intensity of CD112-cherry was oversaturated and it was therefore problematic to analyse or image CD112-mCherry expression patterns, Figure 4.2.

In addition CD112-mCherry showed unexpected localisation, forming intense Intracellular foci throughout the cell making imaging and analysis difficult as the image became over saturated even at low exposure levels, Figure 4.2. While it was not anticipated that CD112 would form these intracellular foci, atypical aggregation of fusion proteins has been described before (Shaner et al., 2005). In contrast cells infected with CD155-mCherry showed expected/typical expression patterns and expression gradually increased throughout the course of infection with optimum detection 3 days p.i., Figure 4.2. At this time-point CD112-mCherry was so intense that it made visualisation near impossible.

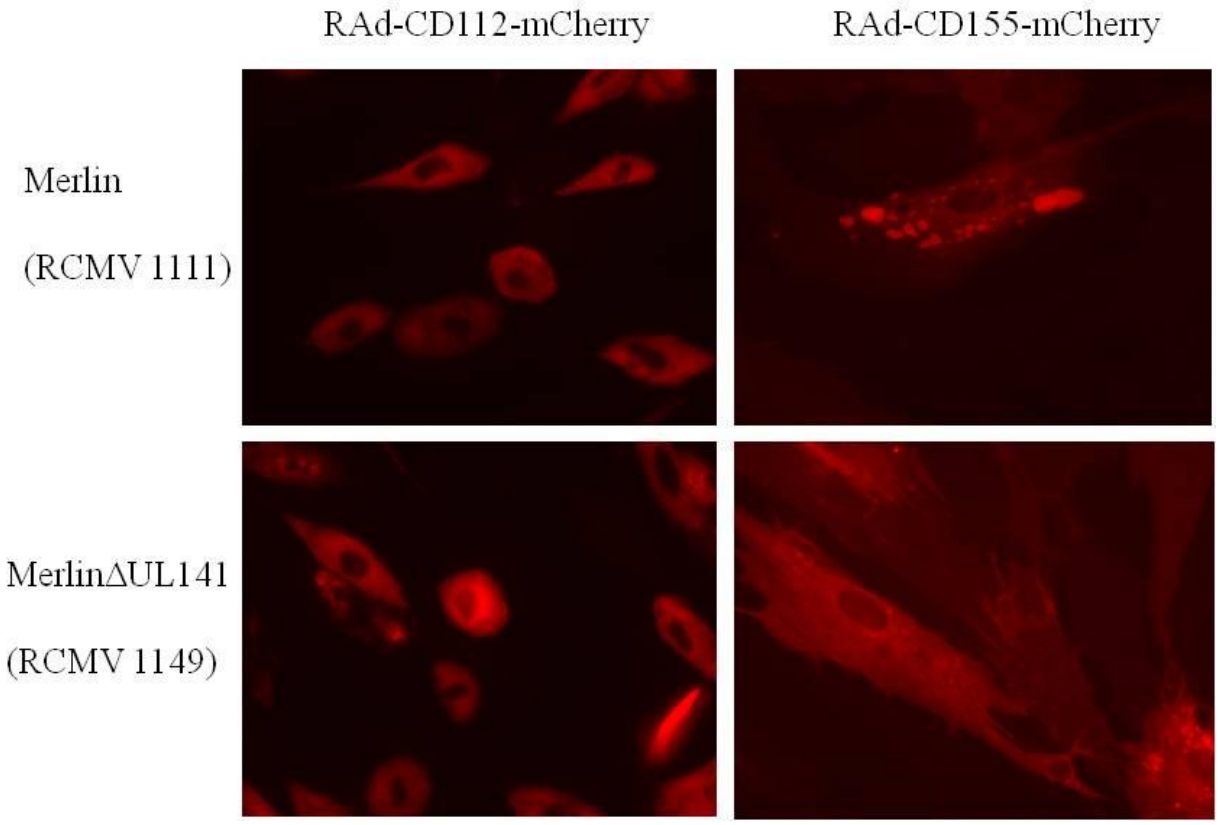


Figure 4.1. Magnetofection ensured the co-infection of HCMV and RAd-mCherry.
HFFF cells were seeded $1x10^6$ / 96well plate. The following day cells were infected with Merlin (RCMV1111) or MerlinΔUL141 (RCMV1149). 2 hours post CMV infection cells were coinfected with either RAd-CD112-mCherry or RAd-CD155-mCherry using magnetofection. CD112 and CD155 expression was monitored by m-Cherry fluorescence. Cells were fixed 3days p.i. in 2% paraformaldehyde and images taken using florescence microscopy.

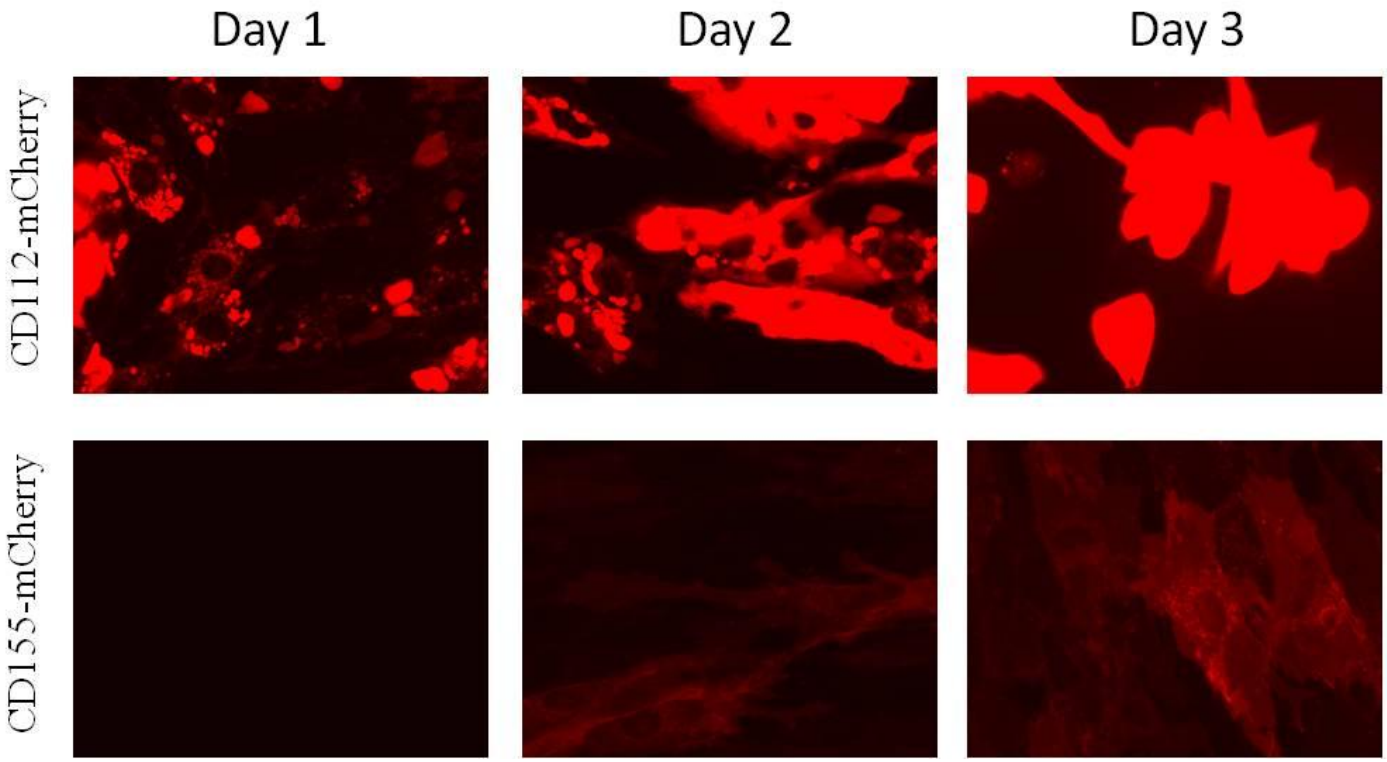


Figure 4.2. Expression of RAd-CD112-mCherry and RAd-CD155-mCherry in uninfected HFFF-hCARs

1 x 10^6 HFFFF-hCARs in 96 well plate were infected with 5PFU/cell of RAd-CD112-mCherry or RAd-CD155-mCherry. Day 1, 2 and 3 post infection cells were monitored for mCherry expression by florescent microscopy. Images are taken with an exposure of 0.015seconds.

It was therefore concluded that this 96-well plate assay format was not suitable for high throughput screening. It was also concluded any screening method involving the RAd-CD112-mCherry was unsuitable due to the intense overexpression and atypical subcellular localisation of CD112-mCherry.

4.3. Commencement of large scale screening project

Flow cytometry had shown to be a reliable method for the detection of cell surface CD112, producing robust and highly repeatable results. Flow cytometry was therefore chosen as the primary screening method for both the HCMV block deletion library and the RAd-HCMV-ORF library as this method allowed for a higher throughput than using immunoblot. Screening began with infection of HFFF's with HCMV mutants that contained block deletions of non-essential HCMV genes, table 4.1.

Table 4.1. HCMV block deletion library

RCMV designation	Comment	MOI
1278	Control RCMV. Containing mutation in RL13 and UL128. UL32-GFP tagged, ΔUL16, ΔUL18. N.B. All other RCMV block deletion mutants were derived from this construct.	20
1293	ΔUL2 - UL11	10
1295	ΔUL22A – UL25	10
1297	ΔUS12 – US17	20
1299	Δ US27 – US28	50
1300	Δ US29 – US34a	15
1318	ΔUS18-US22	50
1332	ΔRL1 – RL6R	25
1333	ΔRL10 – UL1	10

4.3.1. Effect of block deletions in HCMV genome on CD112 expression.

Recombinant HCMVs (RCMVs) with block deletions of non essential ORFs were previously constructed by Dr R Stanton within the laboratory, Figure 4.3/Table 4.1. All Block deletion mutants also included deletions in UL16 and UL18, known immunoevasion functions. UL16 inhibits NK cell cytotoxicity though sequestration of ULBP1,2 and MICB, while UL18 encodes a MHC class 1 homologue. Both these genes protect Merlin infected cells from NK cell cytotoxicity. Deletion of UL16 and UL18 allowed for RCMV infected cells to be utilised for NK assays in a separate laboratory project, while the presence of UL141 facilitated the usage of HCMV block deletions in screening assays for CD112 down-regulation. RCMV block deletions also contained a UL32-GFP fusion which allowed infections to be monitored through GFP expression. The RCMV1278 (MerlinUL32-GFPΔUL16ΔUL18) was the parental strain from which all other block deletions were constructed. RCMV1278 down-regulated CD112, CD155 and MHC-1 and was used as the control for screening the block deletion mutant library, Figure 4.4.

RCMV block deletions were screened for a loss of the ability to down regulate the expression of CD112 (similar to the effect observed with UL141 deletion, figure 3.5) and therefore a loss of function. A positive outcome of this screen would identify a second function targeting CD112 and would also significantly narrow down the number of ORFs (those within a particular block deletion) for follow-up studies.

A total of 8 block deletions were initially screened, table 4.1., spanning regions of HCMV genes non essential for growth in fibroblasts. Infections were optimised to determine MOIs that gave similar levels of GFP expression, as determined by fluorescence microscopy detecting UL32-GFP (data not shown). MOIs for each virus can be found in Table 4.1.

At 3 days p.i. infections were harvested and analysed by two-step cell surface staining via flow cytometry and analysed using FlowJo software. A live cell gate was drawn determined by comparing forward scatter against side scatter according to laboratory protocol. As HCMV block deletions contained GFP tagged UL32, RCMV infected cells were gated on by plotting Side scatter of live cells against FL1 (GFP). Gating in this way revealed two distinct populations of GFP positive, HCMV infected cells. These were classified as GFP low and GFP high populations, Figure 4.4.

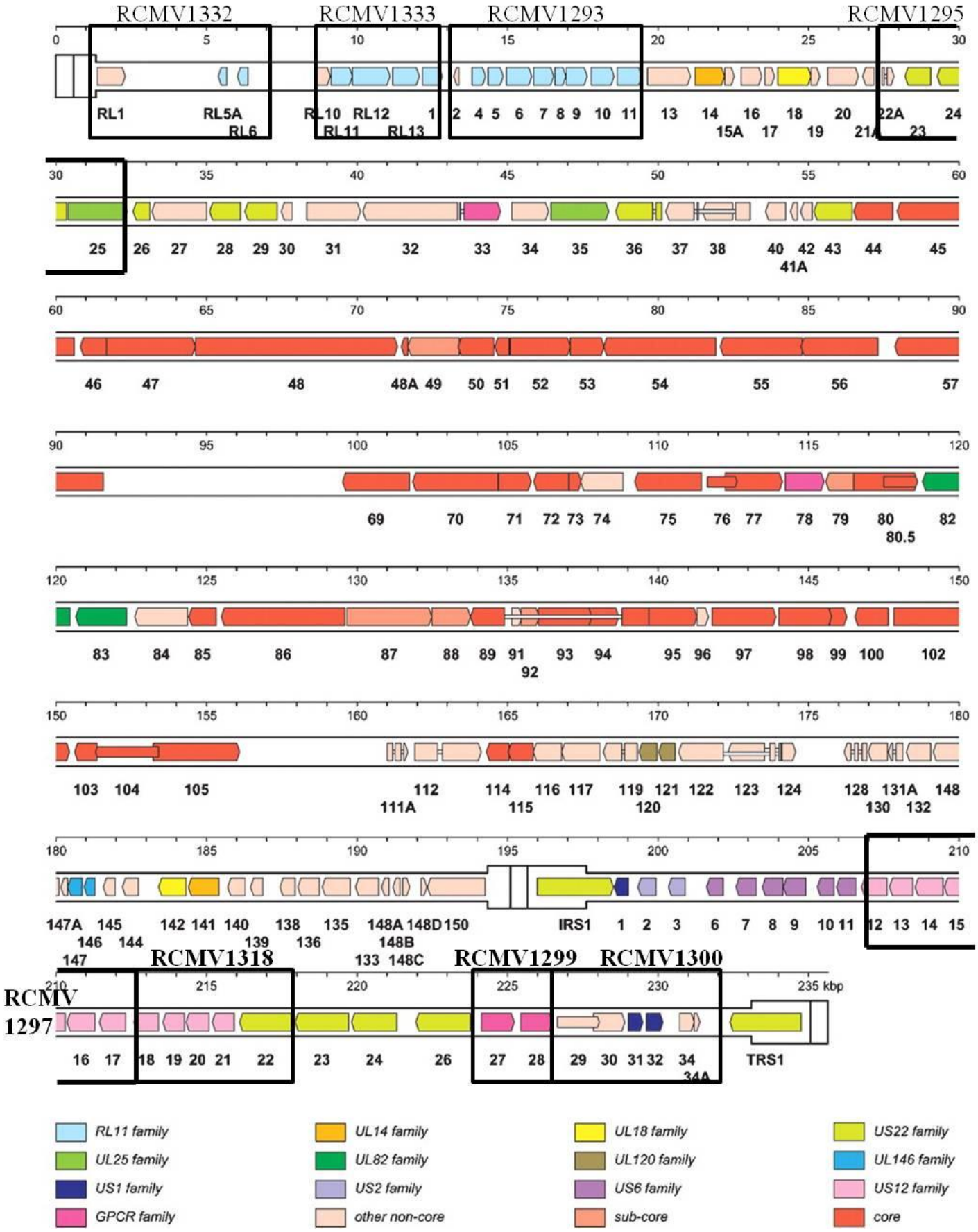


Figure 4.3. Merlin block deletion mutants.
Map of HCMV strain Merlin genome with deleted regions marked. The indicated blocks were individually deleted to generate a panel of viruses, covering between them 46 HCMV ORFs. All deletions were made in the strain Merlin BAC containing mutations in RL13 & UL128. GFP was fused to UL32 to enable tracking of virus. To facilitate functional readouts, UL16 and UL18 were also deleted in all mutants - these have previously been shown to modulate the NK response. Modified from Dolan *et a*l (2004)

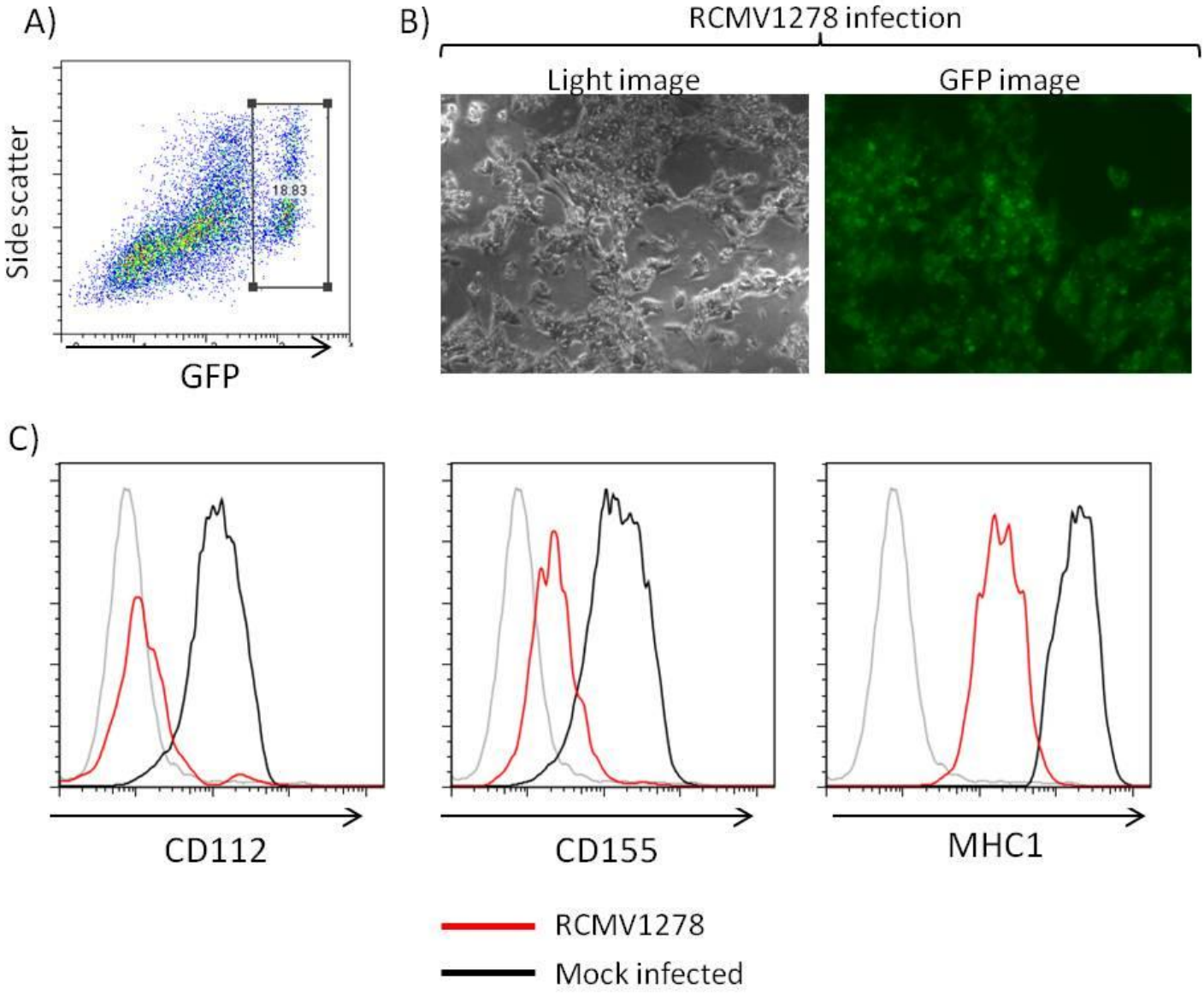


Figure 4.4 Baseline RCMV1278 infection for the screening HCMV block deletion library
HFFF cells were seeded $1x10^6$ in a $25cm^2$ tissue culture flask. The following day cells were infected with HCMV1278, control infections for HCMV block deletions. 3 days post infection cells were harvested and analysed . A) Infected cells were gated on GFP high cells for analysis. B) light image and GFP images of infections were taken to visually confirm infection. C) HCMV1278 infected cells were gated on GFP high population and analysed for cell surface expression of CD112, CD155 and MHC1 shown in red, compared to the cell surface expression of CD112, CD155 and MHC1 on uninfected cells, shown in black.

Cells were infected at MOIs described in table 4.1. The cell surface expression of CD155, CD112 and MHC-1 were analysed on live, GFP-high cells. All 8 block deletion mutants tested were capable of down-regulating both CD155 and CD112 to as similar degree as control HCMV1278 infections, Figure 4.5 and Figure 4.6. As all block deletions were capable of down-regulating cell surface CD112 then a second function targeting CD112 is likely to lie outside of the genes deleted in block deletion mutants.

4.4. Conclusion

Flow cytometry was considered to be the most effective method for use in large scale screening due to its robust and reliable output. A panel off 8 RCMVs containing block deletions of non-essential HCMV genes were screened and all were found to down-regulate cell surface CD112 to as a similar degree as control RCMV1278. If any of the deleted genes were essential in CD112 down-regulation there would have been a loss of function and CD112 expression levels would not be down-regulated as in RCMV1278 infection. CD155 and MHC-1 were also all down-regulated from the cell surface as expected. The deleted genes, totalling 46 were therefore concluded to have a non-essential role in CD112 down-regulation, leaving 121 potential gene candidates for further screening using a recombinant Adenovirus library expressing individual HCMV ORFs.

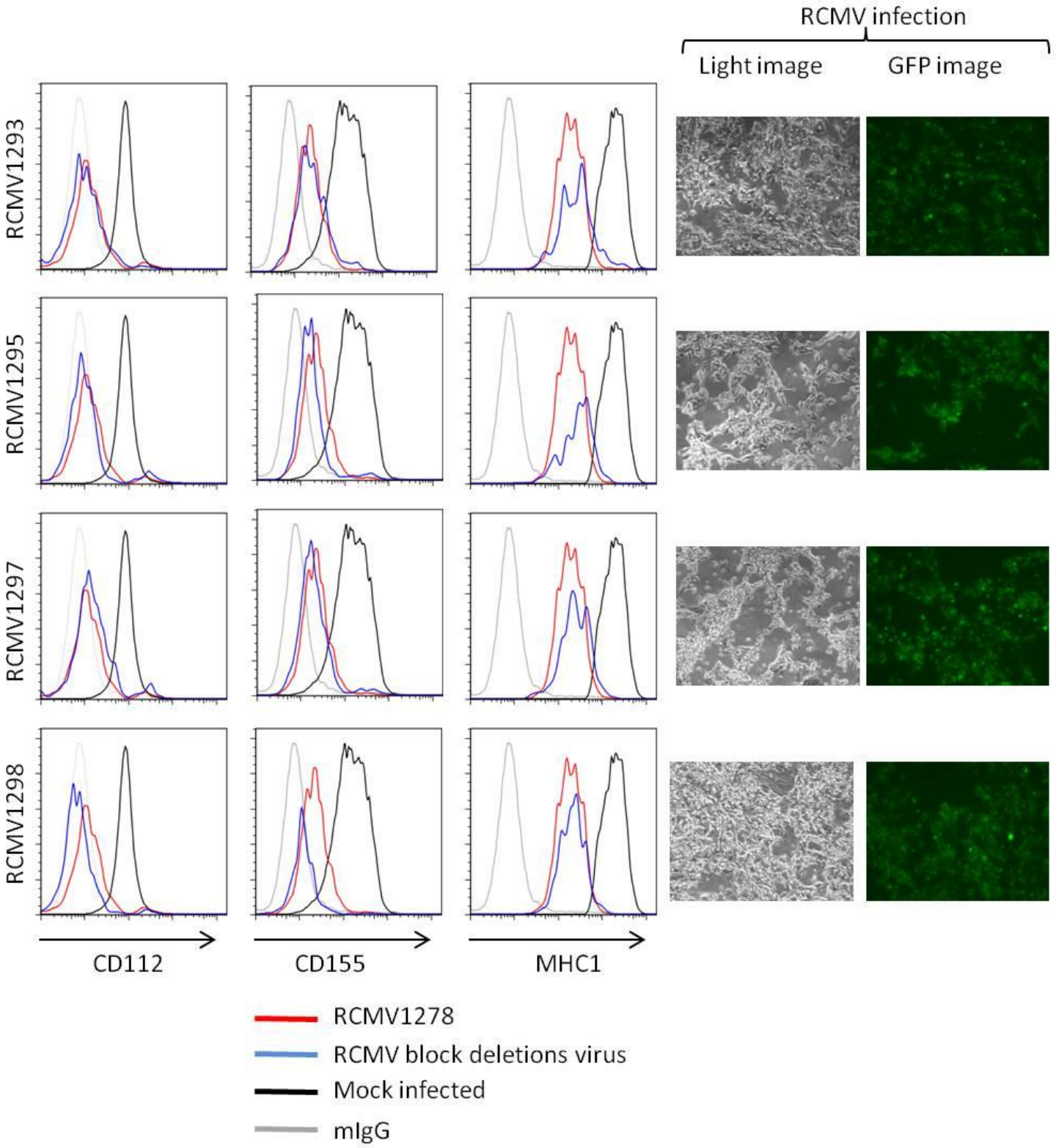


Figure 4.5. Screening results for the HCMV block deletion library: RCMV1293, RCMV 1295, RCMV1297 and RCMV1298

HFFF cells were seeded $1x10^6$ in a $25cm^2$ tissue culture flask, one flask per infection. The following day cells were infected with RCMV1278, RCMV1293, RCMV 1295, RCMV1297 and RCMV1298. 3 days post infection cells were harvested and analysed for cell surface expression of CD112, CD155 and MHC-1. Infected cells were analysed on GFP high population determined be side scatter plotted against GFP. Cell surface expression of block deletion viruses (red) were compared to those for HCMV1278, the control HCMV for the block deletions (blue)

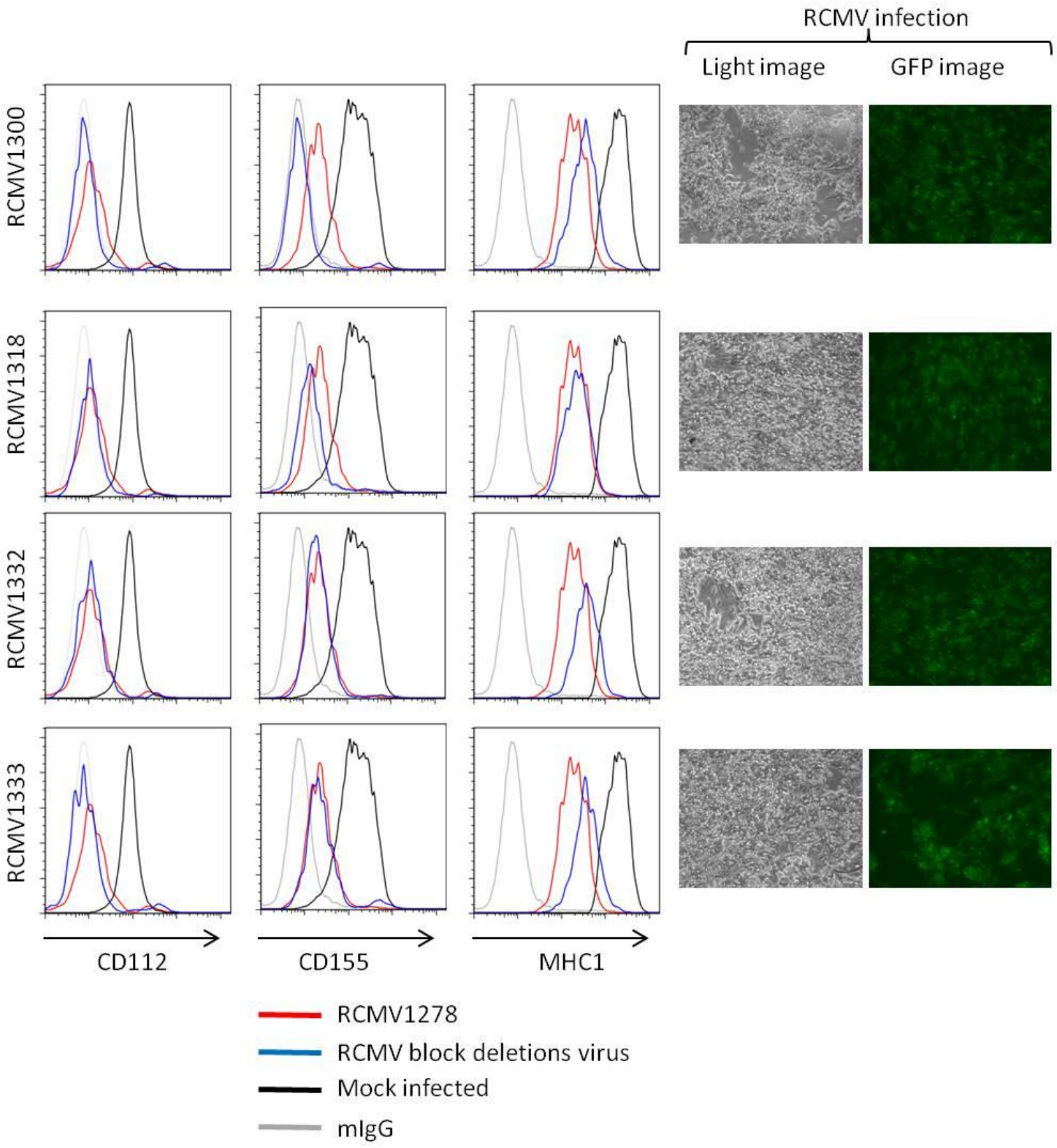


Figure 4.6. Screening results for the HCMV block deletion library: RCMV1300, RCMV 1318, RCMV1332 and RCMV1333.
HFFF cells were seeded $1x10^6$ in a $25cm^2$ tissue culture flask, one flask per infection. The following day cells were infected with HCMV1278, HCMV1293, HCMV 1295, HCMV1297 and HCMV1298. 3 days post infection cells were harvested and analysed for cell surface expression of CD112, CD155 and MHC-1. Infected cells were analysed on GFP high population determined be side scatter plotted against GFP. Cell surface expression of block deletion viruses (red) were compared to those for HCMV1278, the control HCMV for the block deletions (blue)

5.0. CHARACTERISATION OF THE INTRACELLULAR LOCALISATION OF HCMV PROTEINS EXPRESSED FROM RAD BY IMMUNOFLUORESCENCE

As part of a long running research theme within the laboratory into the study of HCMV gene function; work over the last three years by Dr James Davies and Mr Sepehr Seirafian has been centred on the generation of a recombinant Adenovirus library expressing individual HCMV strain Merlin ORFs (RAd-HCMV-ORF library). The HCMV genome was re-annotated by Dr Andrew Davison, Glasgow University, and ORFs were designated as per his most recent annotation (Gatherer et al., 2011). The re-annotation was based on deep sequencing of the Merlin genome, transcriptional profiling during productive HCMV infection and comparison of the HCMV genome with CCMV to determine likely HCMV ORFs (Davison, 2003, Dolan, 2004, Gatherer et al., 2011). This resulted in 170 ORFs being designated and all were cloned into RAd vectors using the recombineering technique developed by Dr Richard Stanton (Stanton, 2008).

The RAd-HCMV-ORF library was to be utilised in the screening for HCMV genes involved in CD112 down-regulation through co-infection with UL141. As this project was the first to use the entire RAd-HCMV-ORF library it was first important to establish transgene expression occurred from these vectors. Since each HCMV ORF is V5-tagged, anti-V5 immunofluorescent staining (IF) of HFFF-hCAR's infected with a RAd from the RAd-HCMV-ORF library was chosen to both confirm transgene expression and also provide qualitative data on the cellular location of each expressed protein.

The screen was carried out in collaboration with Mr Sepher Seiferian, a fellow PhD student in the laboratory who also used the RAd-HCMV-ORF library in a separate project. In order to quickly generate data, the decision was taken for Mr Seiferian and I to each take half of the RAd-HCMV-ORF library. Mr Seiferian screened from RL1-UL40 and UL111A-UL150 and IRS1, figure 4.3, and I screened the remaining RAd-HCMV-ORF's from UL41A-UL105 and all US genes from US1-US34A and TRS1, figure 4.3. The transgene expression data for my section, UL41A-UL105, US1-US34A and TRS1, will be the subsequent focus of this chapter. Mr Seiferian's data for RL1-UL40 and UL111A-UL150 and IRS1 can be found in appendix 1 but will not be analysed in this thesis.

5.1. Transgene detection

Many of the HCMV genes are poorly studied and as a result there are few reagents available that allow the detection of individual HCMV protein products. In order to facilitate transgene expression studies all transgene ORFs in the RAd-HCMV-ORF library were C-terminally tagged with a V5 epitope tag. The V5 tag is a 14 amino acid sequence, GKPIPNPLLGLDST, and was chosen as the epitope is relatively short so should have had minimal impact on transgene protein structure and function. There are also high affinity reagents available that allow the detection of V5, facilitating further downstream studies.

HFFF-hCAR cells were infected with a single RAd from the RAd-HCMV-ORF library at MOI10 in a glass bottomed 96well plate. At 72hrs p.i. cells were fixed and the V5 tag was exploited for immunofluorescent staining of infected cells to test for transgene expression using a monoclonal anti-V5 mAb. A secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect the anti-V5 antibody and staining was observed by fluorescent microscopy using a x40 oil objective. RAd1253 is an empty vector control containing no transgene but contained the V5 sequence. Staining was carried out on uninfected (data not shown) and control RAd-1253 infected HFFF-hCARs as a negative staining control to prevent false positive results due to non-specific binding of the anti-V5 primary or non specific binding of the anti-mouse secondary antibody, figure 5.11. To assess cellular location of detected transgenes the nuclear counterstain DAPI (blue channel), and phalloidin AlexaFluor-488 that stained actin filaments (green channel), were also included.

Of the UL41A to UL105 and US1-TRS1 region screened, protein expression was confirmed and localisation data was obtained for 79 out of 85 transgenes, figures 5.1 – 5.10.

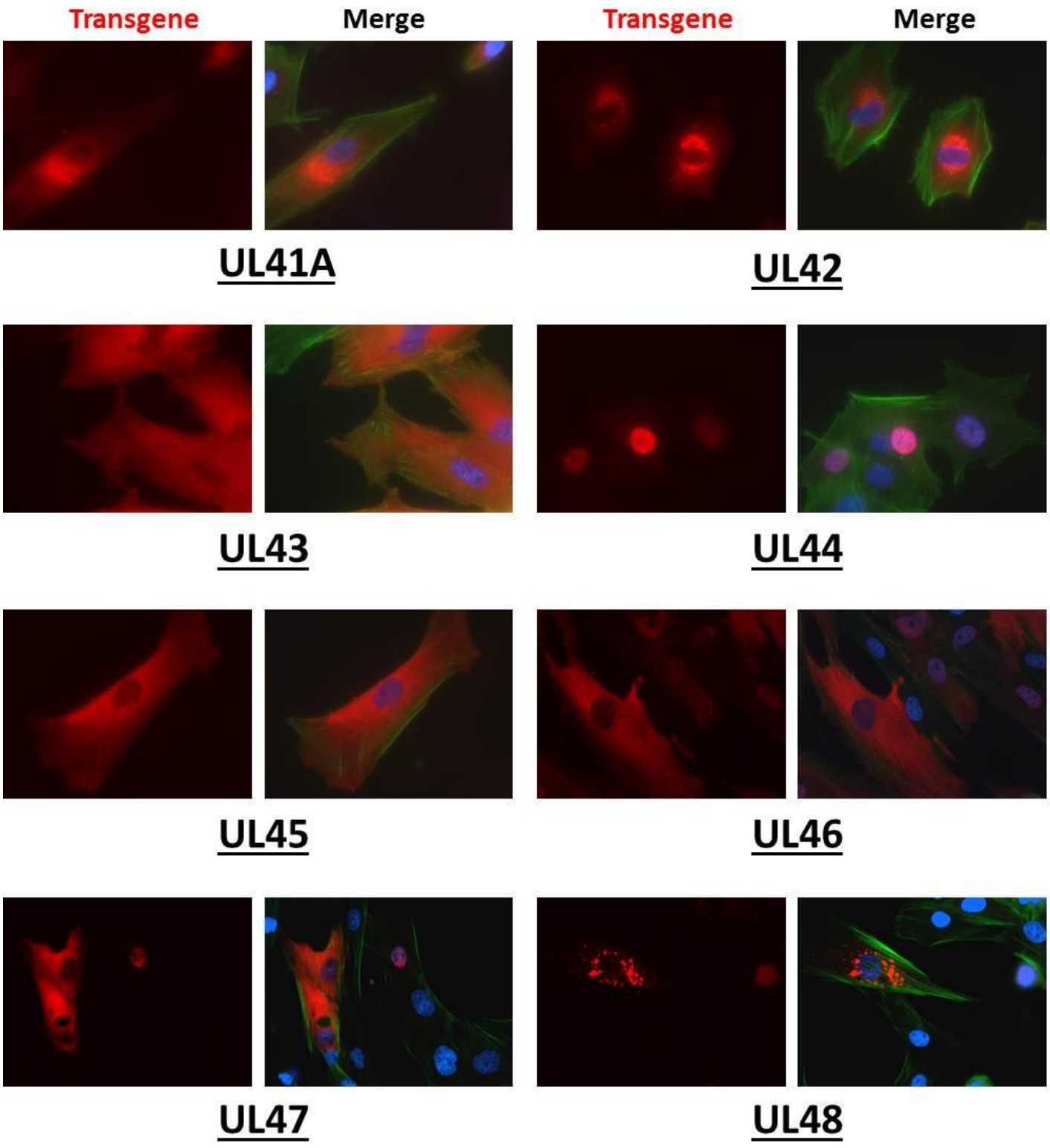


Figure 5.1. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL41A-UL48
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.1 A comparison of transgene localisation to localisation patterns described in the literature, for UL41A - UL48. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
UL41A	Putative membrane protein	Sub cytoplasmic	None identified	N/A
UL42	Putative membrane protein	Sub cytoplasmic	None identified	N/A
UL43	US22 family Tegument protein	Pan cytoplasmic	None identified	N/A
UL44	Processivity subunit of DNA polymerase (ICP36)	Pan-nuclear	Pan nuclear/punctate nuclear Forms punctate nuclear staining colocalising with IE2 in AD169 infected fibroblasts	Sourvinos et al 2007
			Treatment with phosphonoformic acid, inhibited DNA replication resulting in dispersasl of UL44 throughout the nucleus in AD169 infected fibroblasts	Strang et al 2011
UL45	Large subunit of ribonucleotide reductase Lacks catalytic residue and is probably enzymatically inactive Tegument protein	Pan cytoplasmic	None identified	N/A
UL46	Component of intercapsomeric triplexes in capsids (mC-BP)	Either nuclear or cytoplasmic	None identified	N/A
UL47	Tegument protein Possible role in intracellular transport Binds UL48 protien	Either nuclear or cytoplasmic	None identified	N/A
UL48	High molecular weight tegument protein Binds UL47	Sub cytoplasmic	None identified	N/A

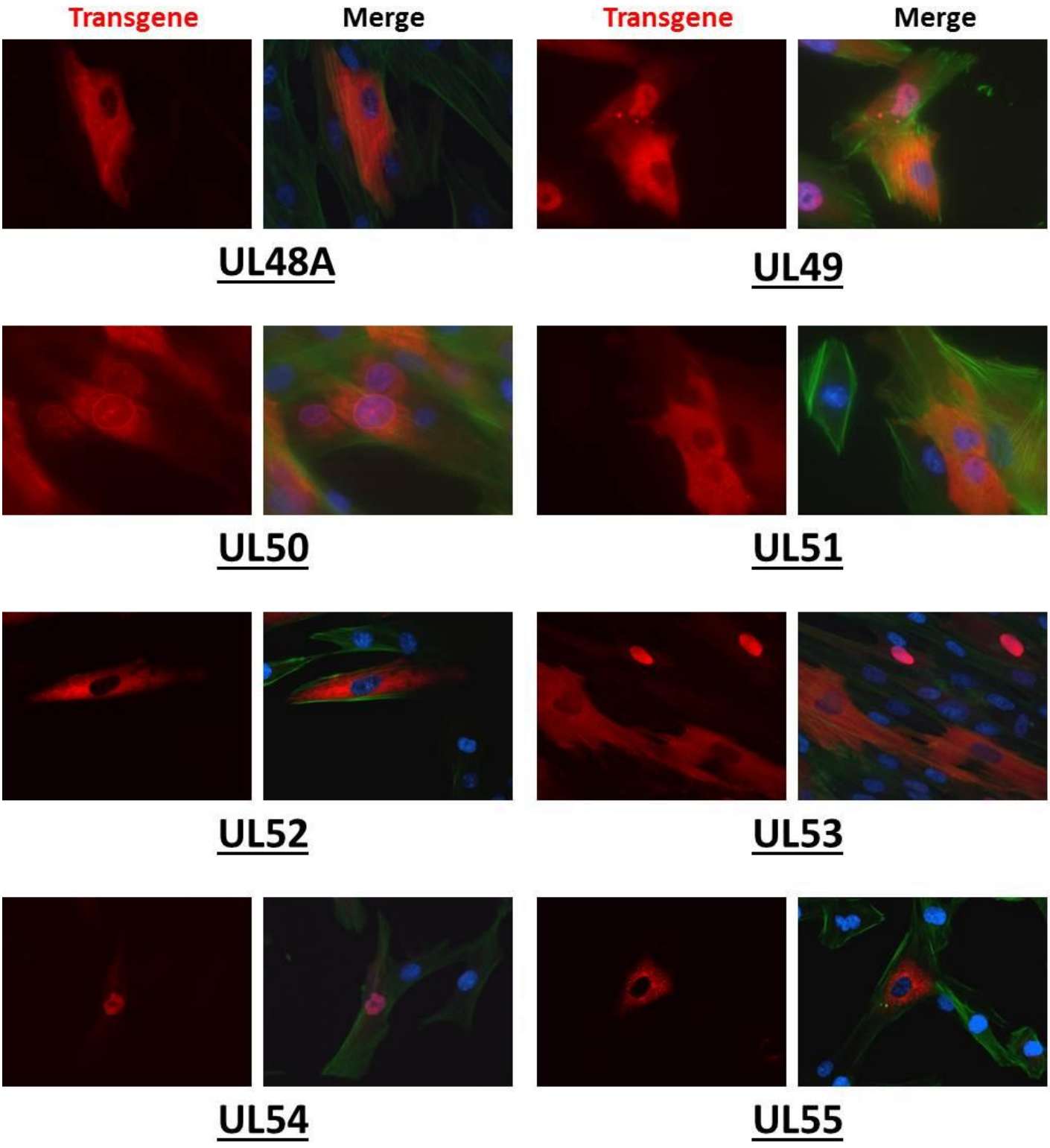


Figure 5.2. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL48A-UL55.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.2 A comparison of transgene localisation to localisation patterns described in the literature, for UL48A - UL55. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
UL48A	Located on tips of hexons in capsids (SCP)	Pan cytoplasmic	Pan cytoplasmic / Pan nuclear In AD169 infected fibroblasts UL48A expression was restricted to the nucleus In cells both infected with Vaccinia virus expressing UL48A and also in transiently transfected COS7 cells, UL48A was detected in the cytoplasm	Lai and Britt 2003 Adamo et al 2004
UL49	Conserved in *Gammaherpesvininae*	Either nuclear or cytoplasmic	None identified	N/A
UL50	Inner nuclear membrane protein Role in egress of capsids from nucleus	Sub cytoplasmic	Sub cytoplasmic and nuclear membrane UL50 localised to the mucleur lamina in transiently transfected HEK293 cells	Miller et al 2010
UL51	Role in DNA packaging	Pan cytoplasmic	Pan nuclear UL51 localised to the nucleus in cells infected with a modified HCMV strain AD169 virus for conditional UL51 knockdown.	Glab et al 2009
UL52	Role in DNA packaging	Pan cytoplasmic	Cytoplasmic and nuclear Expresison was cytoplasmic at 2days. P.i. But at day 3 p.i. UL52HA showed nuclear staining in HCMVΔUL52 infection of a complementing cell line expresisng UL52A.	Borst et al 2008
UL53	Nuclear matrix protein Tegument protein Role of egress of capsids of capsids from the nucleus	Either nuclear or cytoplasmic	Both nuclear and cytoplasmic UL53 localised to the both the nucleus and cytoplasm in transienlty transfected COS7 cells 48 hours p.i. While In HCMV AD169 infected HLEF cells faint staining was observed throughought the nucleus at 48 hours p.i. And in ;arge cutoplasmic formations juxtaposed to the nuceus at 72hours p.i. UL53 localised tboth the nucleus and cytopalsm in transiently transfected HEK293 cells	Dal Monte et al 2002 Miller et al 2010
UL54	Catalytic subunit of DNA polymerase Inhibited by pyrophosphatae (e.g. Forscarnet), nucleaoside (e.g.ganciclovir) and certain non-	Pan-nuclear with some cytoplasmic expression	None identified	N/A
UL55	Virion glycoprotein B (gB) Component of gCI Involved in virus entry	Sub cytoplasmic	Sub cytoplasmic At 48hrs p.i. pUL55 colocalised with pUL71 in VAC in MRC5 fibroblasts infected with HCMV AD169 derived virus Perincuclear staining seen in transiently transfected COS7 cells and during HCMV-AD169 infected fibroblasts	Womack and Shenk 2010 Krzyzaniak et al 2007

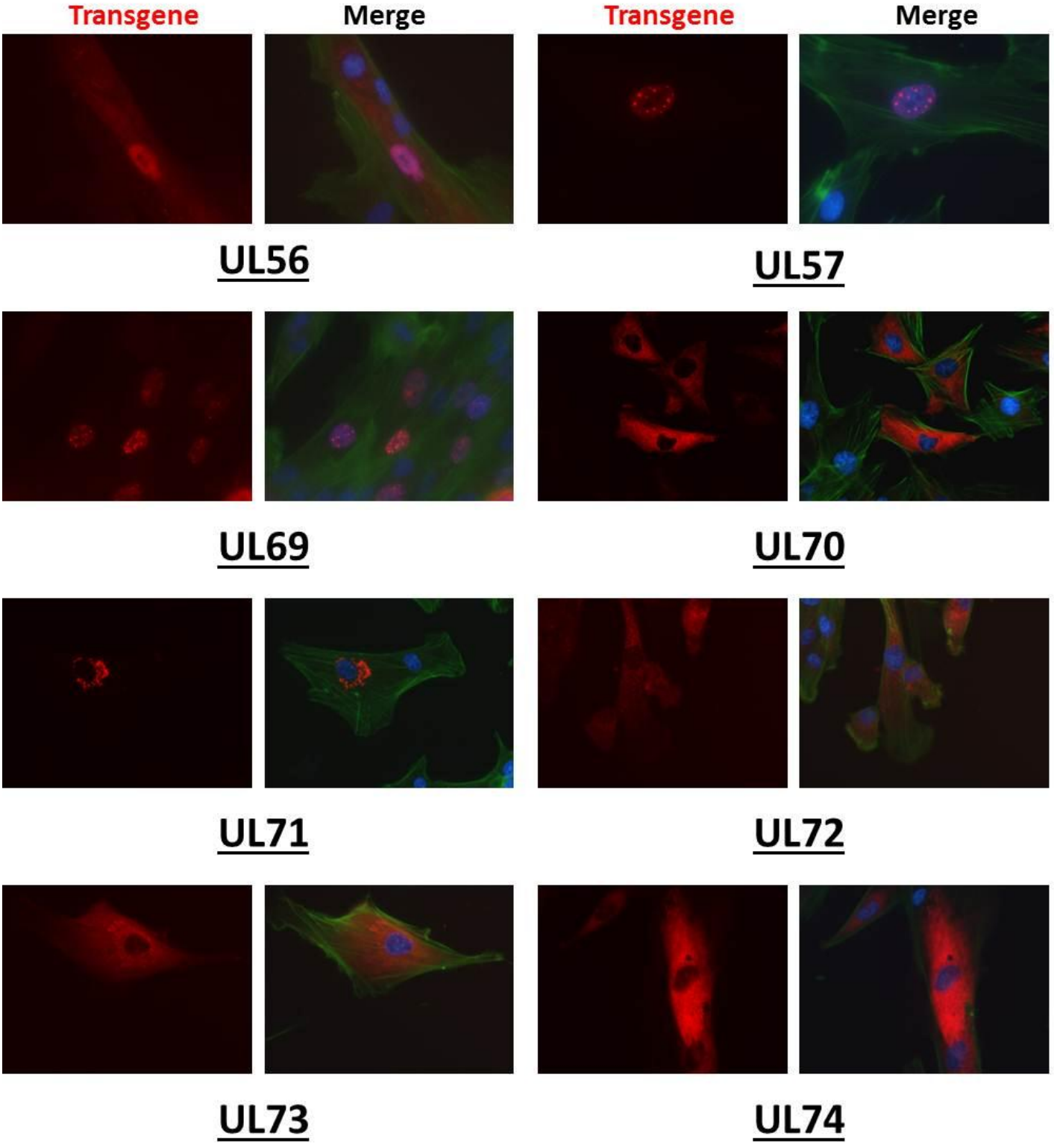


Figure 5.3. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL56-UL74.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.3. A comparison of transgene localisation to localisation patterns described in the literature, for UL456 - UL74. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
UL56	Putative subunit of terminase Binds to DNA binding packaging motif and exhibits nuclease actvity Involved in inhibition of Benzimidazole ribonucleosides and certain non-nucleoside compounds	Pan nuclear with some cytoplasmic expression	Predominanltly nuclear Detected using polyclonal Ab in AD169 infected fibrobalsts	 Bogner et al 1998
UL57	Single stranded DNA binding protein	Punctate nuclear	Punctate nuclear Punctate nuclear staining seen in HCMV AD169 infected HFFs Punctate nuclear staining associating with viral replication compartment in HCVM AD169 infected HFFs	 Tran et al 2009 Strang et al 2011
UL69	Regulatory protein Contibutes to cell cycle block Exhibits nucleocytoplasmic shuffling Tegument protein	Punctate nuclear	Punctate nuclear Punctate nuclear expression seen in transient transfection of 293T cells.	 Salsman et al 2008
UL70	Component of DNA helicase-primase complex; primase	Pan cytoplasmic	Pan cytoplasmic Pan cytoplasmic with some nuclear staining in plasmid transfected U373 and Hela cells. In U373 cells infected with HCMV, UL70 showed a predominantly nuclear location.	 Shen et al 2011
UL71	Putative tegument protein	Sub cytoplasmic	Sub cytoplasmic At 48hrs p.i. pUL71 colocalised with UL99 and UL55 in VAC in MRC5 fibroblasts infected with HCMV Ad169 derived virus	 Womack and Shenk 2010
UL72	Derived from deoxyuridine triphosphatase lacks catalytic residues and is enzymatically inactive	Pan cytomplasmic	Pan cytoplasmic In HCMV AD169 infected HELF cells, and in HELF cells transiently transfected with a UL72 expression vector, UL72 showed diffuse cytoplasmic expression pattern.	 Caposio et al 2004
UL73	Virion glycoprotein N (gN) Component of gCII	Sub cytoplasmic	Sub cytoplasmic Using sera against gM/gN in COS7 transfected cells and in HCMV AD169 infected cells the gM/gN complex showed sub cytoplasmic location	 Krzyzaniak et al 2007
UL74	Virion glycoprotein O (gO) Component of gCIII	Pan cytoplasmic	Sub cytoplasmic Human fibroblast cell infected with HCMV AD169 showed sub cytoplasmic staining at 6 days p.i. And colocalised with TGN38, a marker for vesicles of the TGN	 Theiler and Compton 2002

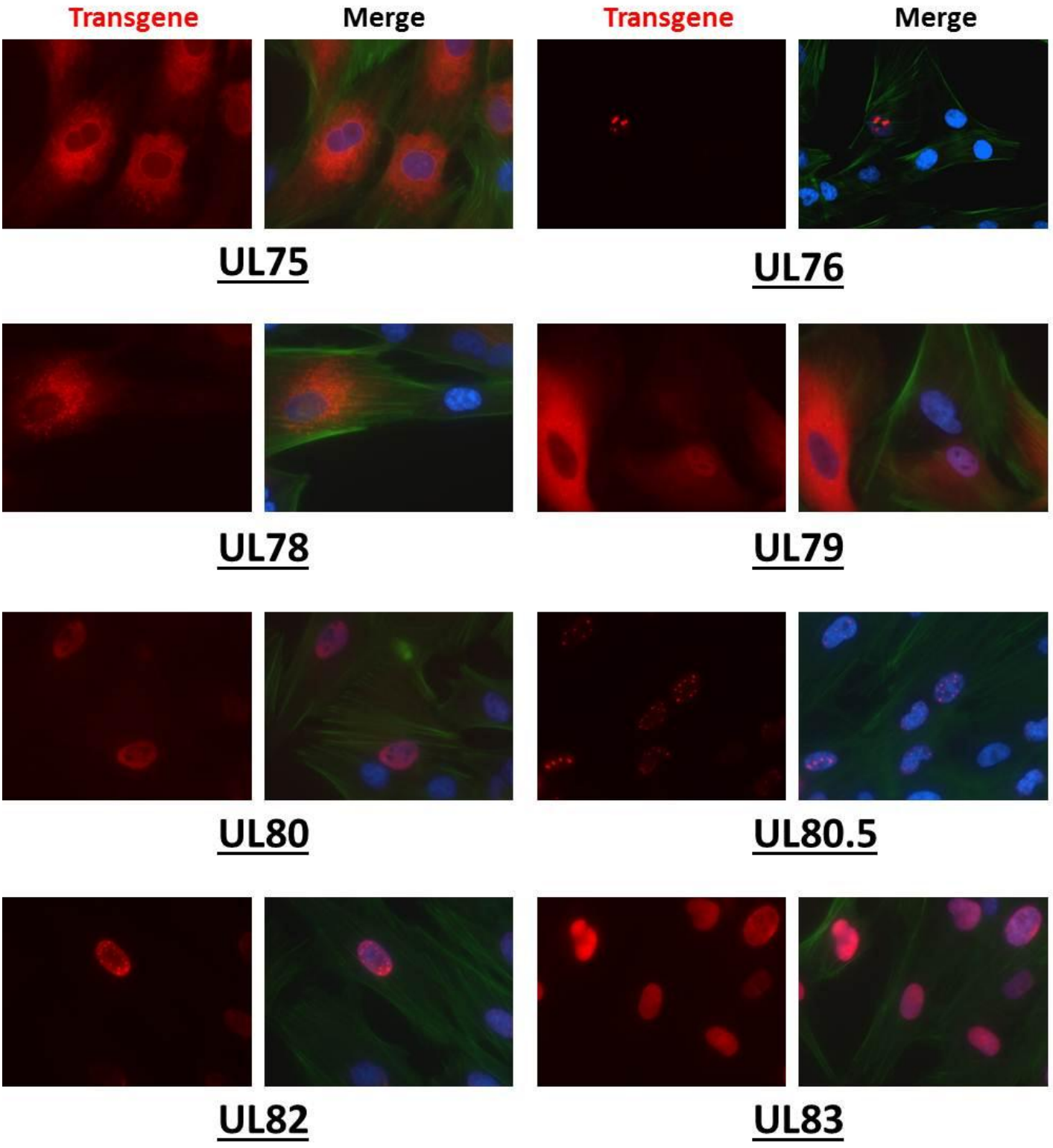


Figure 5.4. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL75-UL83.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.4. A comparison of transgene localisation to localisation patterns described in the literature, for UL75 - UL83. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
UL75	Virion glycoprotein H (gH) Component of gCIII Involved in virus entry	Sub cytoplasmic	Cytoplasmic Cytoplasmic expression seen in transient transfction of Cos7 cells.	Shimamura et al 2006
UL76	Virion-associated regulatory protein	Punctate nuclear	Punctate nuclear Punctate nuclear expression seen in transient transfection of 293T cells. pUL76 expressed in U373 MG, HEL and COS-1 cells all show the same sub-nuclear expression pattern	Salsman et al 2008 Voon-Kwan et al 2009 Wang et al 2000
UL78	GPCR family Putative chemokine receptor	Sub cytoplasmic	None identified	N/A
UL79	Conserved in *Gammaherpesvirinae*	Either nuclear or cytoplasmic	predominantly nuclear but also cytoplasmic. In a pUL79 expressing stable cell line (UL79HA), pUL79 formed primarily in the nucleus but also showed cytoplasmic staining. During AD169ΔUL79 infection of UL79HA fibroblasts UL79 co-localised to virus-induced intracellular structures.	Perng et al 2011
UL80	Protease (N-terminus) and minor scaffold protein	Pan-nuclear	Pan-nuclear Pan-nuclear expression in HF cells transfected with UL80-GFP construct	Ngyen et al 2008
UL80.5	Major capsid scaffold protien	Punctate nuclear	Punctate nuclear Punctate nuclear expression seen in transient transfection of 293T cells.	Salsman et al 2008
UL82	UL82 family tegument phosphoprotein (pp71; upper matrix protein) Transcriptional activator Targeted to ND10 Targets RB proteins for ubiquitin-independant proteasomal degradation	Punctate nuclear	Punctate nuclear and cytoplasmic pUL82 showed punctate nuclear staining in AD169 infected fibrobalsts at IE stages of infection. At 12, 24 adn 72 hours p.i. pUL82 showed cytoplasmic staining. Contransfection of UL82 with UL35 results in UL82 expression shifting from the cytoplams to the nucleus. Punctate nuclear expression seen in transient transfection of 293T cells.	Hensel et al 1996 Salsman et al 2011
UL83	Suppresses IFN response Lower matrix protein pp65	Pan nuclear	Pan nuclear ppUL83 expressed from RAd shows pan-nuclear staining. During AD169 infection ppUL83 forms punctate nuclear staining	Hwang and Kalejta 2009

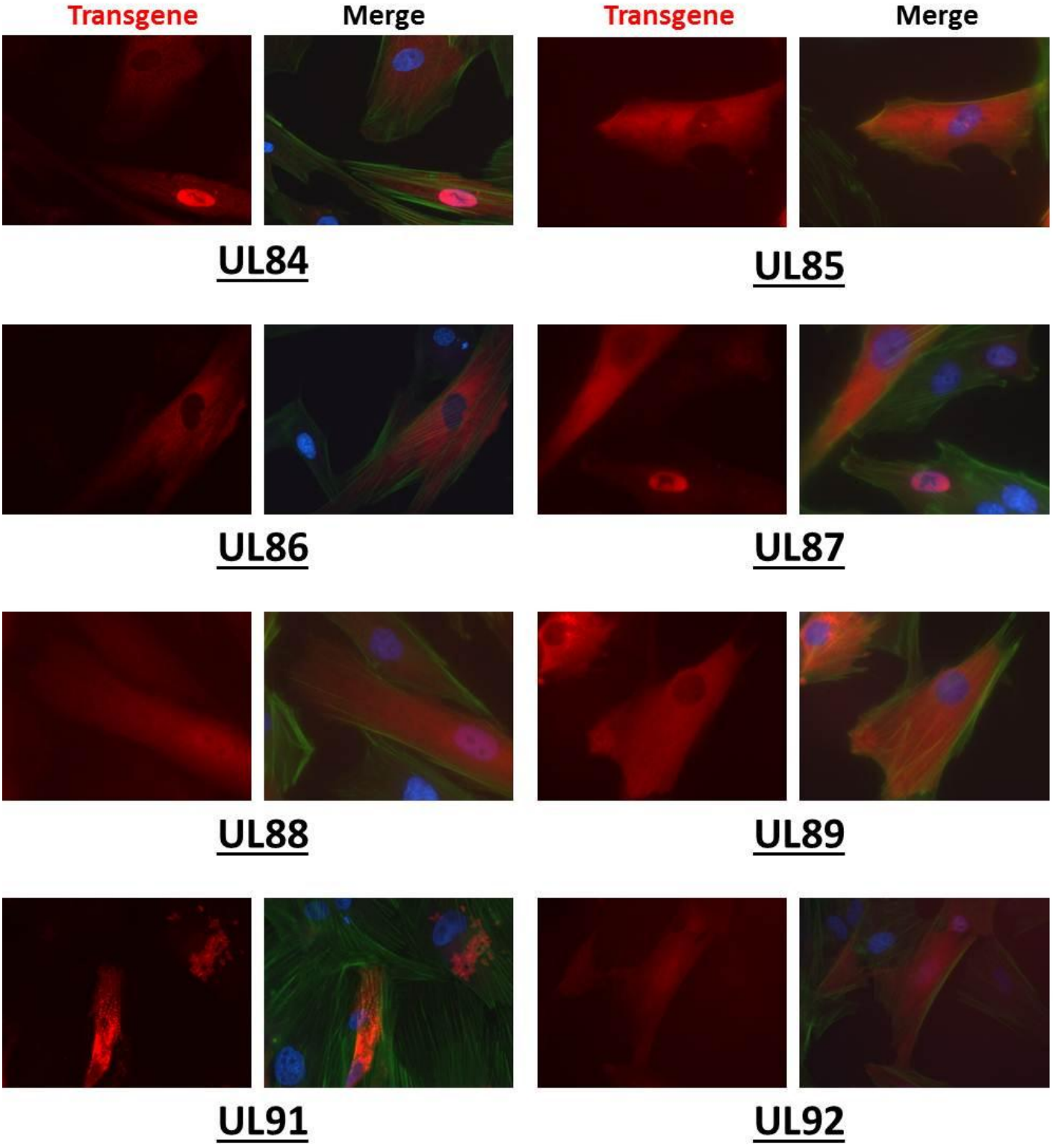


Figure 5.5. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL84-UL92.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.5. A comparison of transgene localisation to localisation patterns described in the literature, for UL84 - UL92. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
UL84	UL82 family Role in DNA replication Exhibits nucleocytoplasmic shuffling Binds to IE2 protein Transdominant inhibitor of IE transcription	Pan nuclear with some cytoplasmic expression	Pan nuclear/ punctate nuclear Forms punctate nuclear staining in the presence of IE1/IE2 or OriLyt, which does not form in the absence of IE1/IE2/OriLyt in cotransfected cells. Colocalised to vRC in AD169 infected cells	Xu et al 2002
UL85	Component of intercapsomeric triplexes in capsids (mCP)	Pan cytoplasmic	Pan cytoplasmic / Pan nuclear In AD169 infected fibroblasts UL85 expression was found in both the nucleus and cytoplasm	Adamo et al 2004
UL86	Major capsid protein, component of pentons and hexons (MCP)	Pan cytoplasmic	Pan cytoplasmic / Pan nuclear In cells infected with Vaccina virus expressing UL86 and also transfected Cos7 cells, UL86 was detected in the cytoplasm In AD169 infected fibroblasts UL86 expression was restricted to the nucleus	Plafker and gibson 1998 Lai and Britt 2003
UL87	Conserved in *Gammaherpesvirinae*	Either nuclear or cytoplasmic	None identified	N/A
UL88	Conserved in Gammaherpesvirinae Tegument protein	Pan cellular	None identified	N/A
UL89	Putative ATPase subunit of terminase Involved in inhibition by benzimidazole ribonucleosides and certain non nucleoside	Pan cytoplasmic	None identified	N/A
UL91	Positinally conserved in *Gammaherpesvirinae*	Either nuclear or cytoplasmic	None identified	N/A
UL92	Conserved in Gammaherpesvirinae	Pan cellular	None identified	N/A

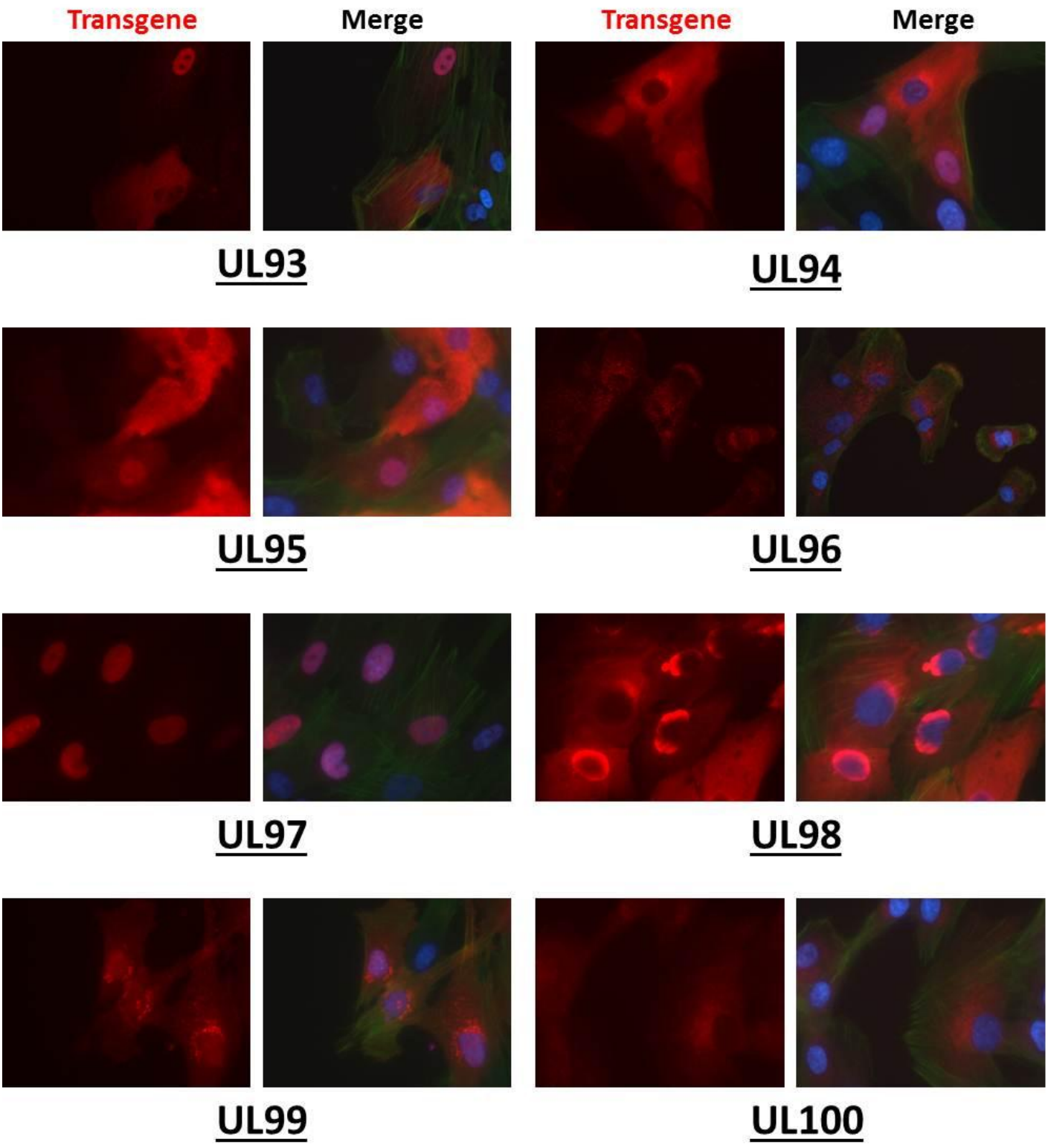


Figure 5.6. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL93-UL100.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.6. A comparison of transgene localisation to localisation patterns described in the literature, for UL93 - UL100. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
UL93	Role in DNA packaging Tegumnet protein	Either nuclear or cytoplasmic	None identified	N/A
UL94	Binds single stranded DNA (ssDNA) Tegumnet protein	Either nuclear or cytoplasmic	None identified	N/A
UL95	Conserved in *Gammaherpesvirinae*	Either nuclear or cytoplasmic	None identified	N/A
UL96	Tegument protein	Sub cytoplasmic	None identified	N/A
UL97	Roles in DNA synthesis, DNA packaging and nuclear egress Phosphorylates UL44 protein Serine-Threonine Kinase Tegument protein Phosphorylates ganciclovir and inhibited by maribavir	Pan nuclear	Pan nuclear Shows pan nuclear expresssion in Cos-7 cells transiently transfected with UL97 expression plamsid	Prichard et al 2005
UL98	Deoxyribose	Both cytompalsmic and sub-nculear	None identified	N/A
UL99	Myristylated tegument phosphoprotein (pp28)	Sub cytoplasmic	Sub cytoplasmic At 48hrs p.i. pUL99 colocalises with pUL71 in VAC In HCMV-AD169 or HCMV-AD169-UL99GFP infected cells, UL99 was found exclusively in the cytoplasm, in particular the perinuclear region	Womack and Shenk 2010 Moorman et al 2010
UL100	Virion envelope glycoprotein M (gM) Component of gCII	Sub cytoplasmic	Sub cytoplasmic During RAd-UL100 infection, pUL100 demonstrated mostly sub cytoplasmic perinuclear expression, although weak expression was seen throughout the cytoplasm.	Krzyzaniak et al 2007 Mach 2000

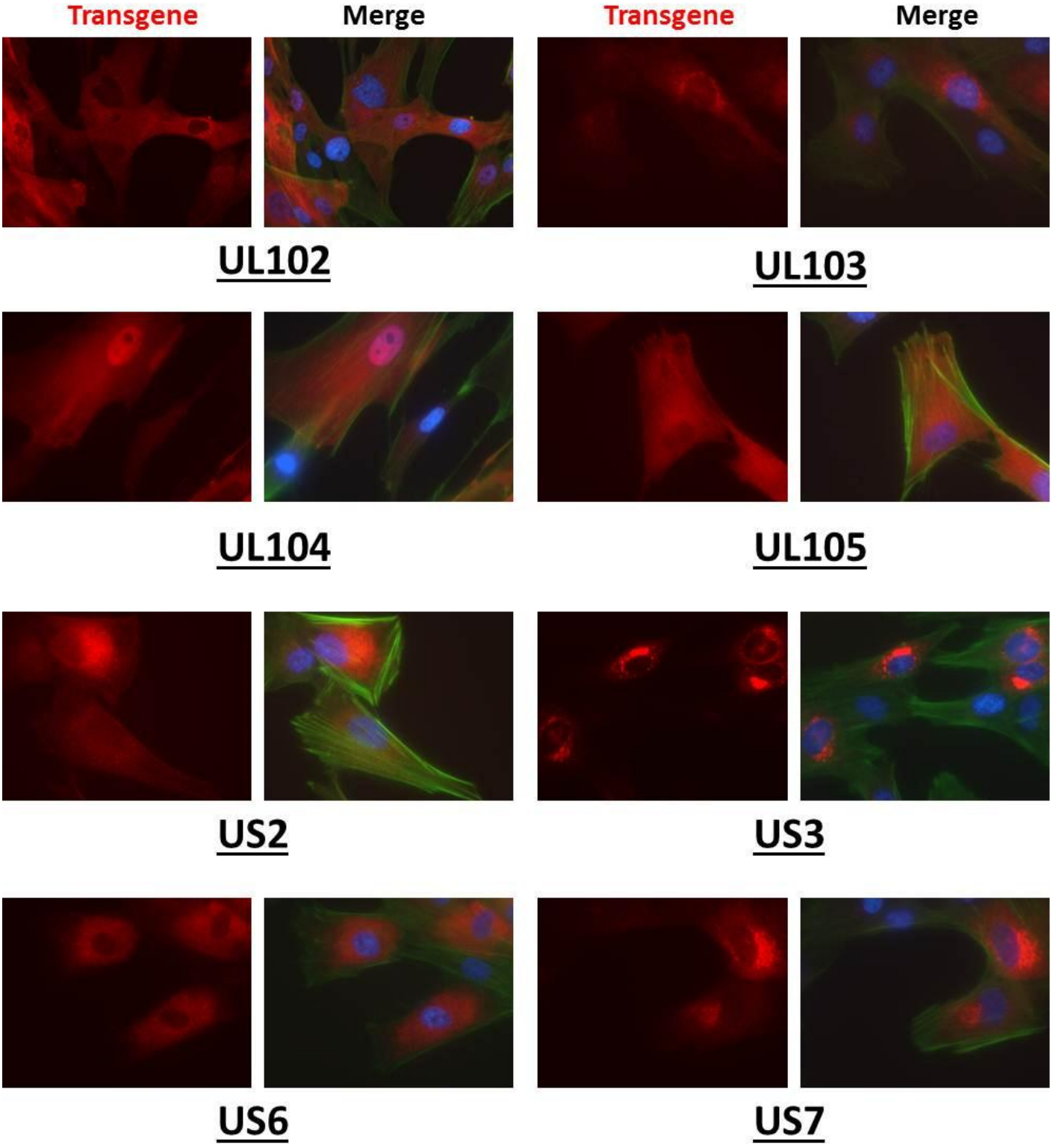


Figure 5.7. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL102-UL105 and US2-US7. HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.7. A comparison of transgene localisation to localisation patterns described in the literature, for UL102 - US7. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
UL102	Component of DNA helicase-primase complex	Pan cytoplasmic	None identified	N/A
UL103	Tegument protein	Sub cytoplasmic	Sub cytoplasmic Accumulates at juxtanuclear sites in uninfected and AD169 infected cells, colocalising with golgi resident protein markers	Ahlqvist and Mocarski 2011
UL104	Portal protein Possibly interacts with terminase	Pan-nuclear with some cytoplasmic expression	Pan-nuclear In AD169 infected fibroblasts pUL104 was diffuse throughout the nucleaus at 24hrs p.i. At 72 hours p.i. pUL104 appeard throughout the cytoplasm as small dots.	Dittmer et al 2005
UL105	Component of DNA helicase-primase complex; helicase	Pan cytoplasmic	None identified	N/A
US2	US2 family Membrane glycoprotein Causes selective degradation of MHC-I and MHC-II	Sub cytoplasmic	Sub cytoplasmic US2FLAG tagged protein colocalises with the ER in perinuclear location in HCMV-Towne infected HEL and EVT cells	Terauchi et al 2003
US3	US2 family Immediate early gene that inhibits processing and transport of MHC-I and MHC-II Membrane glycoprotein	Sub cytoplasmic	Sub cytoplasmic US3FLAG tagged protein forms small aggregates in the ER in the perinuclear region in HCMV-Towne infected HEL and EVT cells	Terauchi et al 2003
US6	US6 family Putative membrane glycoprotein Inhibits TAP mediated peptide transport	Sub cytoplasmic	Sub cytoplasmic US6FLAG tagged protein co localises with the ER in HCMV-Towne infected HEL and EVT cells	Terauchi et al 2003
US7	US6 family Membrane glycoprotein	Sub cytoplasmic	Pan cytoplasmic In HEC-1A cells infected with Ad vectors expressing US7, US7 was found to be diffuse trhoughout the cytoplasm and colocalised with ER markers calnexin and PDI.	Huber et al 2002

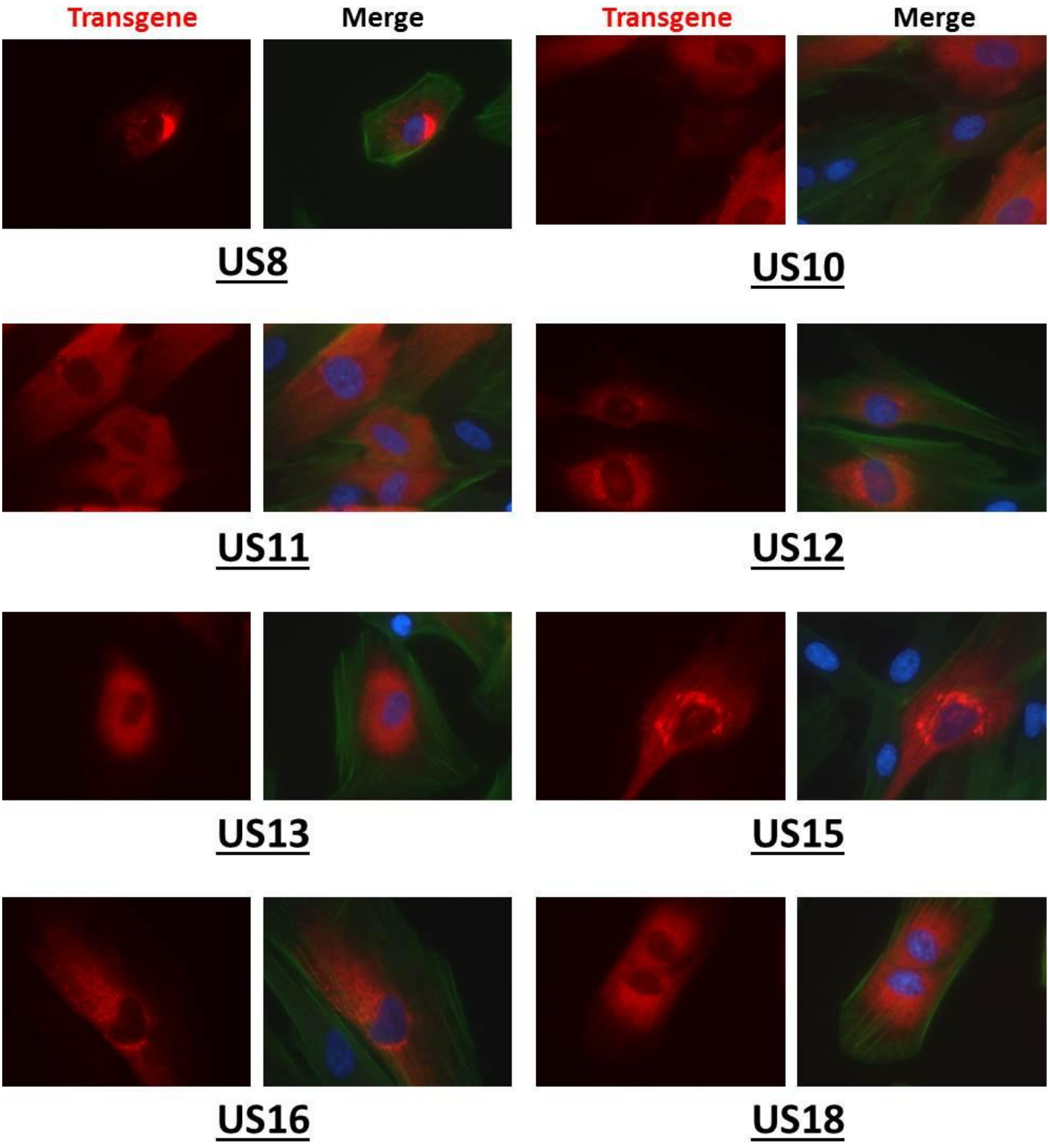


Figure 5.8. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: US8-US18.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.8. A comparison of transgene localisation to localisation patterns described in the literature, for US8 - US18. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
US8	US6 family Membrane glycoprotein	Sub cytoplasmic	Sub cytoplasmic In HEC-1A cells infected with Ad vectors expressing US8, US8 was found to colocalise with markers of the golgi apparatus and trans-golgi network (TGN).	Huber et al 2002
US10	US6 family Membrane glycoprotein	Pan cytoplasmic	Pan cytoplasmic In HEC-1A cells infected with Ad vectors expressing US10, US10 was found to be diffuse trhoughout the cytoplasm and colocalised with ER markers calnexin and PDI.	Huber et al 2002
US11	US6 family Membrane glycoprotein Involved in selective degradation of MHC-1	Pan cytoplasmic	Pan cytoplasmic Co localised to the ER in US11FLAG tagged HEL and EVT cells infected with HCMV TOWNE	Terauchi et al 2003
US12	US12 family Putative multiple transmembrane protien	Sub cytoplasmic	None identified	N/A
US13	US12 family Putative multiple transmembrane protien	Sub cytoplasmic	None identified	N/A
US15	US12 family Putative multiple transmembrane protien	Sub cytoplasmic	None identified	N/A
US16	US12 family Putative multiple transmembrane protien	Sub cytoplasmic	None identified	N/A
US18	US12 family Putative multiple transmembrane protien	Sub cytoplasmic	Cytopalsmic Cytoplasmic but enriched around the nucleus in AD169 infected HLF cells detected using rabbit polyclonal raised against N- and C-terminal fragments of US18	Das et al 2006

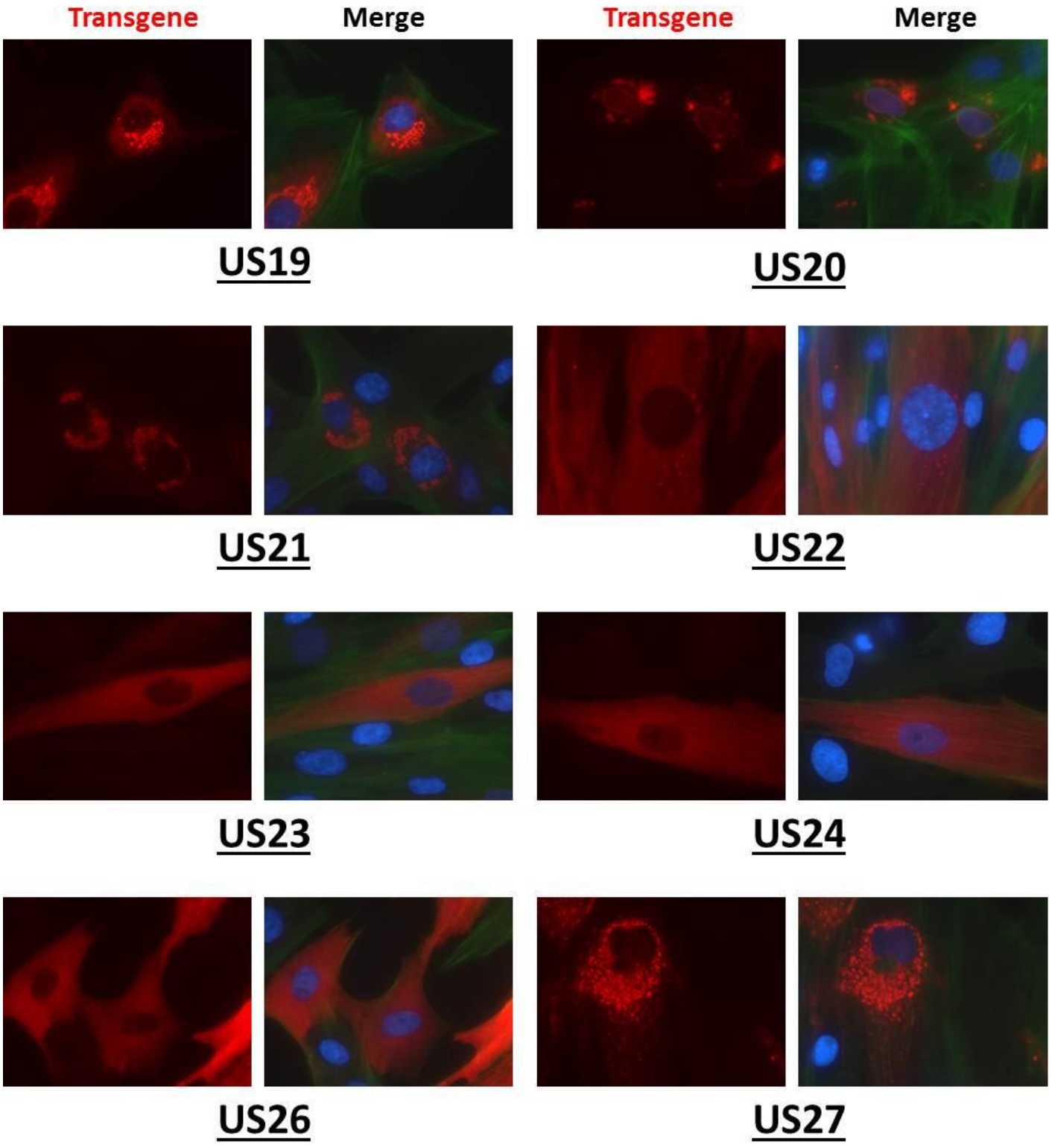


Figure 5.9. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: US19-US27.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.9. A comparison of transgene localisation to localisation patterns described in the literature, for US19 - US27. Gene functions as described in Davison et al (2007).

gene	Function	Noted Location	Location in literature	References
US19	US12 family Putative multiple transmembrane protien	Sub cytoplasmic	None identified	N/A
US20	US12 family Putative multiple transmembrane protien	Sub cytoplasmic	None identified	N/A
US21	US12 family Putative multiple transmembrane protien	Sub cytoplasmic	None identified	N/A
US22	US22 family Tegument protein Released from cells	Pan cytoplasmic	None identified	N/A
US23	US22 family	Pan cytoplasmic	None identified	N/A
US24	US22 family	Pan cytoplasmic	Pan cytoplasmic Pan cytoplasmic expression in HCMV-AD169 infected fibroblasts	Feng et al 2006
US26	US22 family	Pan cytoplasmic	None identified	N/A
US27	GPCR family Membrane glycoprotein	Sub cytoplasmic	Sub cytoplasmic Perinuclear sub cytoplasmic staining similar to US28 in cells infected with modified HCMV strain BFX with US27-FLAG	O'Connor and Shenk 2011

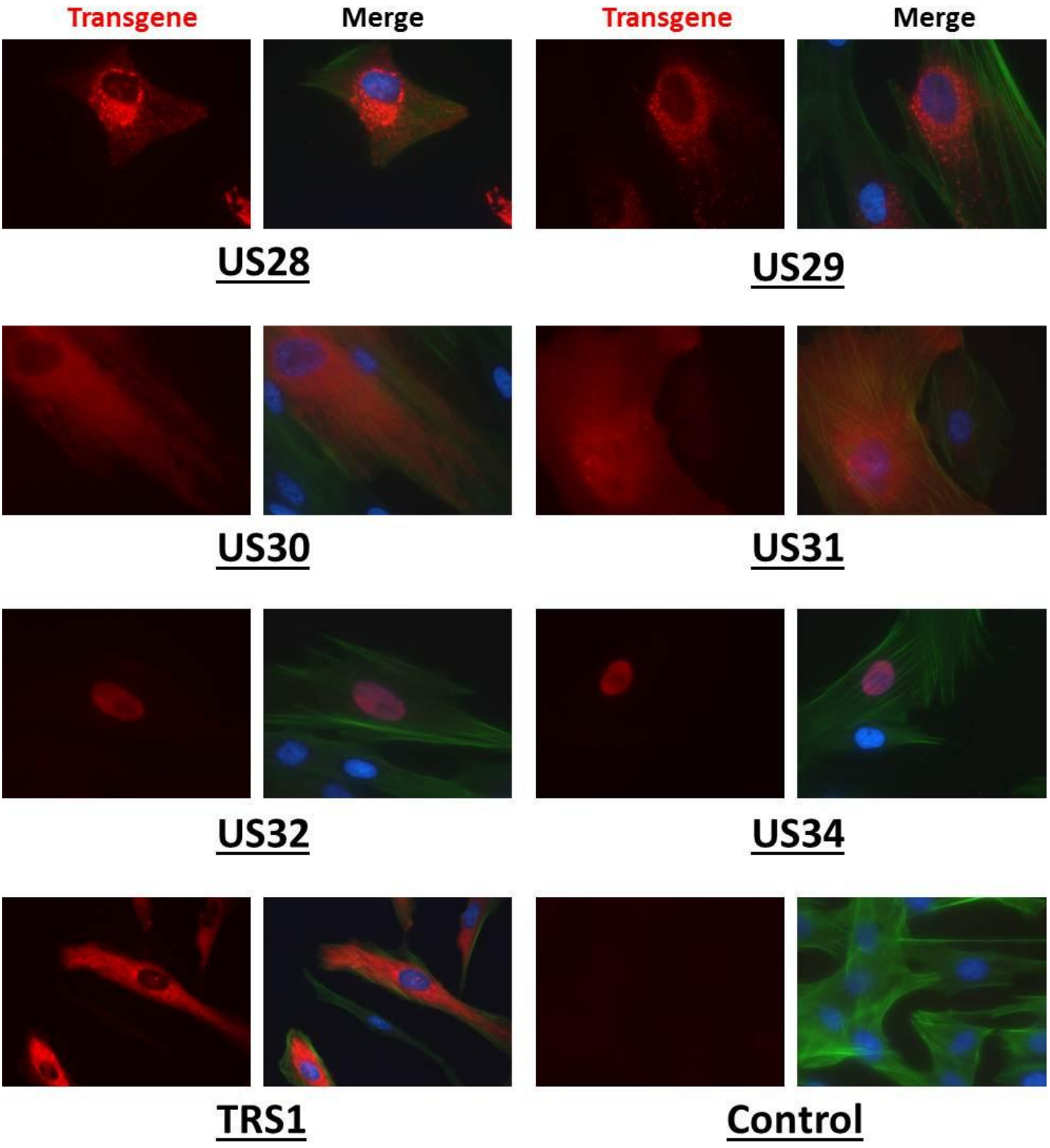


Figure 5.10. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: US28-TRS1
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is **individual transgene expression as detected with anti-V5 primary (red channel**). The second is a merged image consisting of **transgene expression (red channel), phalloidin-488 (green channel)** and **DAPI (Blue channel).**

Table 5.10. A comparison of transgene localisation to localisation patterns described in the literature, for US28 - TRS1. Gene functions as described in Davison et al (2007).

gene	Function	Location notes	Location in literature	References
US28	GPCR family Membrane protein Broad spectrum CC chemokine receptor Mediates cell migration	Sub cytoplasmic	Sub cytoplasmic Showed perinuclear staining in ibroblasts infected with RAd-US28	Streblow et al 2003
US29	Putative membrane glycoprotein	Sub cytoplasmic	None identified	N/A
US30	Putative membrane glycoprotein	Pan cytoplasmic	None identified	N/A
US31	US1 family Duplicated TT virus ORF2 motif	Pan cytoplasmic	None identified	N/A
US32	US1 family Duplicated TT virus ORF2 motif	Pan-nuclear	Punctate-nuclear In transfected 293T cells pUS32 showed punctate nuclear staining associating with PML bodies	Salsman et al 2008
US34	Putative secreted protein	Pan-nuclear	None identified	N/A
TRS1	US22 Immediate early transcriptional factor Involved in shutoff of host protein synthesis and capsid assembly Tegument protein	Pan cytoplasmic	Pan cytoplasmic Pan cytoplasmic staining in transfected MRC5 cells during HCMV-AD169 infection	Chaumorcel et al 2012

5.2. Categorisation of localisation

Proteins were categorised dependant on the type of localisation pattern that they produced. Categories were based on the descriptions found in Salsman et al (2008), which was a comparable study that looked at the subcellular localisation of HCMV proteins. In this study the author expressed multiple proteins from HSV-1, HCMV and EBV and categorised transgene localisation based on the expression pattern that they showed.

In this study there organellar counterstains were not used. Transgene expression that was outside the nucleus could not be distinguished between cytoplasm and orgenelles/vesicles etc. However, there are two distinct types of expression patterns that were outside the nucleus. Salsman et al (2008) referred to these as pan-cytoplasmic and sub-cytoplasmic. Although these definitions are not strictly true, as they do not take into account organelles, these definitions provided the best way of describing observed expression patterns. It was decided that these terms would be used to loosely describe categorisation, with an appreciation for their limitations. Therefore the definitions of categories are as follows:

I. **Pan cytoplasmic**: Refers to expression that appears throughout the cell, excluding the nucleus
II. **Sub-cytoplasmic:** Refers to expression that appears enriched in a particular cellular location outside of the nucleus which could be cytoplasmic, organellar, vesicular etc.
III. **Pan-nuclear:** Refers to diffuse staining throughout the nucleus, as determined by co-localisation with DAPI nuclear counter stain.
IV. **Punctate-nuclear:** Refers to expression that appeared enriched in a particular location within the nucleus.
V. **Miscellaneous:** Refers to expression that does not fall into any of the above categories. This predominantly refered to expression that was both nuclear and cytoplasmic.
VI. **Undetectable:** Where transgene expression could not be detected, or was below the threshold of detection.

Some proteins had more complex localisation categories and fell into multiple categories (e.g. UL54 is both cytoplasmic and nuclear but predominantly showed pan-nuclear expression). In this instance the localisation was recorded in the most relevant category.

Most viral proteins showed cytoplasmic expression with 29 categorised as sub-cytoplasmic and 21 as pan-cytoplasmic, figure 5.1. A further 10 transgenes showed pan-nuclear

A)

Localisation pattern	Number of proteins showing localisation pattern
Sub nuclear	5
Pan nuclear	10
Sub cytoplasmic	28
Pan cytoplasmic	21
Miscellaneous	13
Undetectable	6

B)

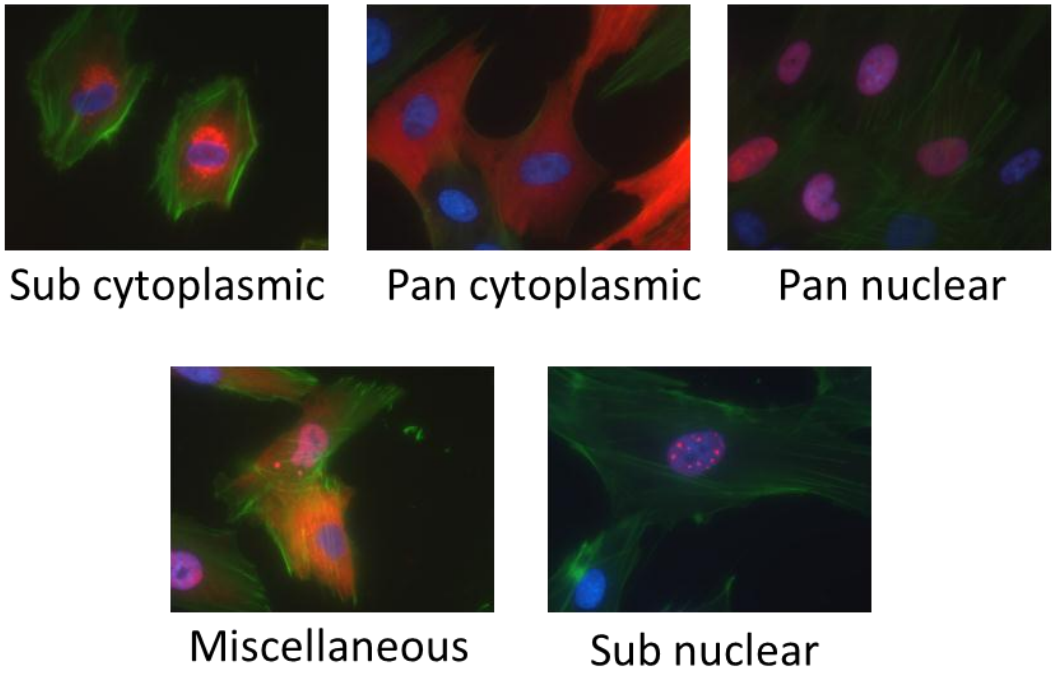


Figure 5.11. Summary of subcellular locations of HCMV transgene expression detected by IF.
HCMV transgenes were expressed from a RAd in HFFF-CARs. The localisation patterns were characterised into five types of subcellular localisation. (A) Summary table indicating the localisations of cytomegalovirus proteins expressed in infected HFFF as determined by IF detection of the V5 epitope tag. (B) IF images of transgenes representative of the five subcellular localisation categories. These include UL42 sub-cytoplasmic, US26, pan-cytoplasmic, UL97 pan-nuclear, UL49 miscellaneous, and UL57 sub-nuclear.

staining and 6 showed punctuate-nuclear staining. 13 were categorised as miscellaneous and 9 were undetectable.

5.3. Comparison of protein localisation to the literature: Differences in localisation

In order to determine whether transgene expression from the RAd-HCMV-ORF library correlated with findings of others; a literature search was carried out for all transgenes, tables 5.1 – 5.10.

A total of 43 out of 79 transgenes had localisations previously described in the literature. Of the 43, our findings correlated with previous literature descriptions for 29, while 14 had partial matches or mismatches. Localisation data could not be found in literature for the remaining 36 transgenes.

The 14 transgenes that had either partial matches (7) or mismatches (7) to the literature were reviewed in further detail.

5.3.1. Genes that showed partial matches with descriptions in the literature

There were a total of six proteins that demonstrated partial matches to descriptions identified in the current literature. These were UL44, UL48A, UL82, UL8, UL84, UL86 and UL98.

UL44

UL44 is a processivity subunit of DNA polymerase (ICP36) and here showed pan-nuclear staining (Ertl and Powell, 1992), Figure 5.1, table 5.1. A literature search showed UL44 to be pan-nuclear when expressed in isolation from an expression plasmid, but was shown to localise with IE2 in punctuate nuclear staining in HCMV-AD169 infected HFFF cells which corresponded to the periphery of viral replication compartments (vRC) (Sourvinos et al., 2007). Phosphonoformic acid is a potent inhibitor of viral DNA synthesis. In HCMV AD169 infected fibroblasts, treatment with phosphonoformic acid resulted in

dispersal of UL44 throughout the nucleus (pan-nuclear). When phosphonofomric acid was washed from the cells, viral DNA synthesis was restored, and the punctuate nuclear staining was re-established (Strang et al., 2011).

Expression UL44 from RAd-HCMV-ORF library was therefore recorded as a partial match as these observations indicate that when HCMV DNA replication is inhibited UL44 formed pan nuclear staining, which correlated with the expression pattern seen during this screen. However, when HCMV is undergoing active viral DNA replication, in the presence of IE2, UL44 formed punctuate nuclear staining corresponding to the vRC.

UL48A and UL86

UL48A and **UL86** interact together during capsid assembly (Gibson et al., 1996b, Chee et al., 1989). UL48A is referred to as the smallest capsid proteins (SCP) and is located on the tips of hexons, while UL86 is referred to as the major capsid protein (MCP). The interaction between UL86 and UL48A is dependent on two linear sequences in pUL48A (Lai and Britt, 2003). In this study both proteins showed diffuse staining throughout the cell excluding the nucleus. Studies using murine mAbs specific for pUL86 or pUL48A in human fibroblasts infected for 5 days with HCMV strain AD169 showed expression from both proteins was localised to the nucleus where signals from both proteins co-localised (Lai and Britt, 2003). When the HCMV capsid proteins were expressed in cells infected with a recombinant vaccinia virus expressing individual capsid proteins, both UL86 and UL48A localised to the cytoplasm of infected cells. Further studies in transfected Cos7 cells (an African green monkey fibroblast cell line) showed UL48A was detected in both cytoplasmic and nuclear compartments. In contrast UL86 was localised only to the cytoplasm in transfected cells. When both plasmids were co-transfected into Cos7 cells nearly complete cytoplasmic co-localisation was observed (Lai and Britt, 2003). The nuclear localisation signals of pUL80 (mCP) was required to translocate pUL86 to the nucleus (Plafker and Gibson, 1998). It is therefore likely that UL86 and UL48A require an interacting protein to translocate to the nucleus.

Expression of UL48A and UL86 independently from RAd-HCMV-ORF library were recorded as partial matches. Both UL48A and UL86 displayed cytoplasmic localisation when individually expressed from either a recombinant vaccinia viral vector or plasmid

transfected COS7 cells, similar to expression patterns identified in this study, FIgrues 5.1 and 5.5, tables 5.1 and 5.5. However, during productive HCMV AD169 infection both UL48A and UL86 were found to be expressed in the nucleus.

UL82

UL82 (pp71) is a transcriptional activator targeted to ND10/PML bodies, known sites of DNA replication, where UL82 interacts with hDaxx to regulate IE expression and viral replication (Hofmann et al., 2002, Ishov et al., 2002). UL82 is also capable of targeting Rb proteins for ubiquitin-independent proteosomal degradation and, along with UL69, is capable of imposing a G1/S-like block that deregulates the expression of many cell cycle related proteins (Kalejta and Shenk, 2003, Kalejta et al., 2003). In this observation expression of UL82 from RAd showed a punctuate-nuclear expression pattern, Figure 5.4, table 5.4. A literature review indentified a time course experiment of pUL82 expression using polyclonal Ab-UL89 in HCMV strain AD169 infected fibroblasts (Hensel et al., 1996). At IE stages of infection pUL82 showed punctuate nuclear staining. At 12, 24 and 72 hrs p.i pUL82 showed cytoplasmic staining (Hensel et al., 1996). Salsman et al (2011) showed that expression of UL82 in transfected U2OS human osteosarcoma cells was nuclear but following co-transfection with a plasmid encoding UL35a, UL82 expression shifted to the cytoplasm.

Expression of UL82 from RAd-HCMV-ORF library was therefore recorded as a partial match as the punctate nuclear expression pattern observed matched expression patterns described in the literature at IE times p.i. but not at E and L times p.i. This is likely due to the interaction of UL82 with UL35a during productive HCMV infection.

UL83

UL83 is known to inhibit the expression of IFN-inducible cellular genes and has been shown to be important for efficient induction of transcription from the IE promoter (Cristea et al., 2010, Browne and Shenk, 2003). In this observation expression of UL83 from RAd showed a pan-nuclear expression pattern, Figure 5.4, table 5.4. A literature search identified UL83 to form large nuclear aggregates in uninfected Cos7 cells

transfected with ppUL83-GFP which possibly formed as a result of dimerisation of pUL83 (Prichard et al., 2005). Coexpression of ppUL83-GFP and ppUL97-V5 eliminated the formation of nuclear ppUL83-GFP aggregates and instead resulted in diffuse nuclear staining of ppUL83-GFP (Prichard et al., 2005). Hwang and Kalejta (2009) showed that ppUL83 expressed from a RAd showed diffuse nuclear staining, which correlated with expression seen in this study, but during HCMV AD169 infection of telomerase immortalised HFFF's formed more punctuate nuclear staining (Hwang and Kalejta, 2009).

Expression of UL83 from RAd-HCMV-ORF library was therefore recorded as a partial match as the pan nuclear expression pattern observed matched expression patterns described by Hwang and Kalejta (2009) who also expressed UL83 from RAd. However, expression did not match that seen by Prichard et al (2005) or expression patterns seen by Hwang and Kalejta (2009) during HCMV AD169 infection.

UL84

UL84 is required for origin-dependant DNA replication, is a transdominant inhibitor of IE transcription and interacts with IE2 in lytically infected cells (Gebert et al., 1997, Sarisky and Hayward, 1996). Expression from RAd-HCMV-ORF library showed UL84 formed predominantly pan nuclear expression patterns with some cytoplasmic staining, Figure 5.5., table 5.5. A literature search found UL84 to show pan nuclear expression patterns in Cos7 cells transiently transfected with UL84 expression constructs (Xu et al., 2002). In UL84-EGFP transfected Cos7 cells co-transfected with all HCMV replication proteins and oriLyt, UL84-EGFP formed punctate nuclear staining in the presence of IE1/IE2 or OriLyt, which did not form in the absence of IE1/IE2/OriLyt (Xu et al., 2002). In the presence of the HCMV replication proteins and oriLyt, UL84 co-localised with UL44 and IE2 in the vRC (Xu et al., 2002).

Expression of UL84 from RAd-HCMV-ORF library was therefore recorded as a partial match as the pan nuclear expression pattern observed matched expression patterns described in the absence of IE1/IE2 or OriLyt but not during HCMV infection when those proteins are present during viral replication.

UL98

UL98, a deoxyribonuclease, is positionally conserved in gammaherpesvirinae (Sheaffer et al., 1997, Davison and Bhella, 2007). UL98 is unusual in that it showed pan-cytoplasmic expression and sub-nuclear expression, with only the periphery of nuclei appeared to show UL98 expression, Figure 5.6, table 5.6. A literature revealed that in HCMV-AD169 infected fibroblasts UL98 showed a pan-nuclear expression pattern, although UL98 did appear to be slightly enriched towards the periphery of the nucleus (Adamo et al., 2004). The authors did not describe any cytoplasmic staining and therefore expression of UL98 form RAd-HCMV-ORF library was recorded as not a partial match.

5.3.2. Genes that did not match descriptions in the literature

There were a total of seven proteins that were classified as mismatches when compared to expression patterns identified in the current literature. These were UL51, UL52, UL53, UL79, UL85, UL87, UL95 and US32.

UL51

UL51 is an essential HCMV protein that plays a role in DNA packaging and here showed pan cytoplasmic expression, Figure 5.2, table 5.2. Glab et al (2009) generated virus mutants that allowed for the conditional and reversible disruption of ddFKBP fusion proteins regulated by the synthetic ligand shield. The conditional knockdown allowed the study of essential herpesvirus proteins such as UL51. UL51 localised to the nucleus in cells infected with a modified HCMV strain AD169 (HCMV- ddFKBP-HAUL51) virus for conditional UL51 knockdown.

The expression of UL51 from RAd-HCMV-ORF library was therefore recorded as a mismatch.

UL52

UL52 has a role in DNA packaging and in this study showed pan cytoplasmic expression, Figure 5.2., table 5.2. Recombinant HCMV's encoding N and C terminally HA tagged

UL52 were constructed in HCMV strain AD169 (Borst et al., 2008).At day 2p.i. some N-terminally tagged pUL52HA was localised to the cytoplasm, but a substantial amount was evenly distributed throughout the nucleus. From day 3 p.i. onwards, the majority of pUL52HA was present in the nucleus. In contrast HCMV containing a C-terminally tagged UL52 protein did not support growth of HCMV and did not produce viral progeny when the purified BAC was transfected into fibroblasts, indicating that the pUL52 C-terminus was crucial for ULl52 function and that a C-terminal tag disrupted this function. In RPE cells transfected with both the C- and N-terminally tagged UL52 HCMV-BACs, N-terminal pUL52HA showed the expected nuclear location of pUL52. While in cells transfected with C-terminal pUL52HA, pUL52 was distributed throughout the cells with a significant proportion being present in the cytoplasm. The authors concluded that the C terminus of pUL52 was crucial for its correct nuclear localisation, which in turn seemed to be required for the essential role of UL52 in the HCMV replication cycle.

The RAd-HCMV-ORF contained C-terminally tagged transgenes. Expression from RAd matched the cytoplasmic expression seen by C-terminally tagged UL52 see by Glab et al (2009). However, expression differed from the nuclear location of N-terminally tagged UL52. The location of N-terminally tagged UL52 is likely to be the correct functional location as this construct has no effect on growth, while C-terminally tagged UL52 is unable to produce viral progeny. The expression of UL52 from RAd-HCMV-ORF library was therefore recorded as a mismatch.

UL53

UL53, a tegument and nuclear matrix protein that has a role in capsid egress from the nucleus (Dal Monte et al., 2002). In this study UL53 showed either pan nuclear or pan cytoplasmic staining in different cells infected with RAd expressing UL53, Figure 5.2, table 5.2. A literature review showed UL53 to have several expression pattern attributed to UL53. In HCMV AD169 infected HELF cells pUL53 showed faint staining dispersed throughout the nucleus at 48hrs p.i. (Dal Monte et al., 2002). At 72 hours p.i. the intensity of the signal increased as was associated with large cytoplasmic formations thought to represent vACs (Dal Monte et al., 2002). In transfected cells, pUL53 is described as being predominantly nuclear (Miller et al., 2010, Dal Monte et al., 2002). Coexpresssion of

UL50 and UL53 induced to relocalisation of UL53 from the nuclear to the endoplasmic reticulum and to the nuclear rim (Miller et al., 2010).

Expression of UL53 from RAd-HCMV-ORF library was therefore recorded as a mismatch.

UL85

UL85 is known as the minor capsid protein (mCP) and is a component of intercapsomeric capsids in triplexes(Baldick and Shenk, 1996). Here UL85 expressed from RAd showed predominantly pan cytoplasmic expression, Figure 5.5., table 5.5. A literature search described that pUL85 in HCMV-AD169 infected fibroblasts, showed both pan nuclear and pan cytoplasmic expression (Adamo et al., 2004). Expression of UL85 from RAd-HCMV-ORF library was therefore recorded as a mismatch as there was no pan nuclear expression of UL85.

UL87 and UL95

As yet, **UL87** and **UL95** have no identified function but both have been described as being conserved in gammaherpesvirinae. In this study both proteins showed either pan nuclear or pan cytoplasmic expression in different cells, Figures 5.5. and 5.6, tables 5.5. and 5.6. A literature search showed that fibroblasts infected with a recombinant HCMV TOWNE (UL95HA UL87myc), showed punctuate nuclear staining for both proteins (Isomura et al., 2011). UL95 and UL87 did not appear to co-localise as UL95 foci were distinct from UL87 foci and 1 day p.i. (Isomura et al., 2011). However, at 2 and 3 days p.i. both UL95 and UL87 were recruited into distinct foci where they co-localised (Isomura et al., 2011). Both UL95 and UL87 showed co-localisation with UL44 to vRC (Isomura et al., 2011).

Expression of UL87 and UL95 independently from RAd-HCMV-ORF library were recorded as mismatches as both of these proteins did not show punctuate-nuclear staining when expressed from RAd.

US32

US32 is a member of the US1 family and contains a duplicated TT virus ORF2 motif. In this study US32 showed a pan nuclear expression pattern, Figure 5.10, table 5.10. A literature search showed that in transfected 293T cells pUS32 showed punctuate-nuclear staining associated with PML bodies, sites of virus replication (Salsman et al., 2008). Although nuclear, this does not match expression of US32 from RAd-HCMV-ORF library and was recorded as not a match.

5.3.3. Potential causes for difference in localisation described in the literature compared to localisation in this study.

A total of 43 transgenes had localisations described in the literature. Of those 43, 29 had localisations that were consistent with results observed in this thesis. The remaining 14 proteins had expression patterns that differed from those described in the literature. Differences in localisation could be due to a number of reasons:

1) The transgene requires other HCMV proteins for correct function/location.

During HCMV infection, proteins interact to recruit additional proteins to cellular locations appropriate for their function. The most notable of these are proteins involved in capsid assembly which demonstrate predominantly nuclear location during viral infection, corresponding to the site where capsids are assembled. It is therefore possible that a negative hit could be achieved when the RAd-HCMV-ORF library is used in a functional screen due to the lack of an interacting protein.

2) Time point visualised post infection

This is particularly important when proteins are looked at in the context of viral infection. At IE timepoints p.i. proteins might show one type of expression pattern and a different localisation pattern at a later timepoints. This is likely due to other interacting HCMV proteins which only become expressed at later timepoints post infection. An example of

this is UL82, which is described as showing a punctate nuclear localisation pattern at IE p.i. but demonstrates cytoplasmic expression patterns at E and L timepoints p.i.

3) Potential transgene overexpression

All transgenes in this study are driven from the HCMV MIE promoter, which is one of the strongest promoters in mammalian biology. Because of the strength of the MIE promoter it is possible that the natural site of protein localisation or its interactions with chaperones may get saturated and mis-localisation of the protein may occur. In order to address this the experimental protocol could be amended to use reduced viral tires used and reduce incubation times and visualise transgene expression earlier than 3 days p.i.. Additionally using a weaker promoter might result in a more representative expression pattern for some proteins.

4) C-terminal tag prevents correct protein function/location

In this study both RAd-UL51 and RAd-UL52 showed cytoplasmic expression, but during HCMV infection these show a nuclear location. A C terminally tagged UL52 resulted in atypical pan cytoplasmic expression as the tag prevented the correct cellular location by blocking the nuclear localisation signal. Therefore adding a tag may alter a protein's function and location. To address this problem the transgene could be expressed without a tag and an antibody produced to verify its expression and location.

5) Cell line used

This particular screen was carried out in HFFF-hCARs. However, many different cell types have been used throughout the literature to express HCMV genes including human fibroblasts, such as HFFF's, African Green Monkey fibroblasts, such as Cos7 cells, and human embryonic kidney cells, such as HEK 293T cells. Proteins could localise to different cellular compartments depending on the cell line they are expressed in. If for example, a HCMV protein interacts with a cellular protein, that protein might be expressed

differently in different cell type or not at all. Human fibroblasts are permissive to HCMV *in-vivo* and *in-vitro* and so these provide a physiologically relevant cell line for study.

6) Different tags could results in different locations

The RAd-HCMV-ORF library uses a V5 tag. Many other tags have been used in the literature. For example; myc, FLAG, HA, proteinA and EGFP have all been used. Large tags, such as EGFP, could result in atypical location or aggregate formation if overexpressed. In order to avoid this possibility untagged protein could be expressed and detected with a specific monoclonal antibody.

7) Method of transgene delivery

The method of transgene delivery varies between studies. Many different methods have been used including DNA plasmids, RAd, recombinant HCMVs and recombinant vaccinia viruses, which may have an impact on the resultant protein. Ideally transgenes would be delivered using multiple vectors to confirm the same expression pattern was observed.

8) Different viral sequences used

All sequences in the RAd-HCMV-ORF library are derived from the BAC clone of HCMV-strain Merlin. A variety of other HCMV strains have been used in the literature, with HCMV strains AD169 and Towne being the most common. The genomes of HCMV trains AD169 and Towne are known to differ significantly to strain Merlin due to the tendency of HCMV to acquire genetic mutation over prolonged *in-vitro* passage. It is possible that HCMV strains could have acquired a mutation in a localisation signal which would affect localisation. Therefore protein localisation could vary between viral strains.

9) Types of detection reagents

Monoclonal or polyclonal antibodies were used to either detect the protein directly or to recognise an epitope tag. Epitope tags could potentially be cleaved from the mature protein and consequently the expression pattern would not be indicative of transgene expression. Additionally polyclonal antibodies could be non-specific and could be detecting other proteins. Monoclonal antibodies raised against the transgene of interest would produce a more robust result.

5.4. Undetectable proteins

The localisation of six viral proteins could not be determined, UL77, US1, US9, US14, US17 and US34A. Four of these have previous expression patterns identified in the literature, table 5.11. UL77 is described as pan-nuclear and US9 is described as pan-cytoplasmic (GlaB et al., 2009, Mandic et al., 2009, Huber et al., 2002). US14 is described as having granular staining throughout the cytoplasm, while US17 is described as being pan-nuclear during HCMV strain AD169 infection (Das et al., 2006).

There are several possibilities as to why expression could not be determined for these proteins. As transgene expression was detected using an antibody against the V5 tag. It is possible that with the anti-V5 mAb there was an inability to detect the V5 tag resulting in the transgene being undetectable. This could have been due to:

1) **No transgene expression from the vector**

2) **Steric hindrance** of the V5 tag due to protein structure so the V5 tag is 'not seen'.

3) **Homo- or hetero-dimerisation** of the transgene with itself or a cellular protein making the V5 tag inaccessible.

4) **Cleavage** of the C-terminal V5 tag.

5) **Degradation** of the transgene product and the tag before detection.

Table 5.11 List of Transgenes that were undetectable by immunofluorescence when expressed from recombinant adenoviral vector

gene	Function	Location notes	Location in literature	References
UL77	Role in DNA packaging	Undetectable	Pan nuclear in cells infected with a modified HCMV strain AD169 virus for conditional UL77 knockdown.	Glab et al 2009
US1	US1 family Duplicated TT virus ORF2motif	Undetectable	None identified	N/A
US9	US6 family Membrane glycoprotein	Undetectable	Pan cytoplasmic GFPUS9-Toledo infected HEK293, Hela and HF cells, US9-GFP distributed to both the ER and Mitochondria In HEC-1A cells infected with Ad vectors expressing US9, US9 was found to colocalise with ER markers calnexin and PDI.	Mandic et al 2009 Huber et al 2002
US14	US12 family Puatative membrane glycoprotein	Undetectable	Pan cytoplasmic Uniform granular staining throughout the cytoplasm in AD169 infected HLF cells detected using rabbit polyclonal raised against N- and C-terminal fragments of US14	Das et al 2006
US17	US12 family Puatative membrane glycoprotein	Undetectable	Pan nuclear colocalises to infected cells nculei in AD169 infected HLF cells detected using rabbit polyclonal raised against N- and C-terminal fragments of US17	Das et al 2006
US34A	Putative membrane glycoprotein	Undetectable	None identified	N/A

Antibodies against these specific ORF products would need to be generated to definitively determine whether a protein product is produced from these expression vectors.

5.5. Conclusion

The majority of transgenes (79 out of 85) from the RAd-HCMV-ORF library could be detected using reagents against the V5 epitope. A literature review allowed expression patterns for 43 of these transgenes to be compared to our observed expression patterns. 29 correlated, while 7 showed partial matches and 7 mismatches when compared to the current literature. Differences in expression patterns could be due to a number of reasons, with the most notable being differences in the timepoint expression was visualised post infections or whether the transgene was expressed in isolation from a vector or expressed in the context of viral infection. Some discrepancies would be expected with localisations determined in the context of viral infection since interactions with other viral proteins could alter location. For such genes it is likely that additional viral genes interact to traffic proteins to their alternate cellular location to carry out function.

The RAd-HCMV-ORF library was concluded to be suitable for the large scale screening for HCMV genes involved in the down-regulation of CD112 as most proteins appeared to show locations relevant to their function and in agreement with the literature. However, these results indicate that the interplay between HCMV genes can affect their subcellular localisation and likely their function throughout infection and these should be considered in the context of screening for function.

Finally genes were grouped into categories based on their expression patterns. These expression patterns appear to tie in with function and perhaps could be used to narrow down functional screening in the future. For example if screening for a protein thought to be involved in transcriptional regulation then it would be logical to screen those proteins that demonstrated pan- or sub-nuclear expression patterns as a priority over those that show pan-cytoplasmic expression patterns.

6.0. SCREENING OF THE RECOMBINANT ADENOVIRUS LIBRARY FOR CD112 DOWN-REGULATION.

With the generation of the RAd-HCMV-ORF library and the verification of expression, summarised in chapter 5.0., the resource was utilised for the screening of CD112 down-regulation. Since the expression of UL141 in isolation had no effect on CD112 cell surface expression, but was essential for CD112 down-regulation, it was necessary to co-infect two RAds to attempt to reproduce the Merlin phenotype. The screening method of choice was the co-infection of RAd-UL141 plus a RAd from the RAd-HCMV-ORF-Library. The cell surface expression of CD112 was then monitored by Flow cytometry.

6.1 Confirming CD112 down-regulation in HFFF-CAR's.

In-vitro, fibroblasts are one of the few cell lines that are permissive for productive HCMV infection. As screening the RAd-library of HCMV ORFs would require a large volume of cells it was decided that primary fibroblast cell lines were unsuitable due to their finite lifespan. Ad5 normally uses the CAR (coxsackie and adenovirus receptor) as a primary receptor and an αv integrin as a secondary receptor to gain entry into cells. Whilst human fibroblasts are routinely used in HCMV research, they have low levels of cell surface CAR. Instead, screening was carried out in the immortalised fibroblast cell line HFFF-hCAR's. The HFFF-hCAR cell line was previously generated in-house through the retroviral insertion of the telomerase hTERT and the Coxsakie Adenovirus receptor (CAR). The telomerase renders this cell line immortalised, while CAR enhances for Adenovirus entry. The expression of CAR allows for approximately 10x less Adenovirus to provide the equivalent level of infection in primary HFFF's. The switch from HFFF's to the HFFF-hCAR cell line therefore, allowed lower MOIs to be used and thus conserving RAd stocks. It was therefore important to show that cell surface CD112 expression levels were down-regulated in HFFF-hCAR's as well as in primary HFFF's.

HCMV infections were carried out in HFFF-hCARs and cell surface CD112, CD155 and MHC-1 detected by flow cytometry. As expected, during Merlin infection of HFFF-hCAR's, CD155, MHC-1 and CD112 were all down-regulated 3 days p.i Figure 6.1.

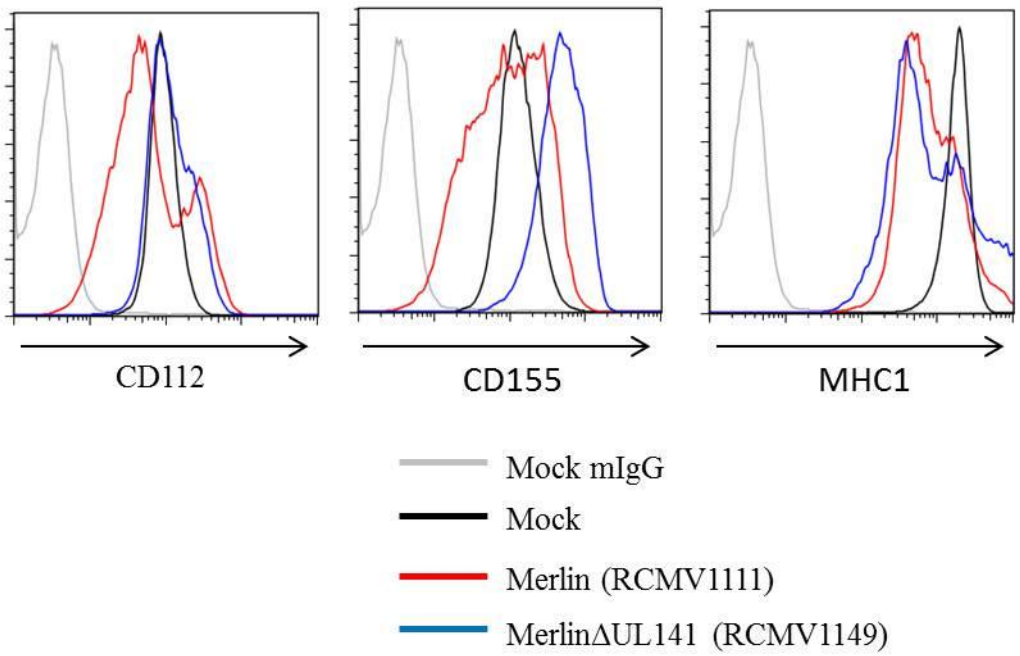


Figure 6.1. Cell surface CD112 is downregulated on HCMV infected HFFF-hCAR's
HFFF-hCARs were infected with HCMV at MOI 10 and the cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry.

MelrinΔUL141 infection resulted in the expected down-regulation of MHC-1 but not CD155 nor CD112. This result meant that HFFF-hCARs were suitable for large scale screening of the RAd-HCMV-ORF library.

6.2. Screening RAd library by flow cytometry

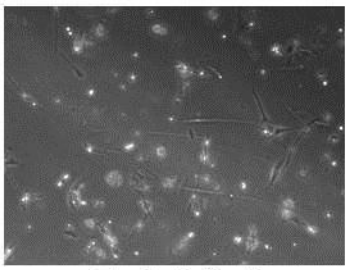
Screening of the HCMV block deletion mutants had discounted 46 genes from being essential in HCMV down-regulation of CD112, Chapter 4.0. Initial screening of the RAd-HCMV-ORF library focused on the remaining 124 ORFs. The 46 genes were also screened as a low priority, appendix 2.0.

Screening began with infecting HFFF-hCAR's with RAd-UL141 plus a RAd from the RAd-HCMV-ORF library, and analysing cell surface expression of CD112 by flow cytometry. Optimising began with screening with RAds spanning the UL13-UL20 region. Initial infections at a MOI of 10 identified CD112 as being down-regulated by several HCMV genes (data not shown). However, this was quickly attributed to rounding up of cells due to toxicity of RAd over-expression, Figure 6.2a. This was an important observation as such screening conditions would provide a false positive result and practical steps were required to be taken to reduce the amount of toxicity and therefore false positive results. This was achieved in several ways:

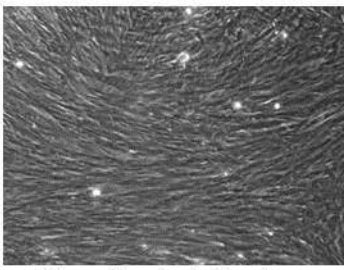
1) If signs of cell rounding occurred, experiments were abandoned and some were optimised for reduced cytotoxicity by reducing MOI's.
2) Infections were thoroughly washed to remove as many rounded up or dying cells as possible.
3) During analysis strict gating on a live cell population was used to try and limit false positive results.

Toxicity of infections was defined as the rounding up of infected cells which led to their detachment from the tissue culture flask so that cells were in suspension, and could have been due to a number of reasons. The transgene could have been toxic when expressed in HFFF-hCAR's. There could have been a miscalculation during titration meaning that far too much RAd was added to infections (e.g. MOI 100 instead of MOI 10). Or RAd could

A)



Toxic infection
(RAd-UL34)



Non-Toxic infection
(RAd-CTtl)

B)

RAd-HCMV-ORFs that demonstrated signs of toxicity
RL8A, RL10, RL11, UL2, UL4, UL5, UL15A, UL16, UL17, UL19, UL20, UL23, UL26, UL34, UL37, UL41A, UL138, US14, US28, US29

Figure 6. 2. RAd infections that demonstrated signs of toxicity. HFFF-hCARs were infected with RAd from the RAd-HCMV-ORF library at MOI 10 and analysed 3 days p.i. by phase contrast microscopy for signs of toxicity. A) Phase contrast image of RAd-UL34 + RAd-Ctrl infection, which was deemed to be toxic and also RAd-Ctrl infection, which was non-toxic B) table of all RAd's that demonstrated signs of toxicity during infection of HFFF-hCAR's infected at MOI 10.

have picked up E1 region from the packaging cell line, restoring the E1 region to the RAd genome resulting in the generation of replication competent virus.

For the majority of RAd infections, MOI 10 proved to show little or no signs of toxicity. However, a total of 20 RAds showed signs of toxicity, Figure 6.2b. Of these, 9 ORFS RL8A, RL10, RL11, UL2, UL4, UL5, US14, US28 and US29 are found in regions deleted in HCMV block deletion mutants. As these block deletion mutants were still capable of down-regulating cell surface CD112, individual HCMV ORF's in these regions were considered to not be essential in CD112 down regulation and no attempts were made to optimise infections to avoid toxicity. UL15A, UL16, UL17 and UL19 all showed signs of toxicity but a titration of toxicity revealed no signs of toxicity at MOI2 and for these infections a reduced MOI was used. Immunofluorescence was undertaken to ensure that expression was detectable at these reduced MOI's. For the remaining 8 ORFs; UL20, UL23, UL26, UL34, UL37, UL41A and UL138 reduced MOI's made no effect on toxicity and these viruses could not be used in an assay to analyse their role in CD112 down-regulation.

All infections were carried out in HFFF-hCAR's. All viral infections were controlled for total amount of viral load. For example, if RAd-UL141 was used at an MOI10 and RAd-UL45 was used at MOI10, making a total of MOI20, then empty control RAd-1253 would be used at MOI 20.

Control conditions included; a) mock (uninfected), b) control RAd (RAd-Ctrl) and c) RAd-UL141 + RAd-Ctrl, Figure 6.3. The experimental condition consisted of RAd-UL141 + RAd-X where RAd-X denotes any ORF in the RAd-HCMV-ORF library and RAd-X + RAd-Ctrl to check whether RAd-X had any effect.

Three days post infection, samples were stained for mIgG (Negative control), CD112 (experimental test), CD155 (to test for the action of UL141) and finally MHC-1 (a positive control for staining, as well as a control for off target down-regulation, Figure 6.4 – 6.35. Each RAd-X was analysed a minimum of three times and following figures 6.4-6.35 show representative results.

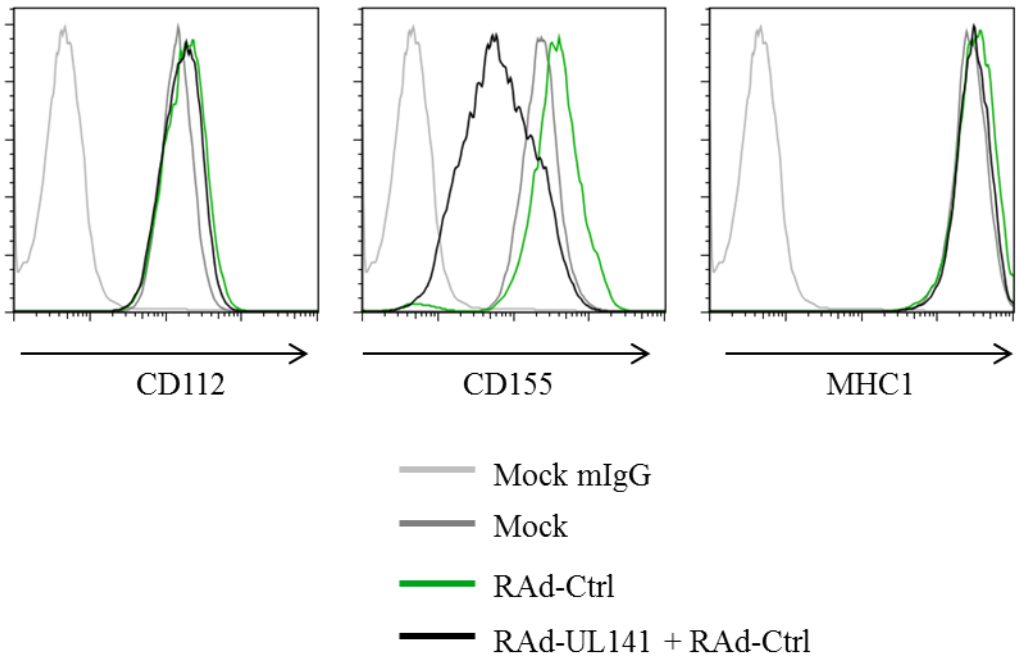


Figure 6.3. Control infections for screening RAd-HCMV-ORF library. HFFF-hCARs were infected with **RAd-Ctrl (MOI 20),** **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)** or **mock infected**. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry.

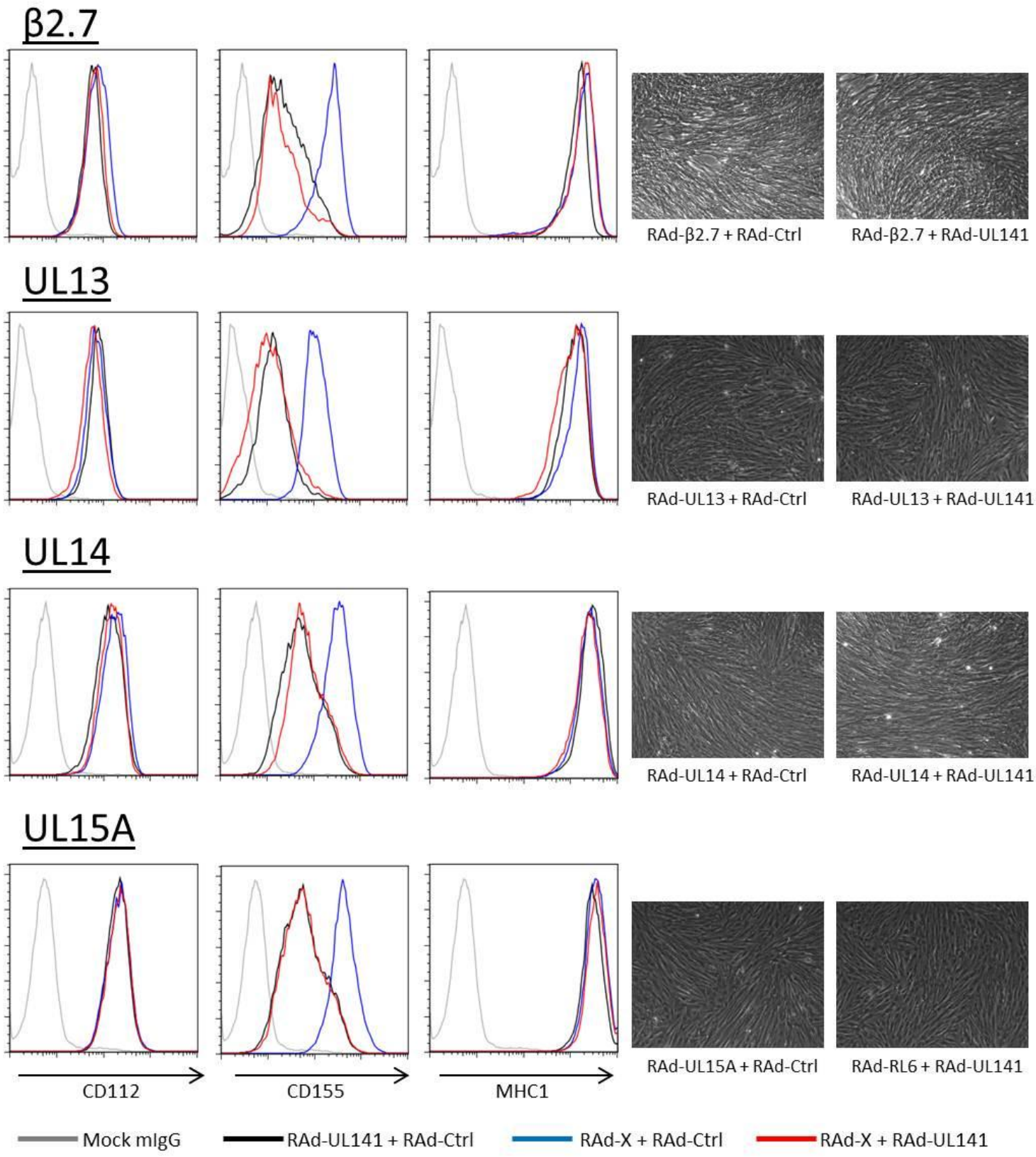


Figure 6.4. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-β2.7, RAd-UL13, RAd-UL14 and RAd-UL15A.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene labelled in the figure. An exception was RAd-UL15A which was infected at MOI2 due to toxicity seen at higher MOI's. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

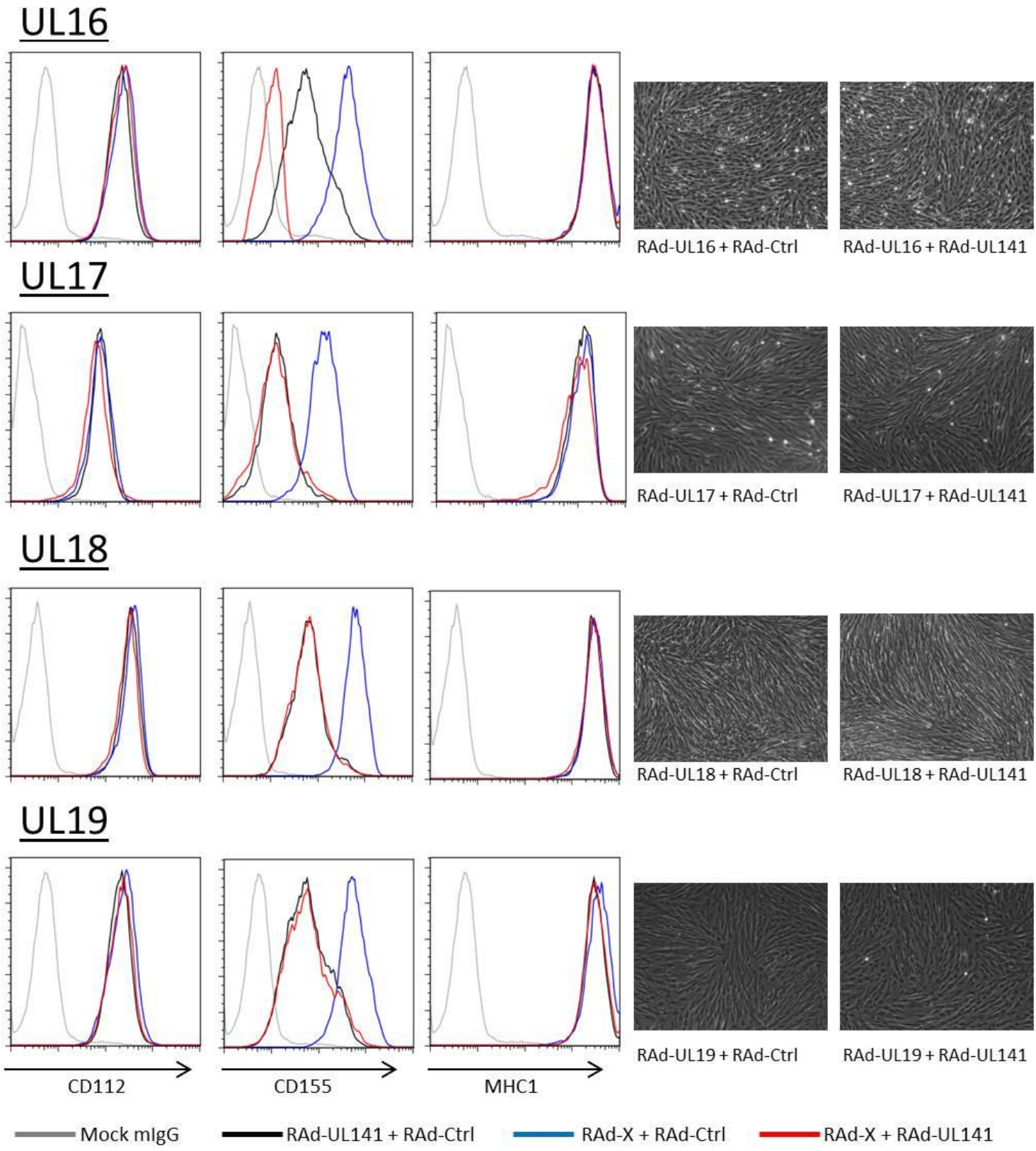


Figure 6.5. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL16, RAd-UL17, RAd-UL18 and RAd-UL19.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene labelled in the figure. An exception was RAd-UL15A which was infected at MOI2 due to toxicity seen at higher MOI's. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

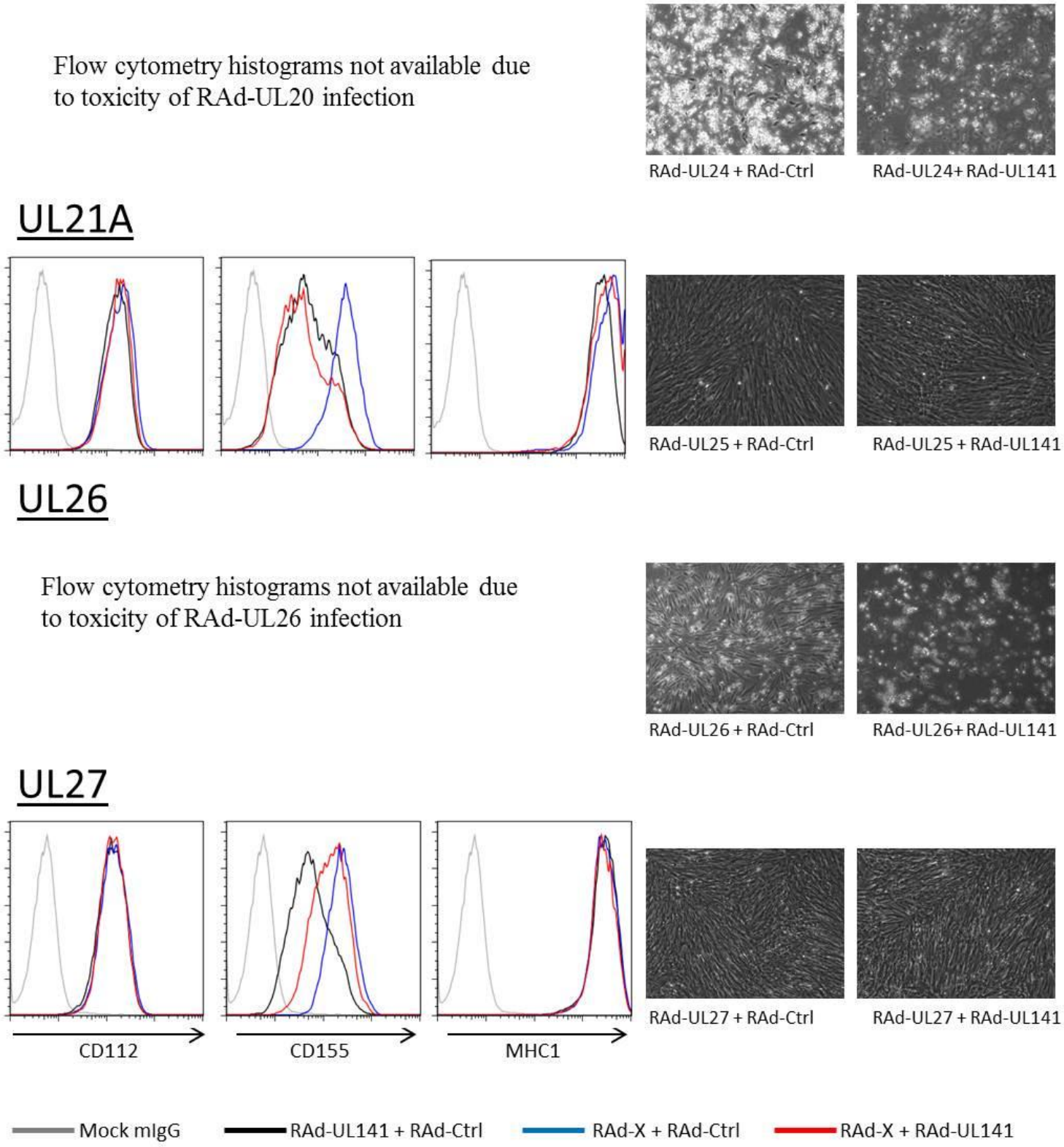


Figure 6.6. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL20, RAd-UL21A, RAd-UL26 and RAd-UL27.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-UL20 and RAd-UL26 due to toxicity.

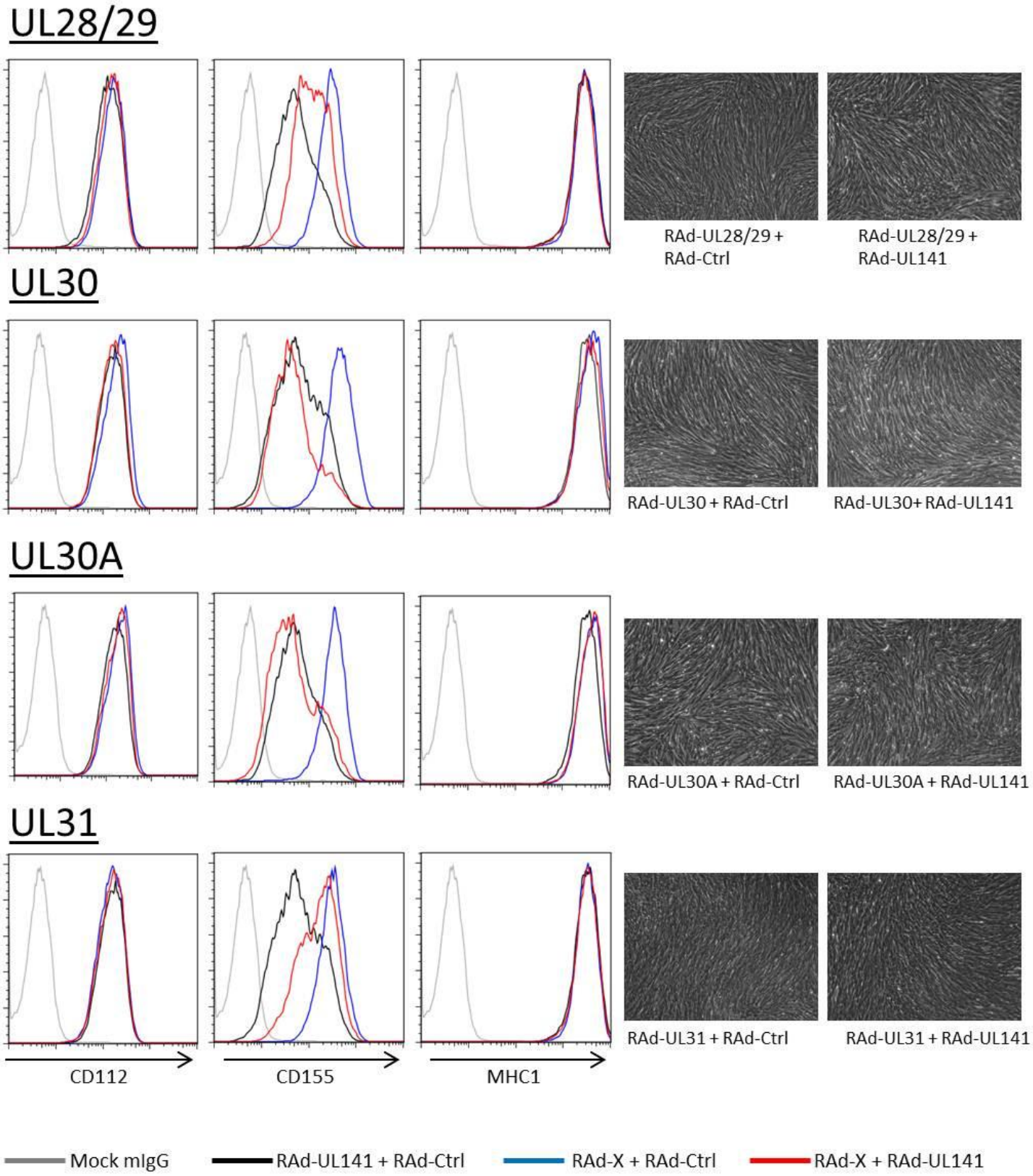


Figure 6.7. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL28/29, RAd-UL30, RAd-UL30A and RAd-UL31.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

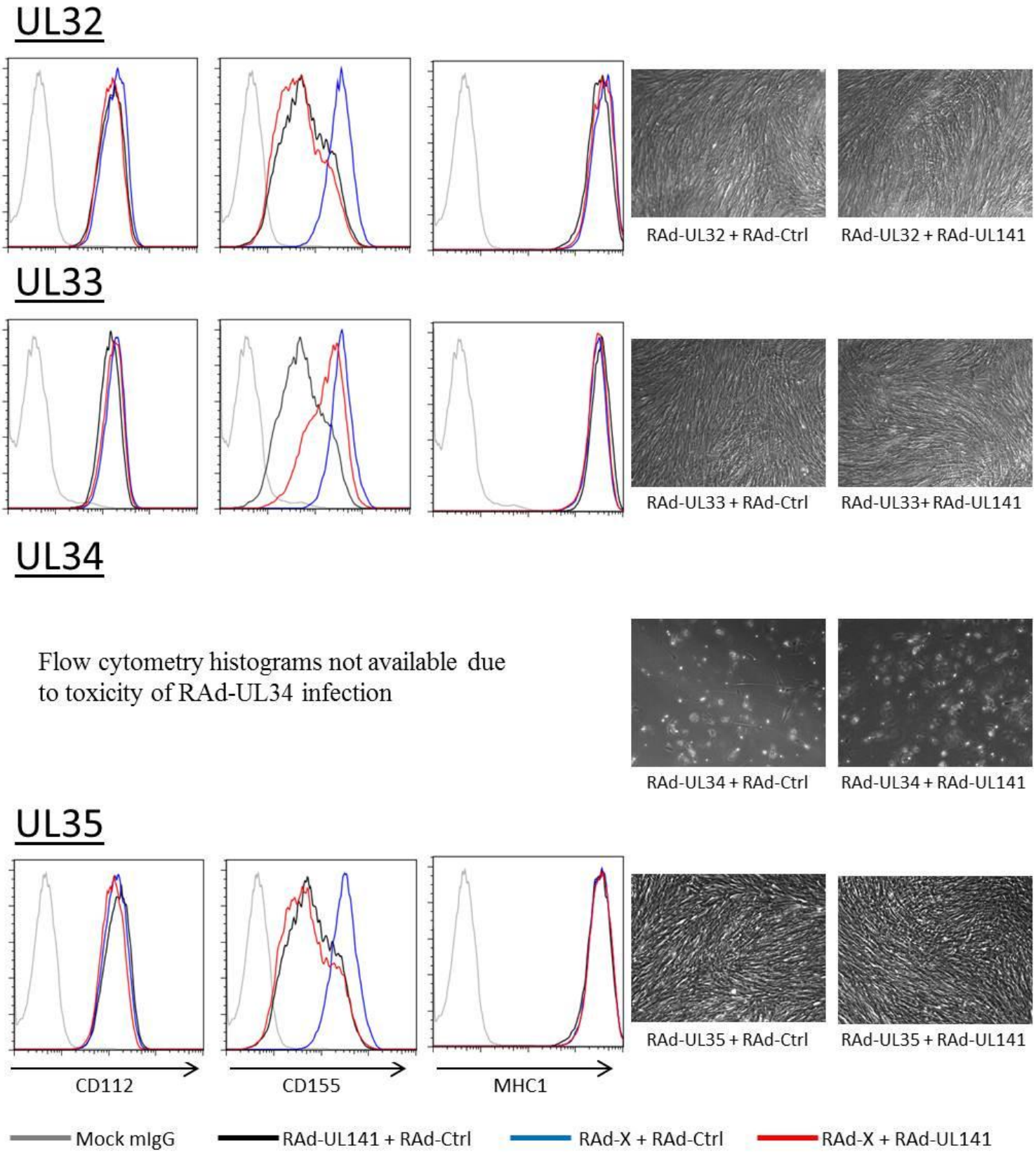


Figure 6.8. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL32, RAd-UL33, RAd-UL34 and RAd-UL35.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-UL34 due to toxicity.

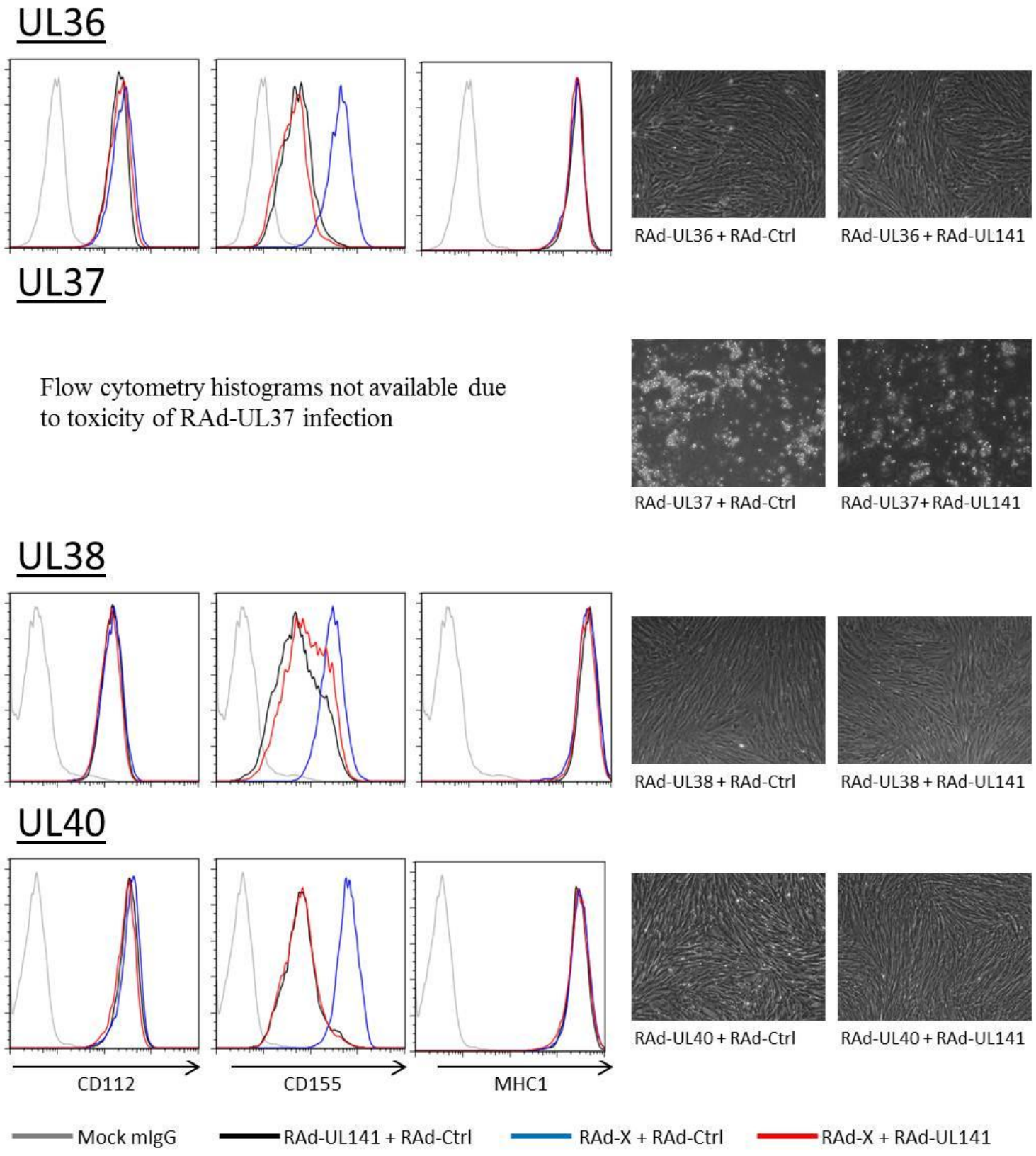


Figure 6.9. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL36, RAd-UL37, RAd-UL38 and RAd-UL40.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-UL37 due to toxicity.

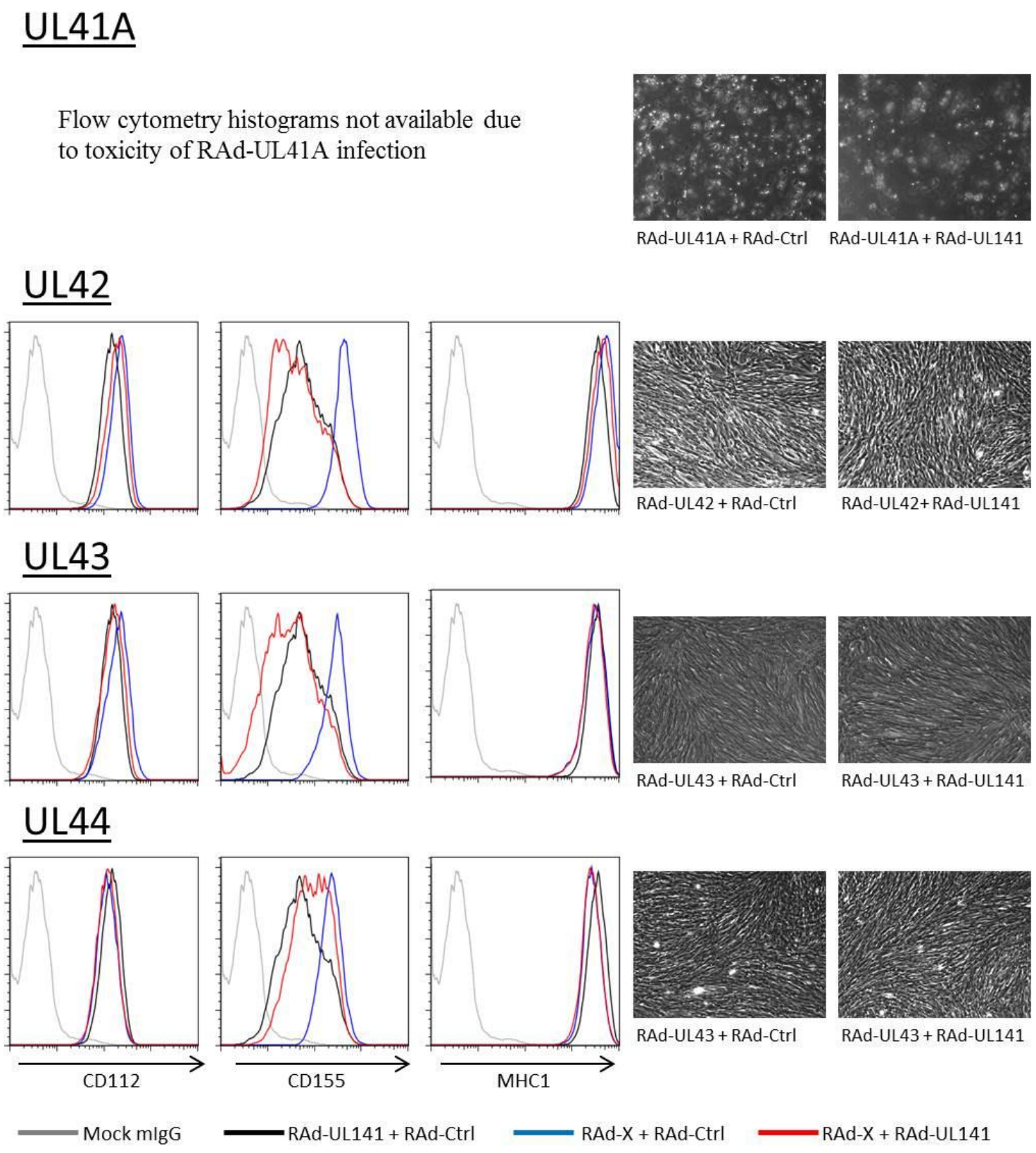


Figure 6.10. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL41A, RAd-UL42, RAd-UL43 and RAd-UL44.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-UL41A due to toxicity.

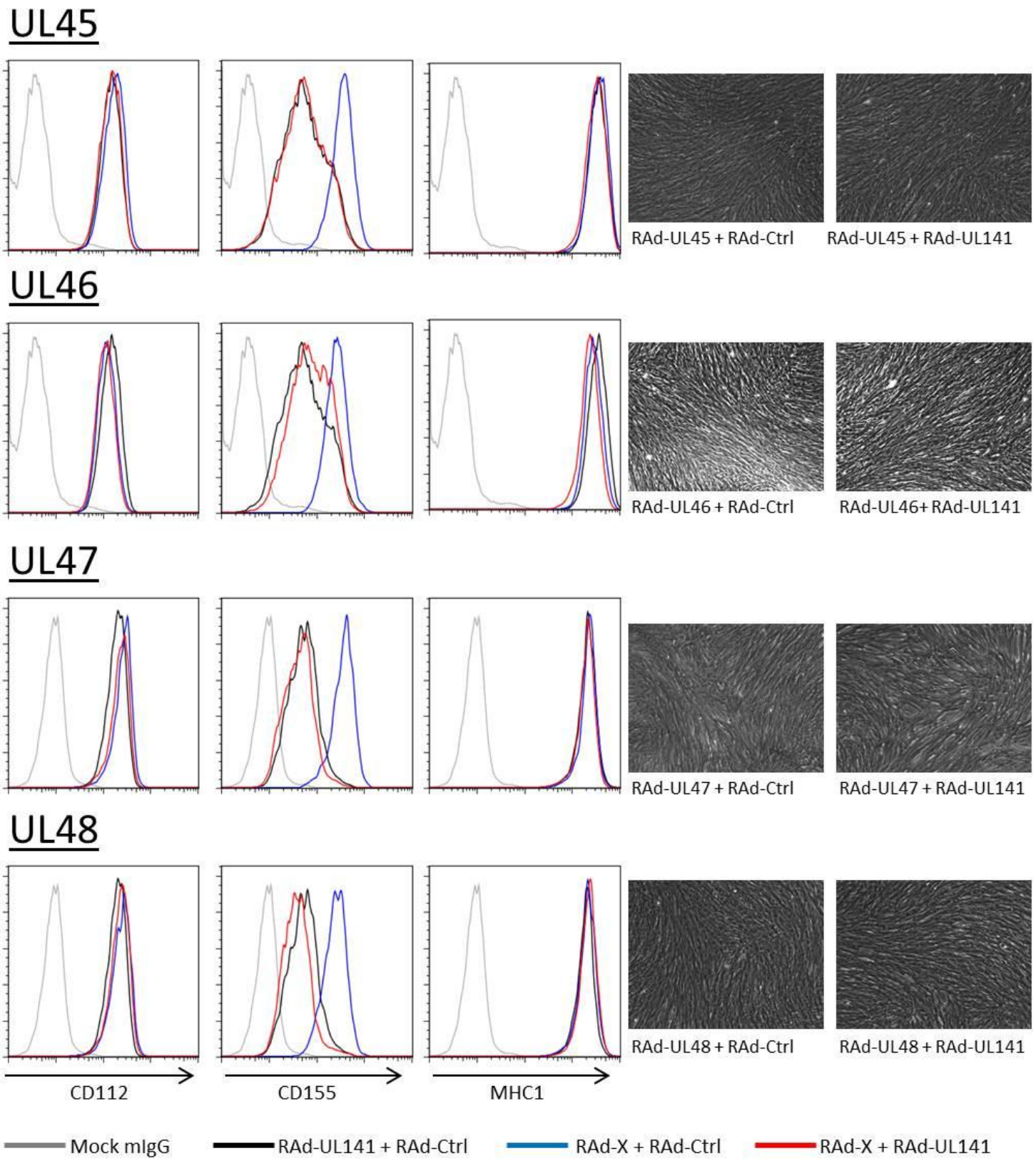


Figure 6.11. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL45, RAd-UL46, RAd-UL47 and RAd-UL48.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

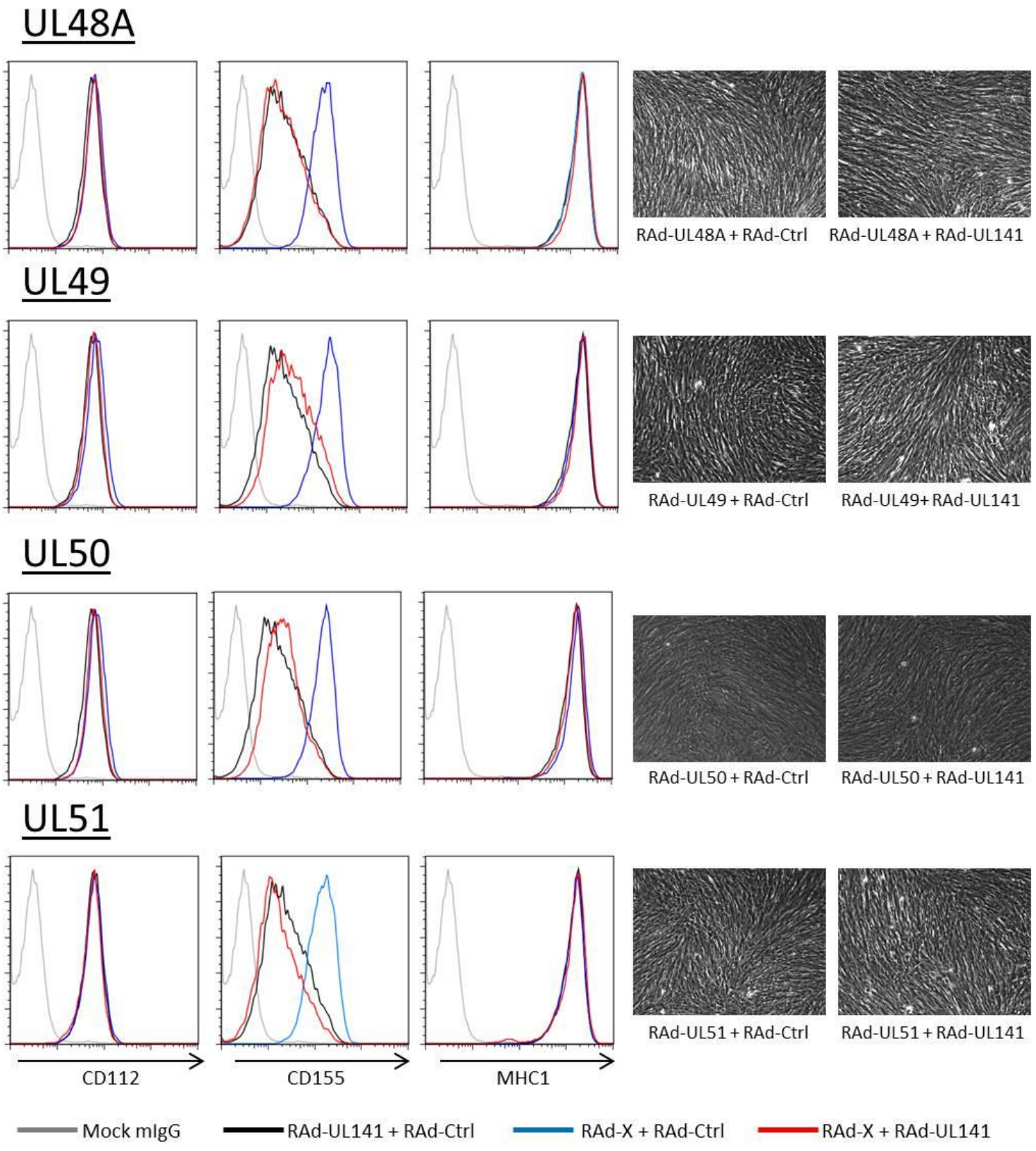


Figure 6.12. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL48A, RAd-UL49, RAd-UL50 and RAd-UL51.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy

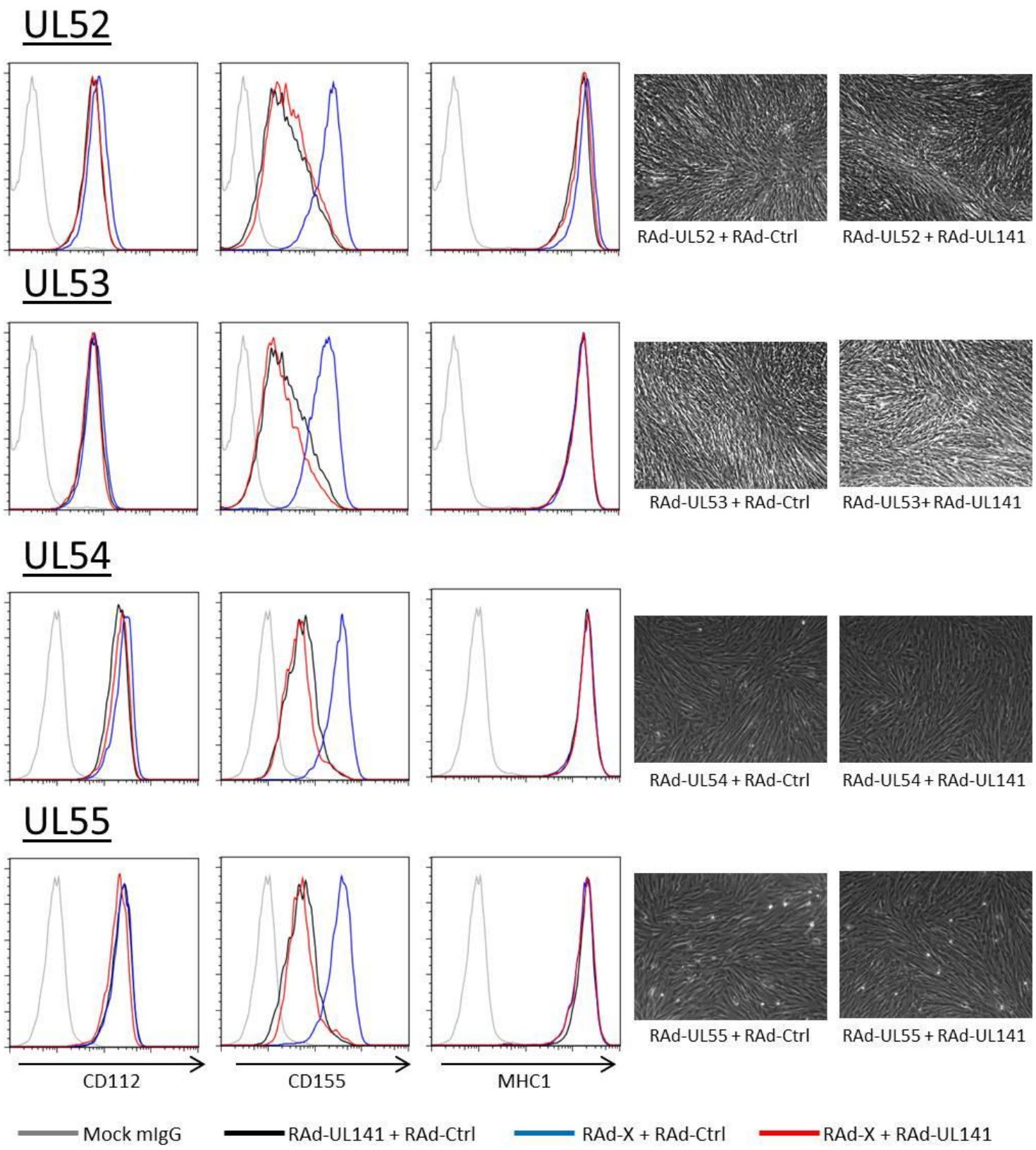


Figure 6.13. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL52, RAd-UL53, RAd-UL54 and RAd-UL55.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

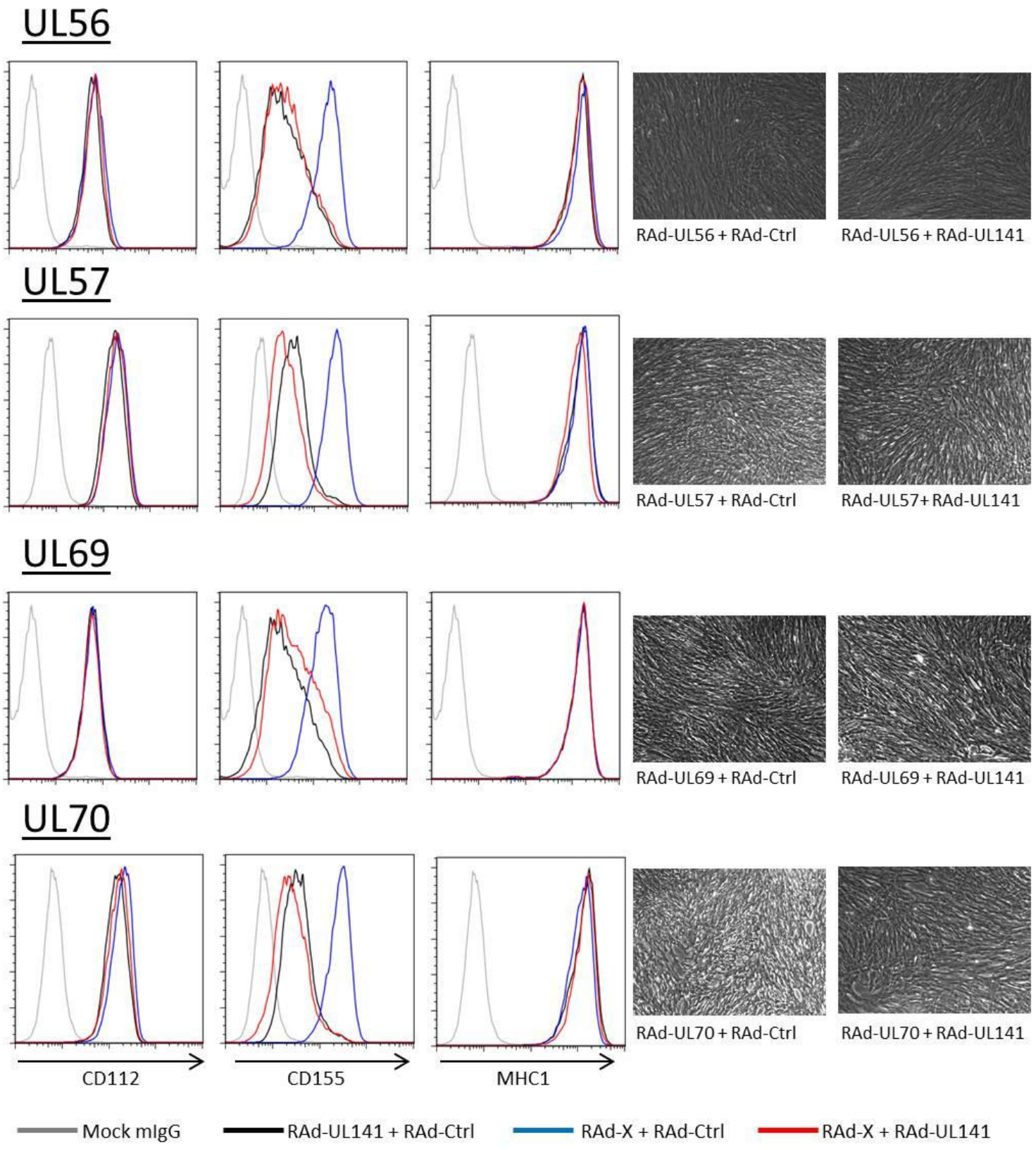


Figure 6.14. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL56, RAd-UL57, RAd-UL69 and RAd-UL70.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

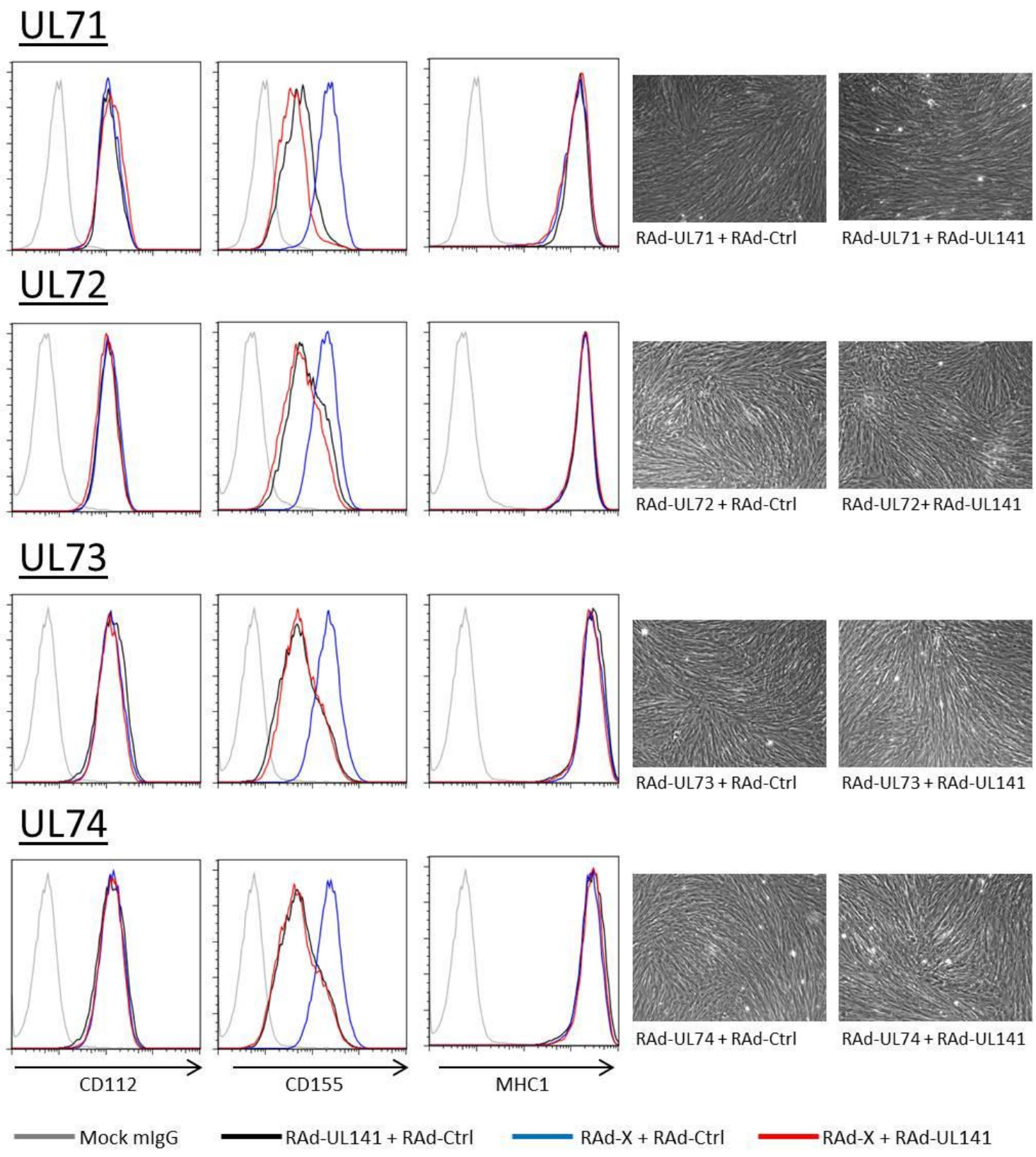


Figure 6.15. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL71, RAd-UL72, RAd-UL73 and RAd-UL74.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

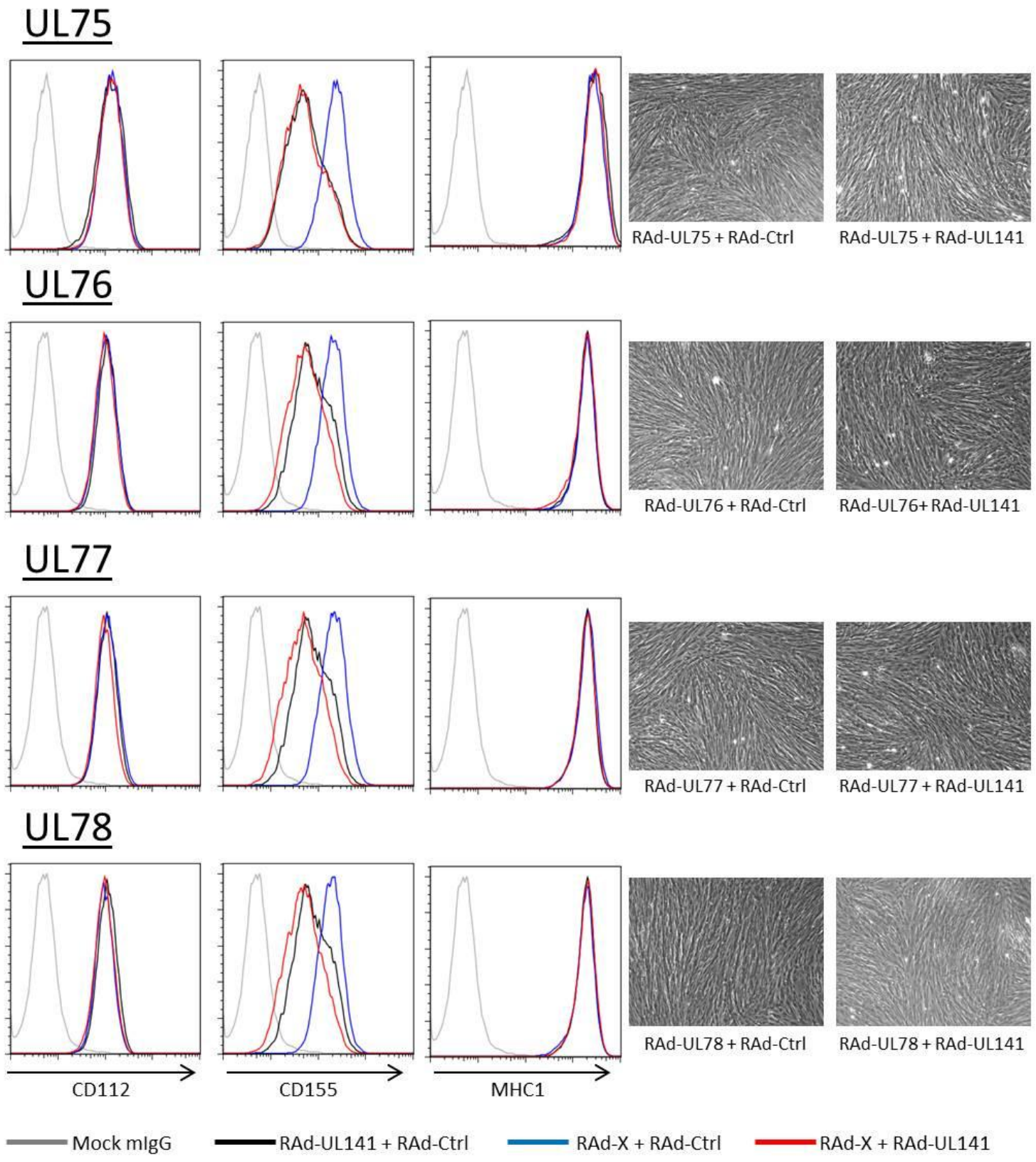


Figure 6.16. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL75, RAd-UL76, RAd-UL77 and RAd-UL78.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

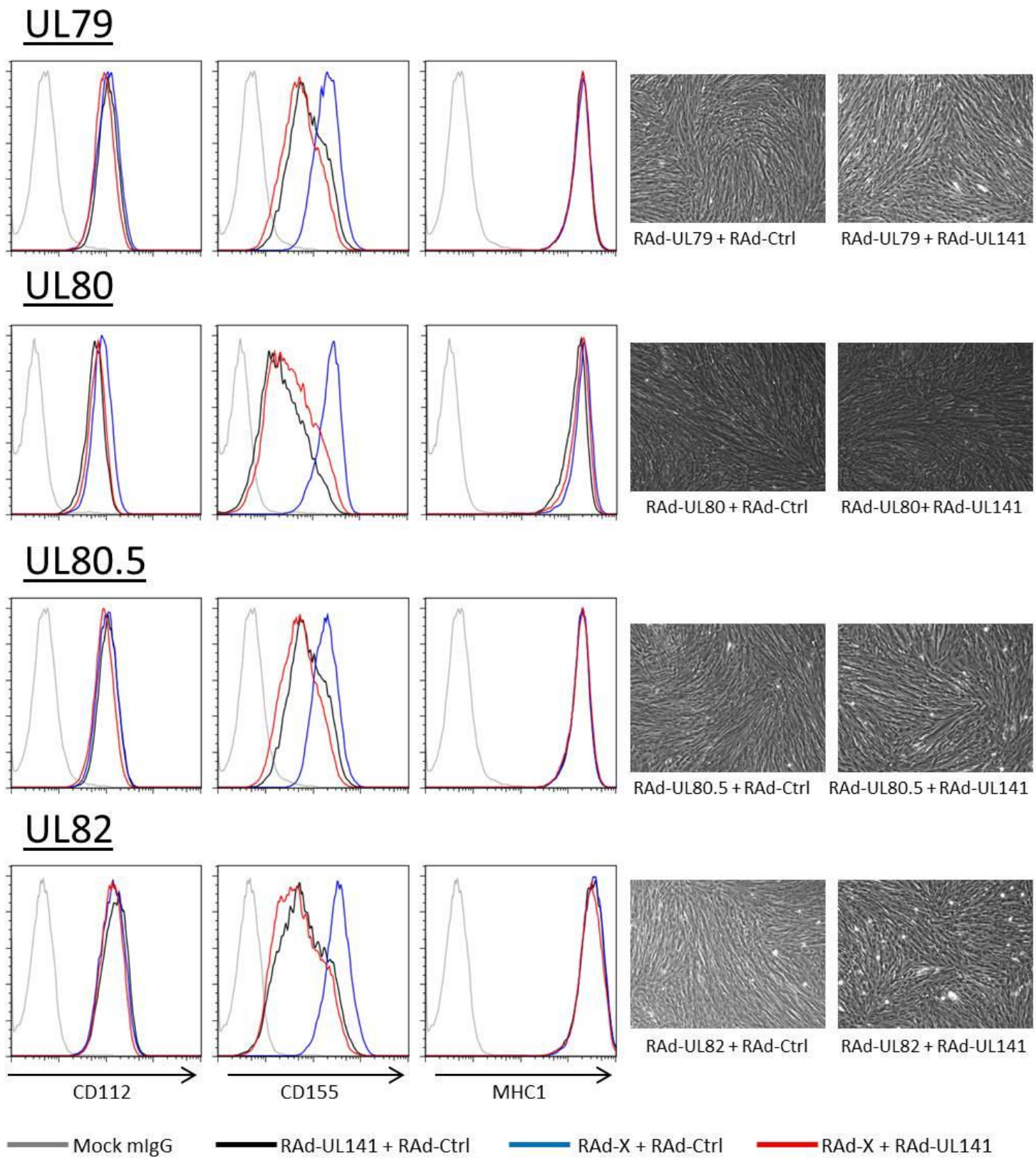


Figure 6.17. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL79, RAd-UL80, RAd-UL80.5 and RAd-UL82.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

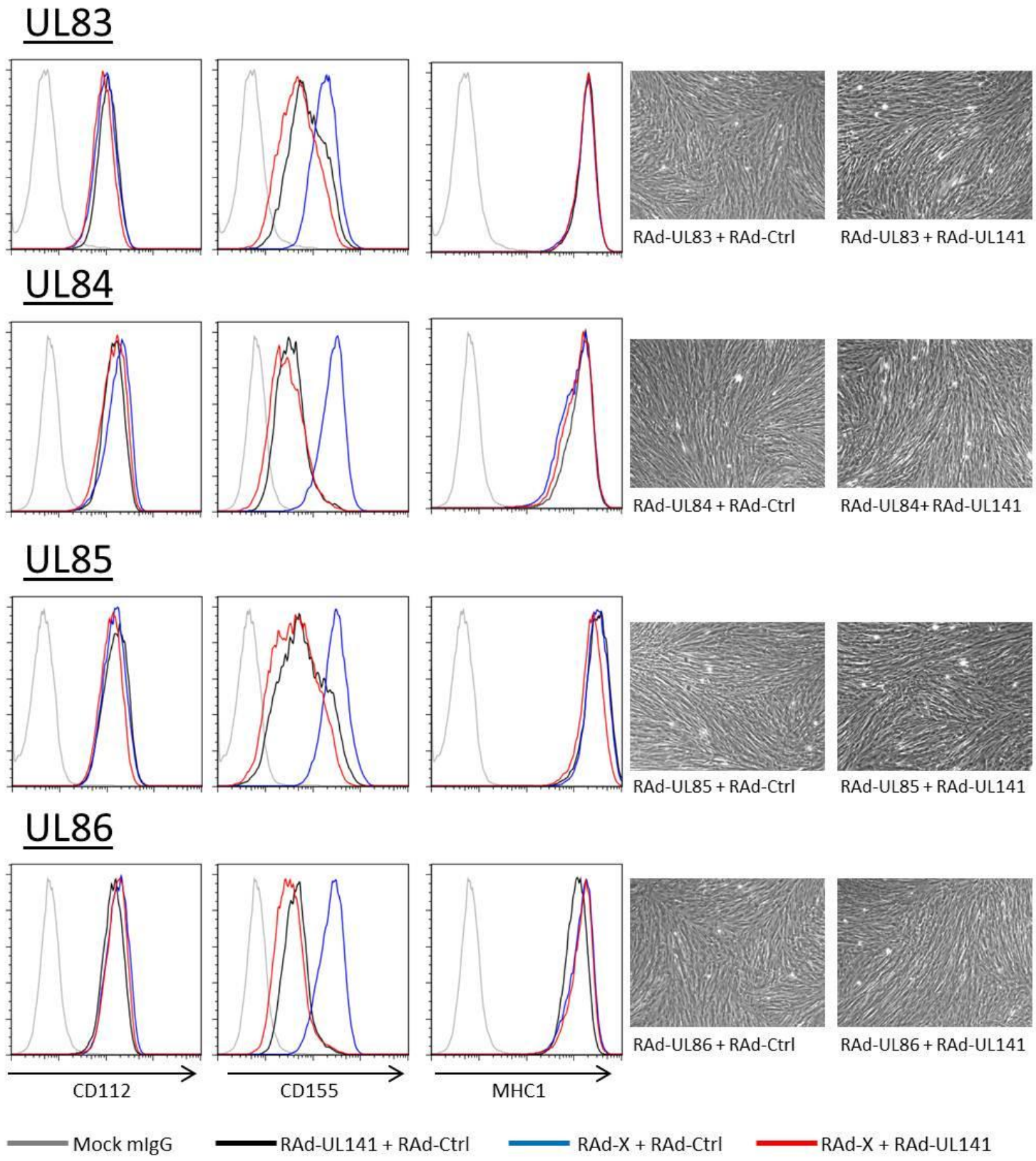


Figure 6.18. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL83, RAd-UL84, RAd-UL85 and RAd-UL86.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

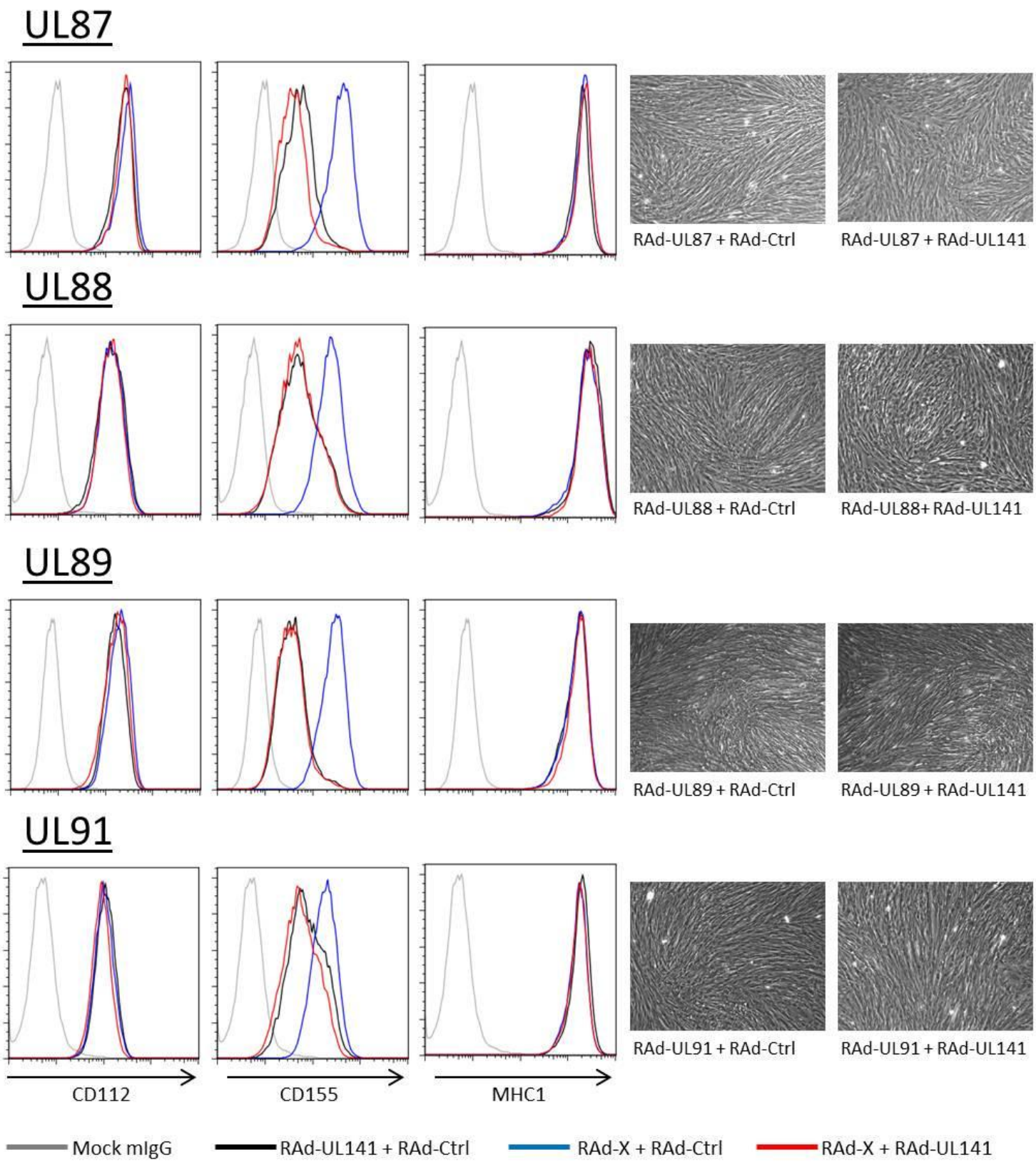


Figure 6.19. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL87, RAd-UL88, RAd-UL89 and RAd-UL91.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

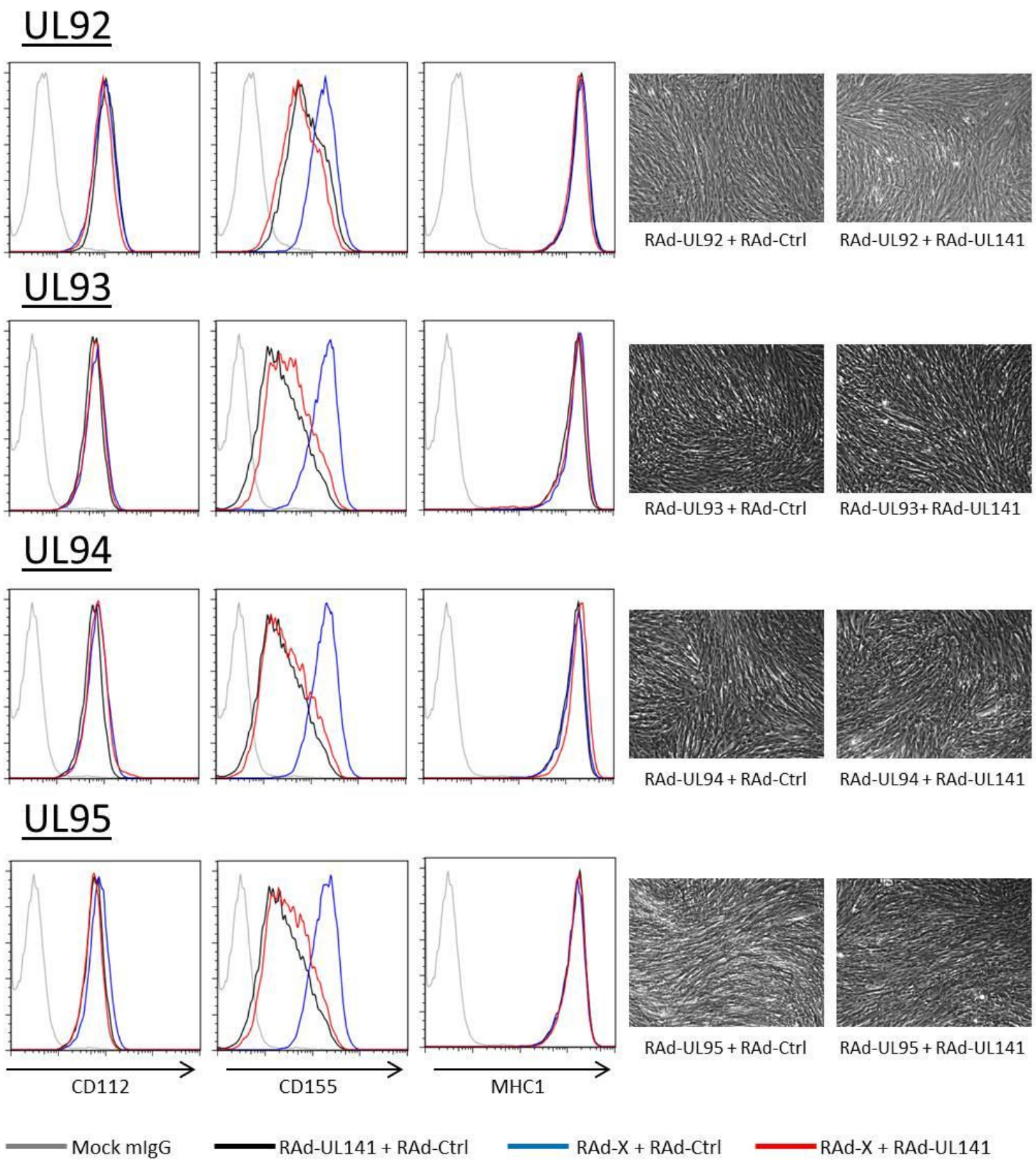


Figure 6.20. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL92, RAd-UL93, RAd-UL94 and RAd-UL95.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

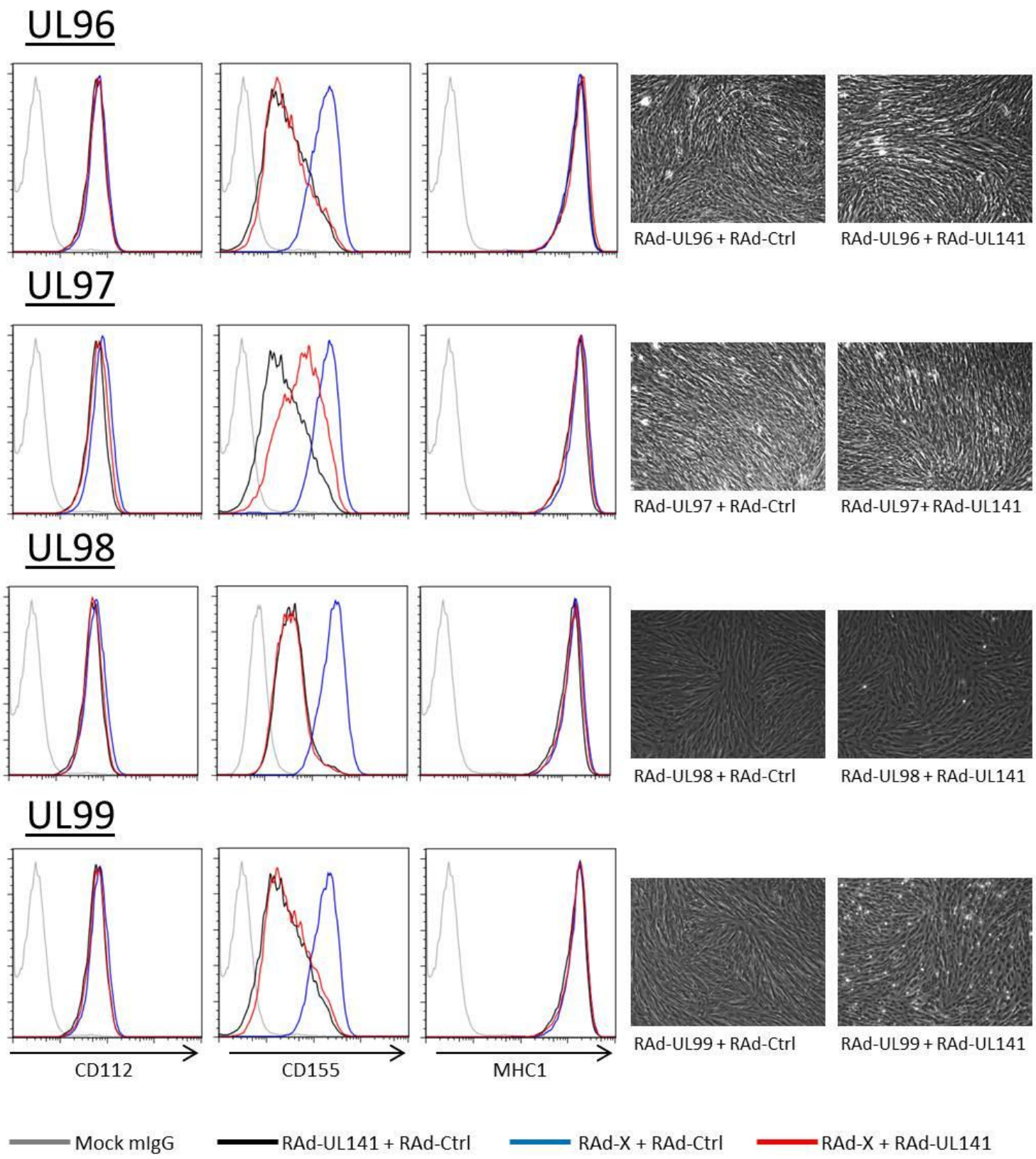


Figure 6.21. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL96, RAd-UL97, RAd-UL98 and RAd-UL99.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

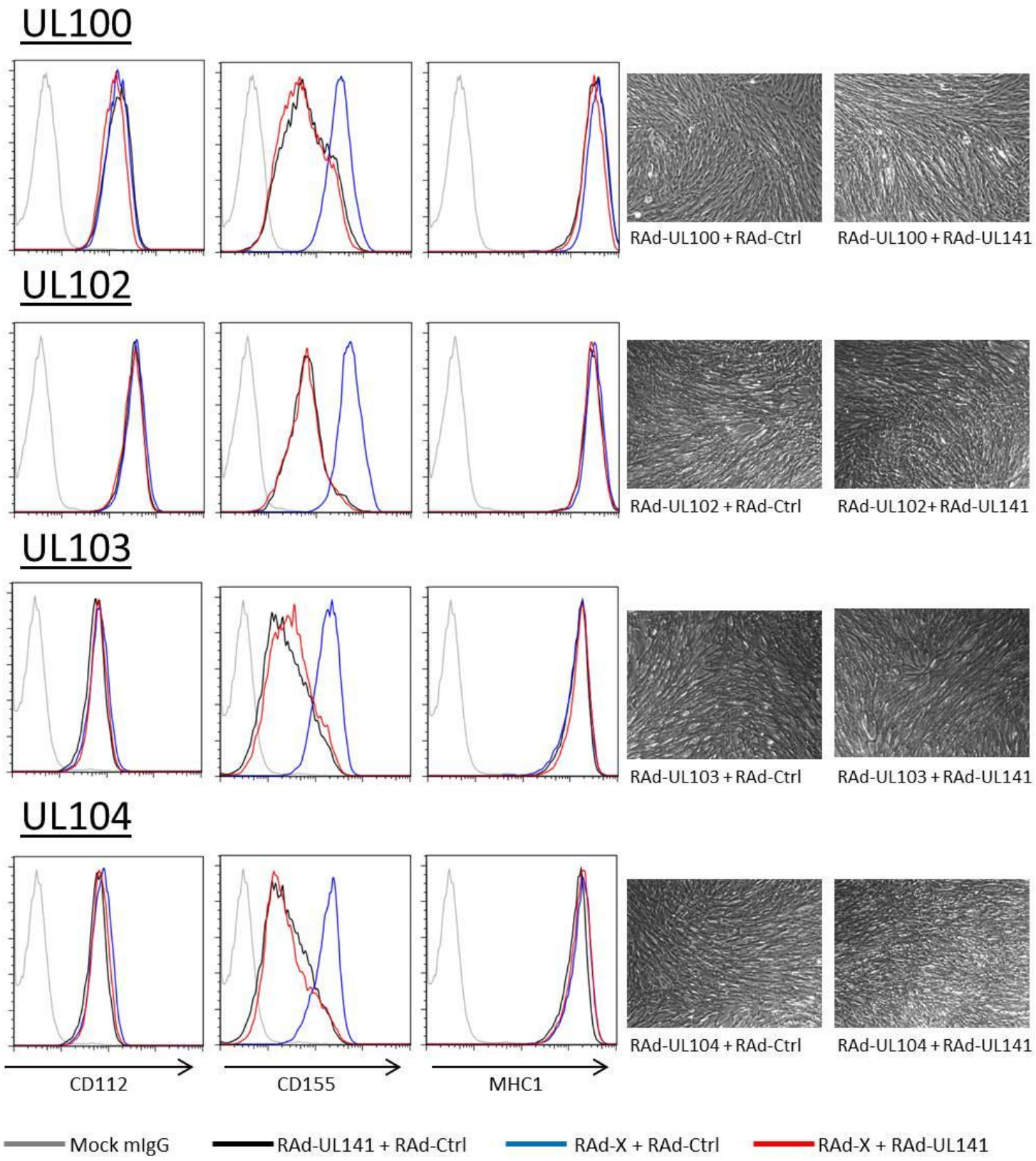


Figure 6.22. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL100, RAd-UL102, RAd-UL103 and RAd-UL104.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

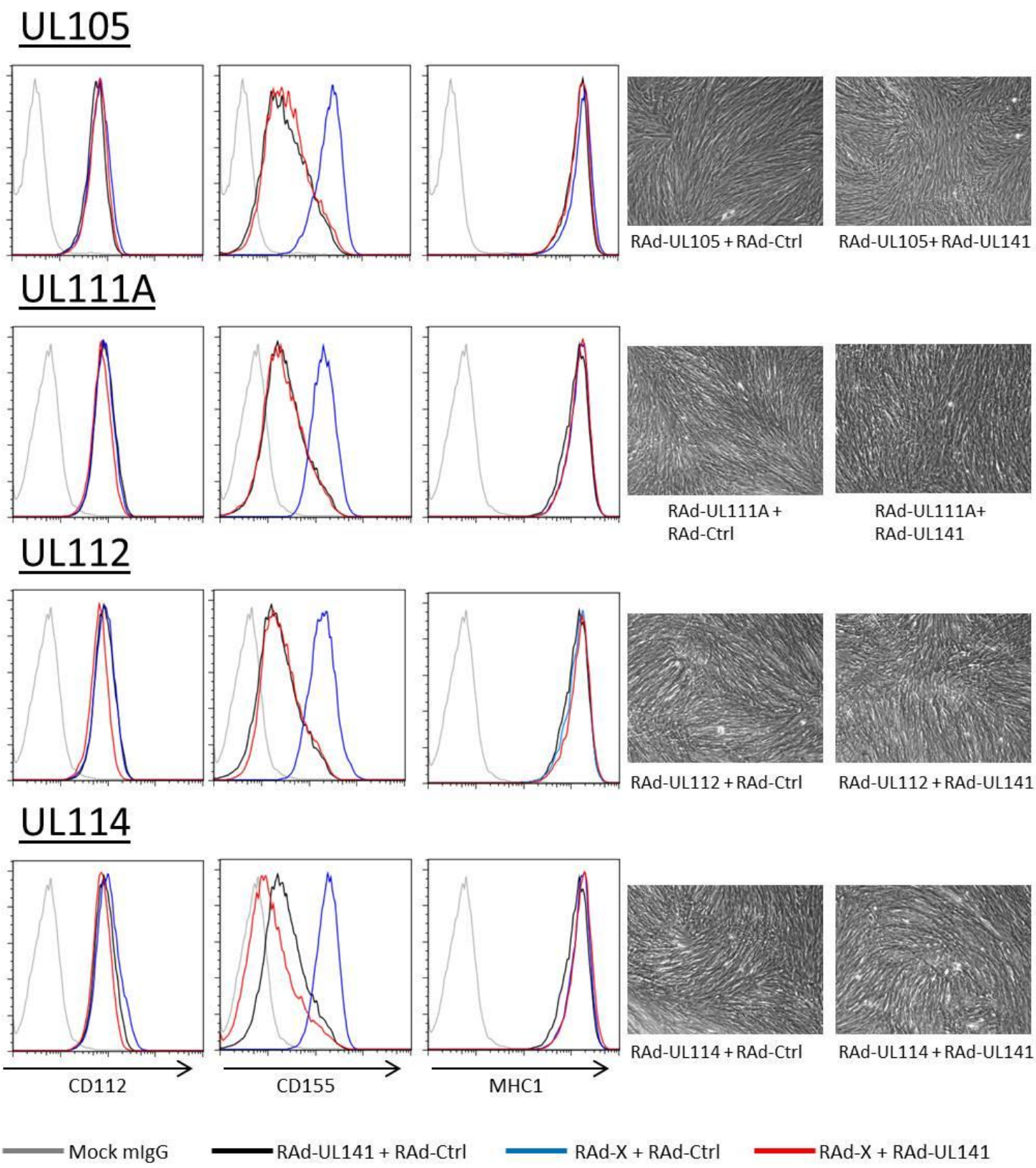


Figure 6.23. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL105, RAd-UL111A, RAd-UL112 and RAd-UL114.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

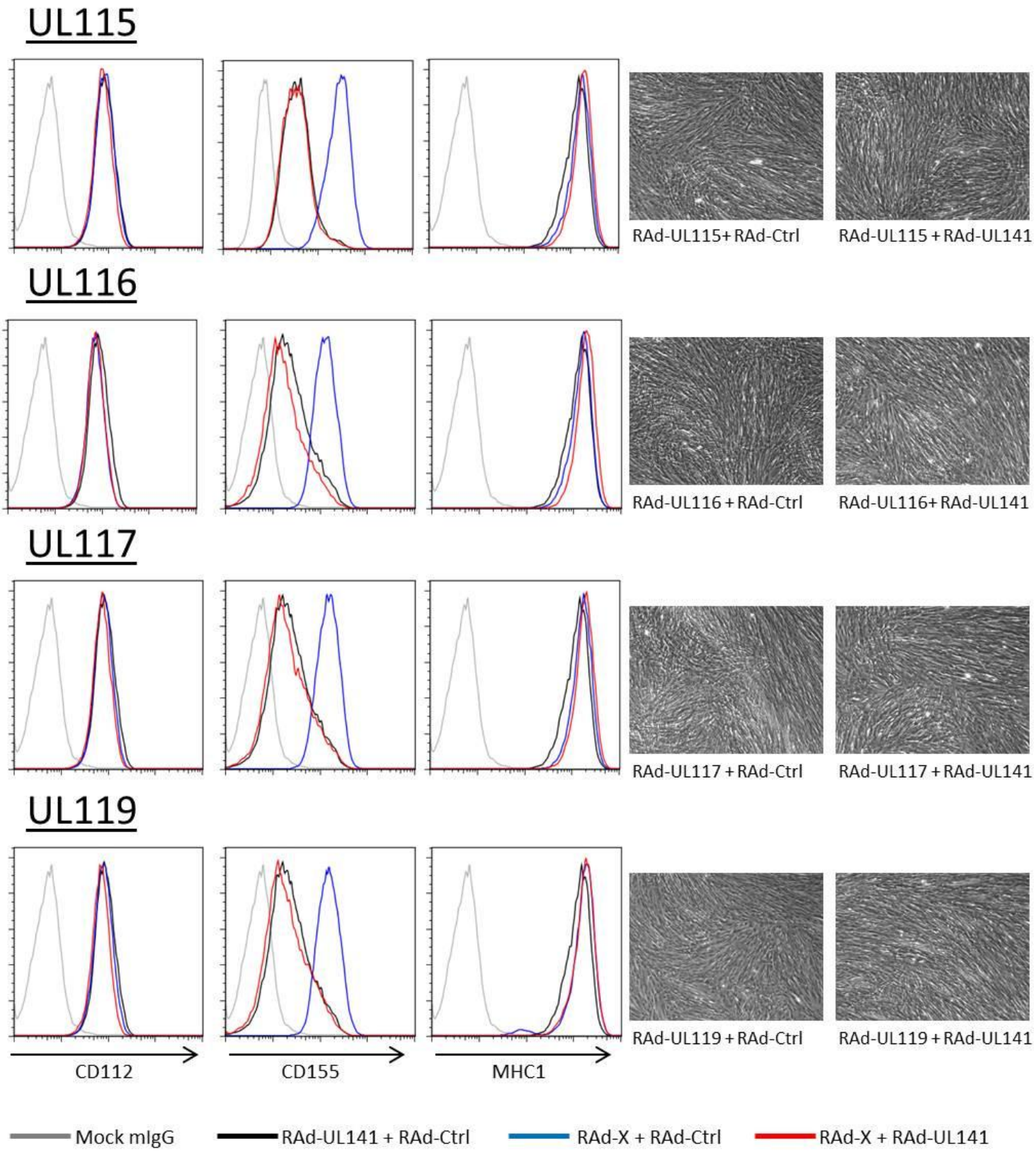


Figure 6.24. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL115, RAd-UL116, RAd-UL117 and RAd-UL119.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

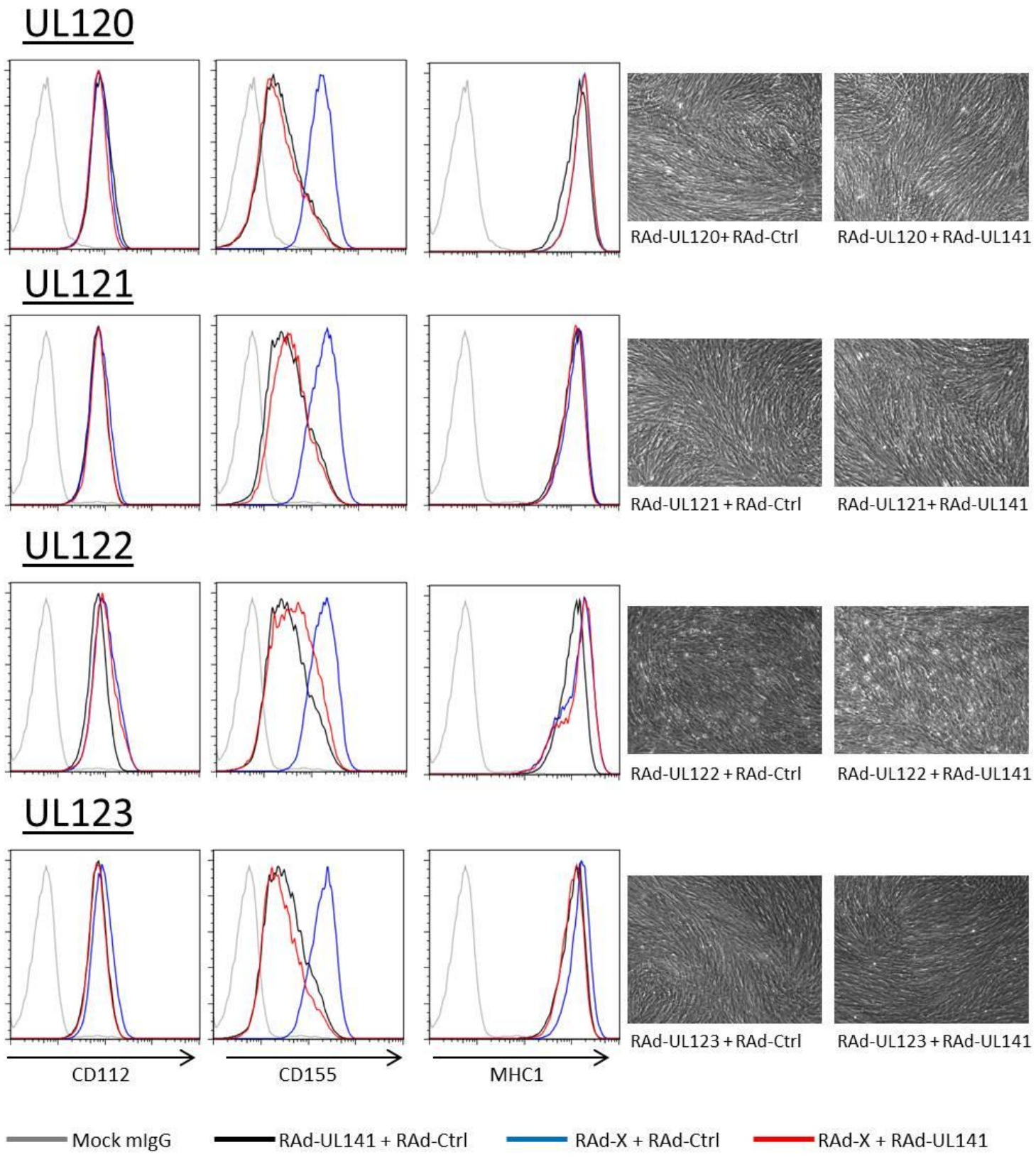


Figure 6.25. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL120, RAd-UL121, RAd-UL122 and RAd-UL123.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

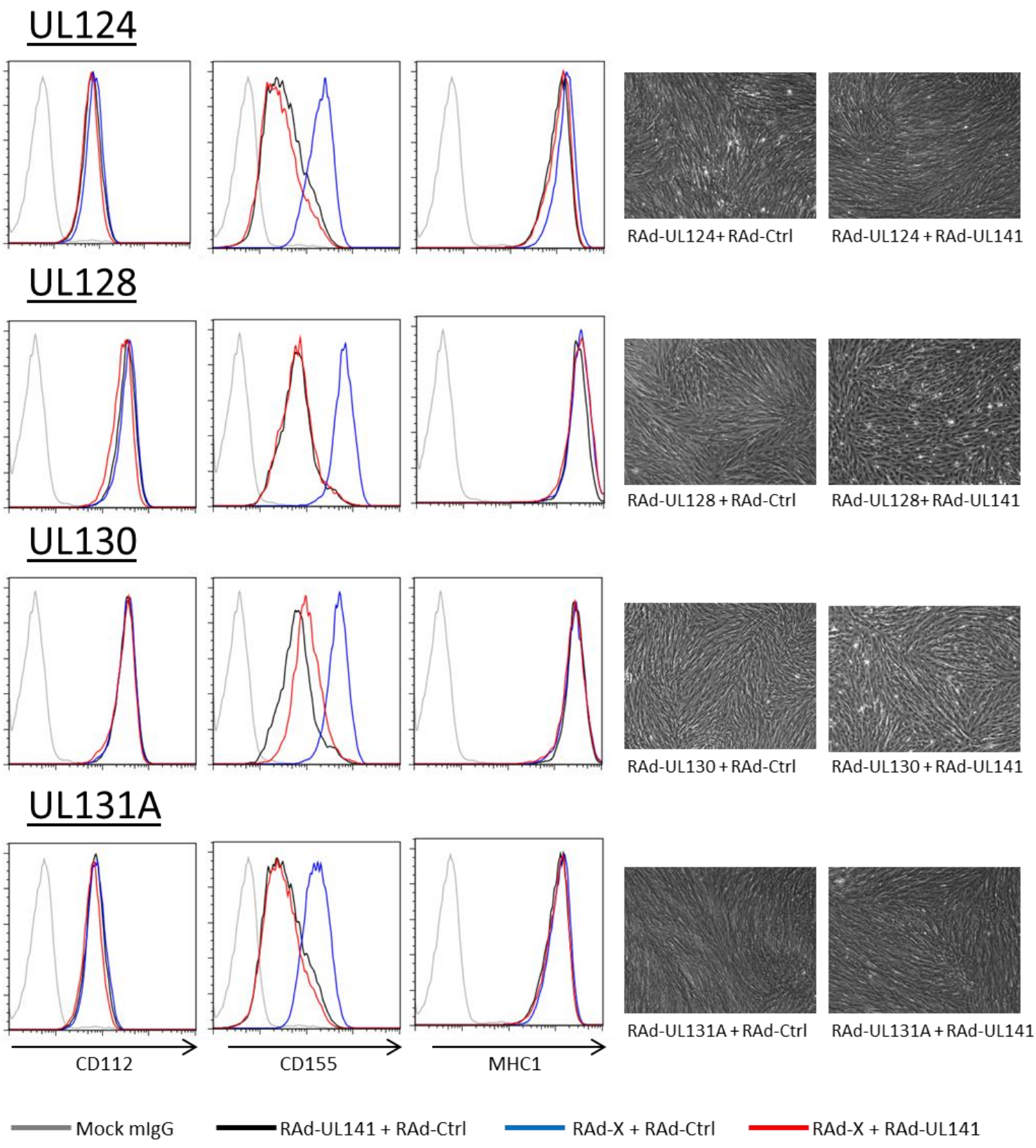


Figure 6.26. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL124, RAd-UL128, RAd-UL130 and RAd-UL131A.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

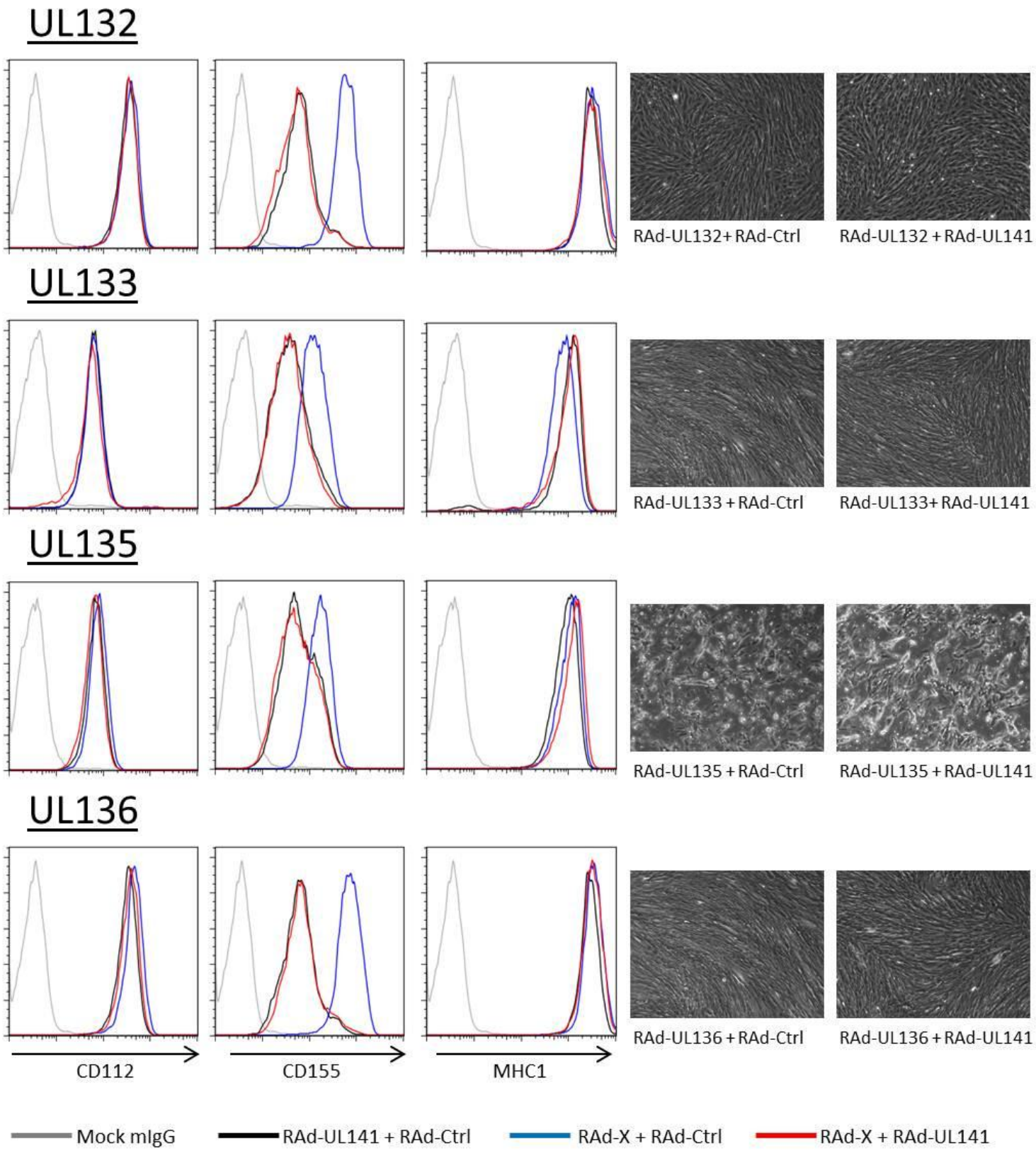


Figure 6.27. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL132, RAd-UL133, RAd-UL135 and RAd-UL136.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

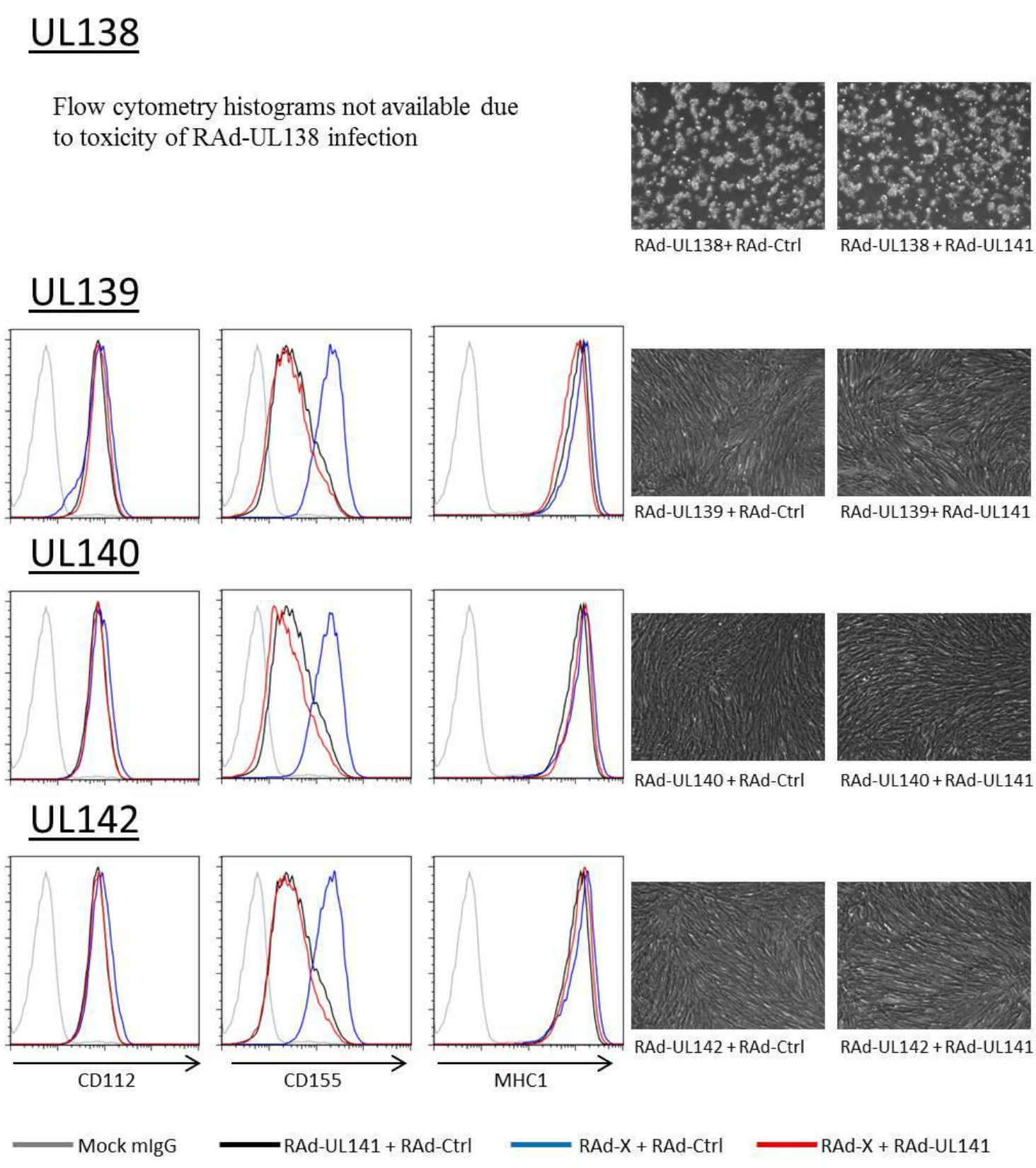


Figure 6.28. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL138, RAd-UL139, RAd-UL140 and RAd-UL142.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-UL138 due to toxicity.

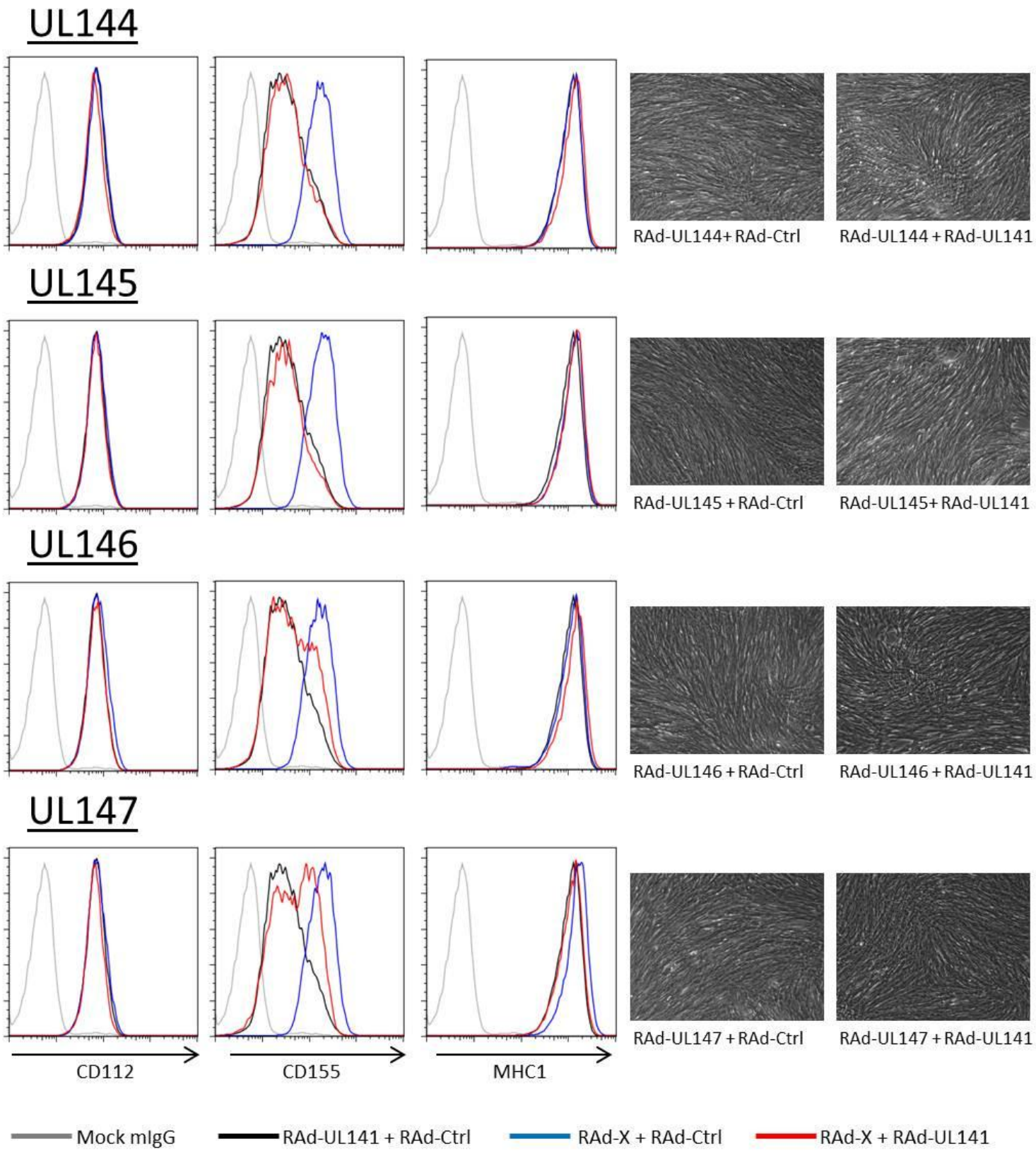


Figure 6.29. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL144, RAd-UL145, RAd-UL146 and RAd-UL147.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

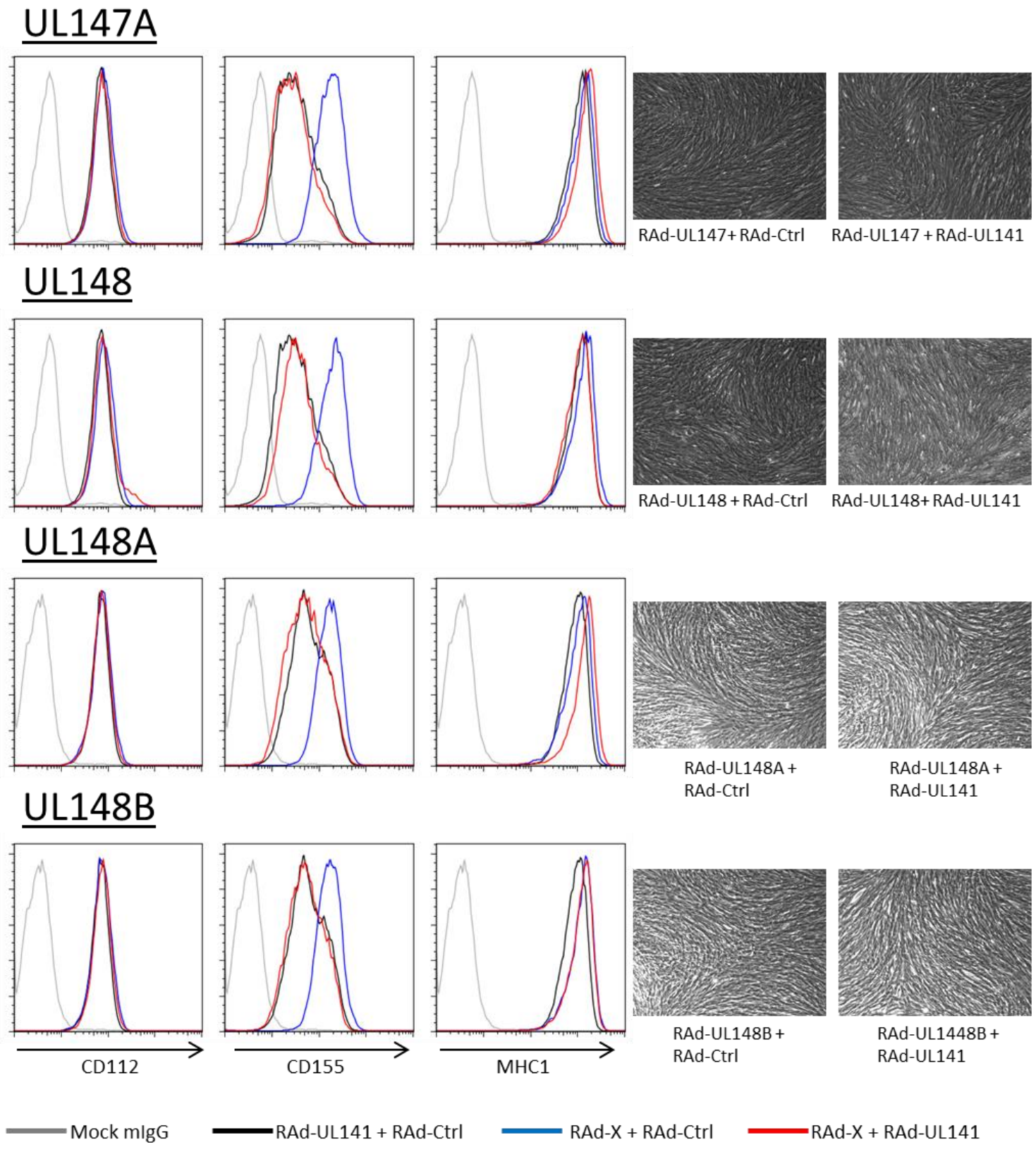


Figure 6.30. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL147, RAd-UL148, RAd-UL148A and RAd-UL148B.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

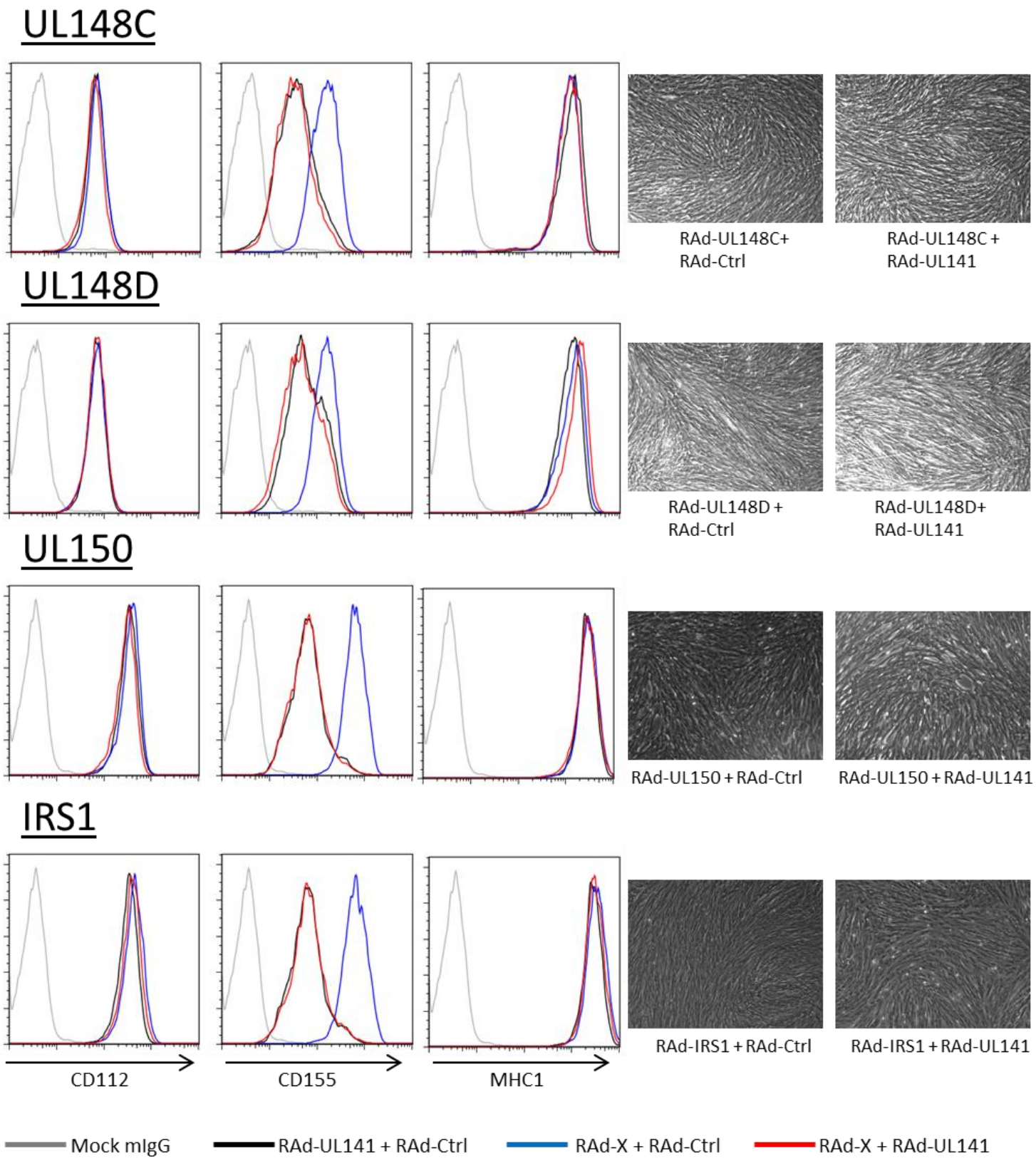


Figure 6.31. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL148C, RAd-UL148D, RAd-UL150 and RAd-IRS1.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

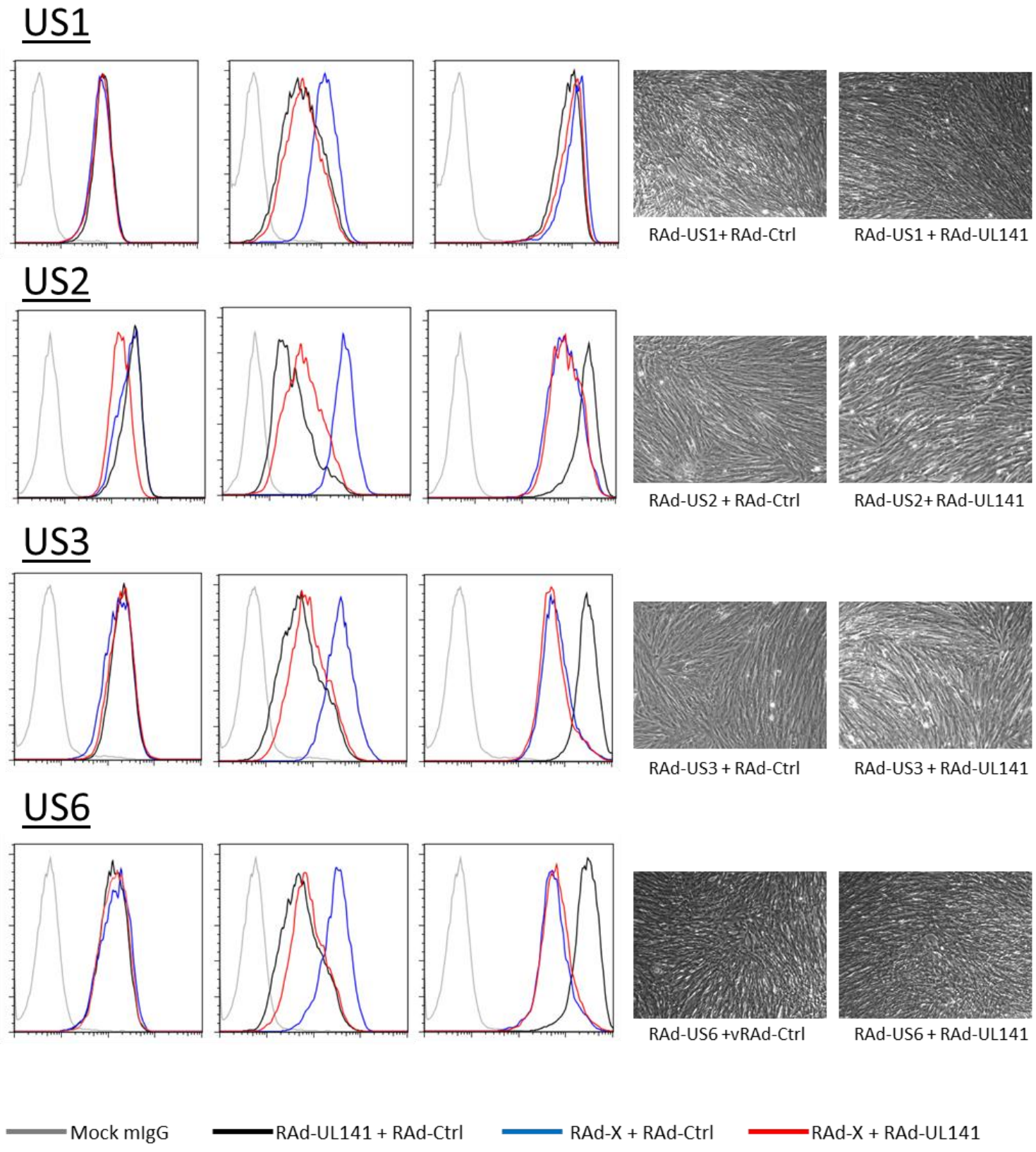


Figure 6.32. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-US1, RAd-US2, RAd-US3 and RAd-US6.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

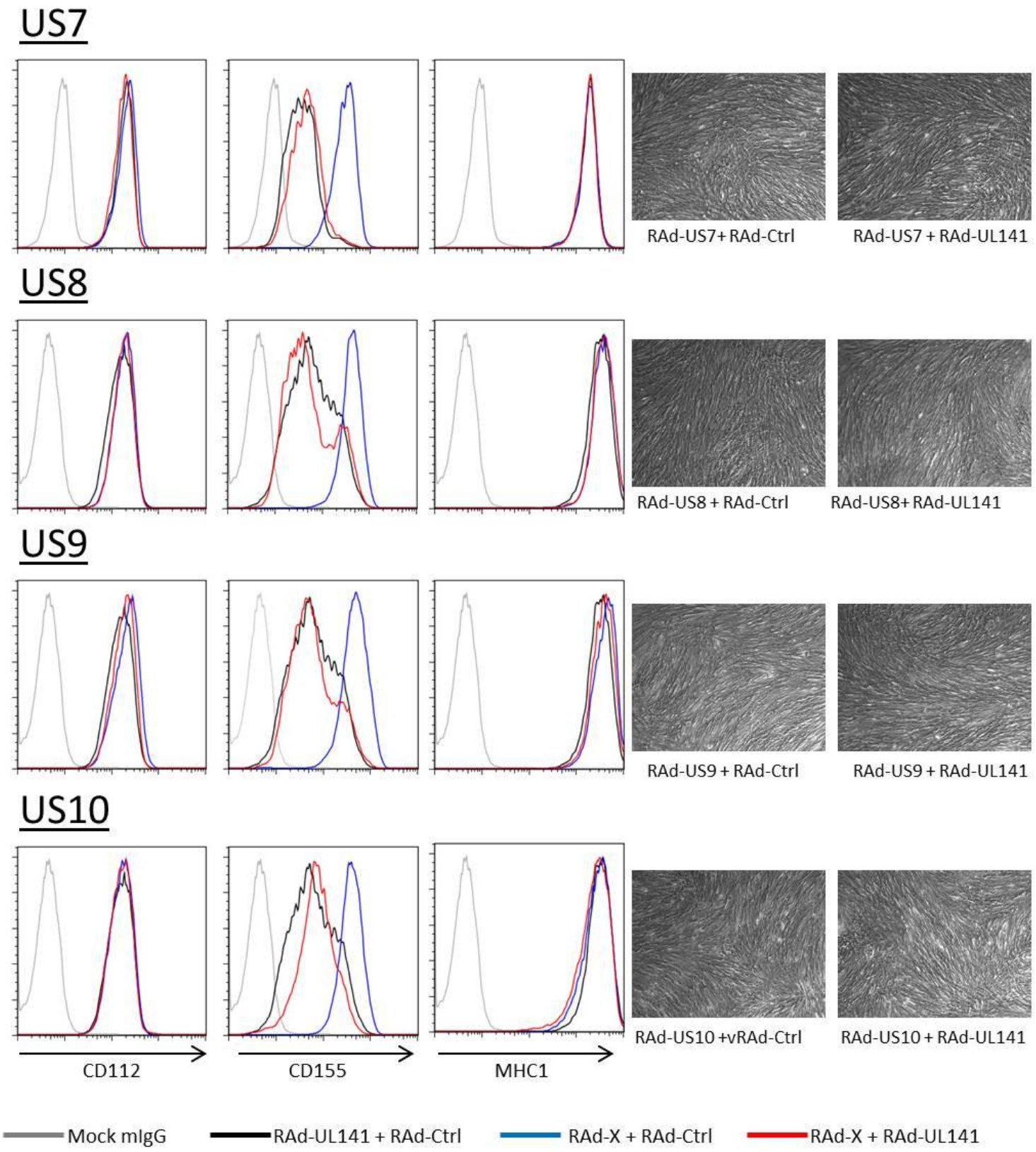


Figure 6.33. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-US7, RAd-US8, RAd-US9 and RAd-US10.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

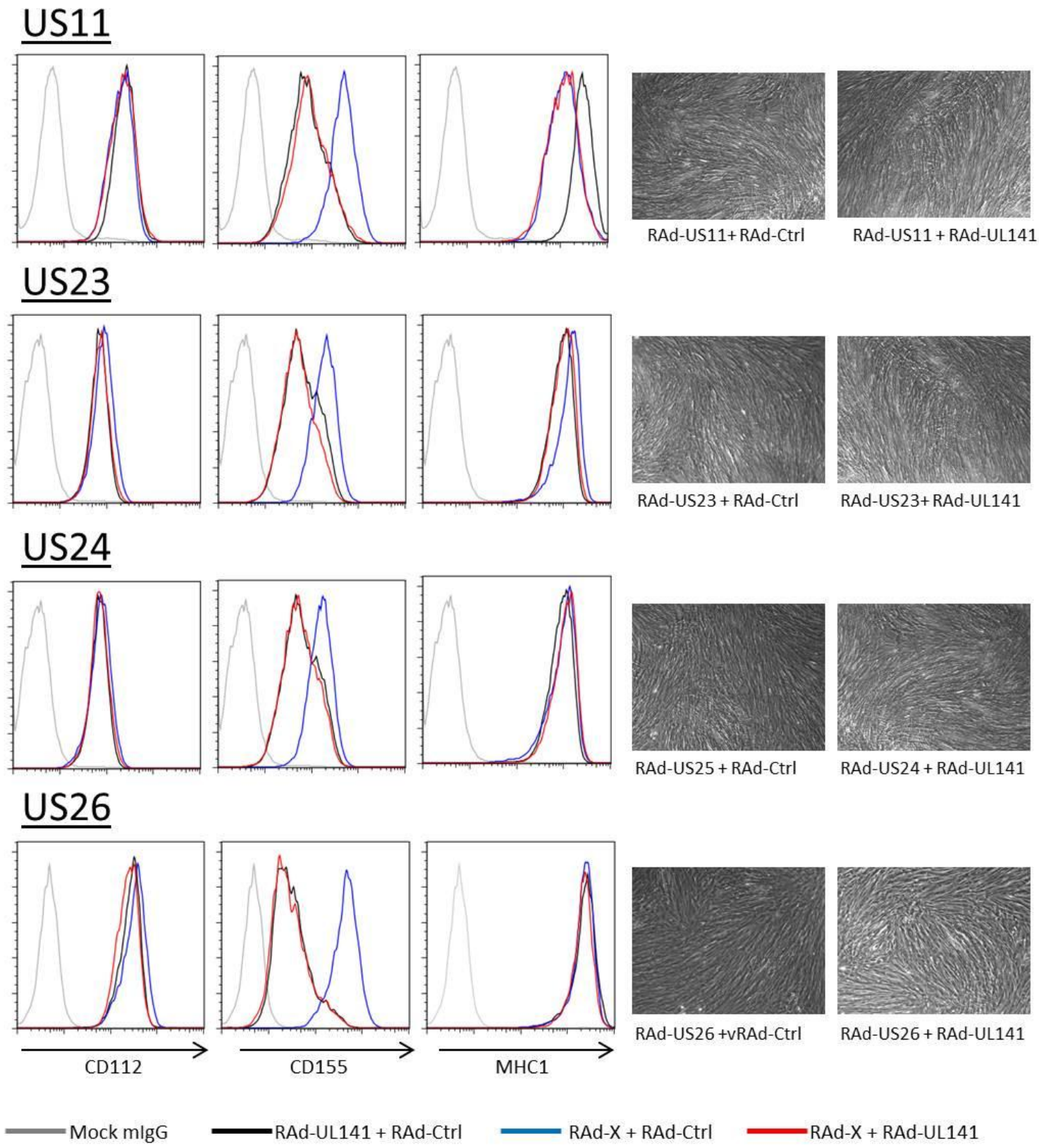


Figure 6.34. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-US11, RAd-US23, RAd-US24 and RAd-US26.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

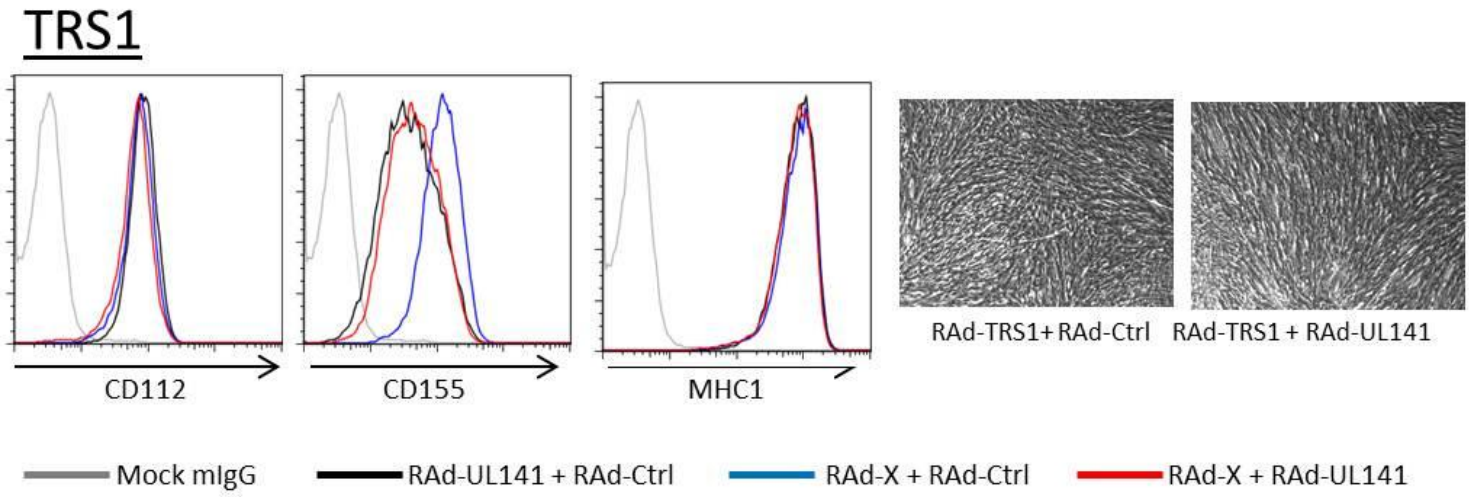


Figure 6.35. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-TRS1. HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy.

6.3. RAd-HCMV-ORF library screening results: CD155

Cell surface CD155 staining of infected cells was used as a positive control for the action of UL141, as CD155 is notably down-regulated from the cell surface during RAd-UL141 infection.

For the majority of conditions CD155 was down-regulated from the cell surface in infections where UL141 was present, namely UL141 + control RAd-1253 and the experimental condition of RAd-UL141 and RAd-X. Where UL141 was not present CD155 remained at similar levels as compared to control RAd-1253. However, there were two groups of genes with noticeable exceptions:

The first group consisted of seven genes that, when co-infected with UL141, showed a further decrease in cell surface expression of CD155 than when UL141 was expressed in isolation. These were RL12, RL13, UL7, UL16, UL22A, UL43 and UL114. These genes had no effect on CD155 expression levels when expressed on with empty RAd control (RAd-1253), which was important as it showed that the effect was specific and not a result of over-expression of these proteins. This was an interesting observation as it is possible that the expressed proteins may interact with UL141 to retain CD155 in the ER. UL16 has already been shown to sequester several proteins inside the ER. Alternatively these genes could be acting as positive regulators of the MIE promoter, which is used to control expression of UL141 in the RAd construct. Most of these genes have no described function in the transcriptional regulation. UL114 is a Uracil-DNA glycosylase, and is the only gene with described roles in the temporal regulation of DNA replication. UL22A is a secreted immunoregualtory RANTES binding decoy receptor. The remaining proteins have as yet no assigned function. UL43 is a tegument protein with no current function attached, while RL12, RL13 and UL7 are putative membrane glycoproteins and members of the RL11 family. All of these genes had no affect on CD112 or MHC-1. Further analysis of these onservations will now be the subject of an independent project.

The second group consisted of eight genes that, when co-infected with UL141, did not down-regulate CD155 to the same extent as RAd-UL141 + RAd-1253. These were UL25, UL27, UL28/29, UL31, UL33, UL44, UL97 and UL147. It is possibly that these proteins could either be directly or indirectly interacting with UL141 to prevent UL141 sequestering CD155. They could be acting as negative regulators of the MIE promoter, preventing UL141 expression and therefore reducing CD155 sequestration. Or there was

an titration error altering the ratio of viruses (UL141/X) from 10/10 to, for example, 10/100 which could mean there was less UL141 in the system. However, functions associated with these proteins do not appear to coincide with these hypotheses. Only two proteins have functions associated with DNA; UL44 a processivity subunit of DNA polymerase and UL97 Serine-threonine protein kinase that has roles in DNA synthesis, DNA packaging and nuclear egress. Another two proteins have assigned function. UL33 GPCR family and a membrane protein putative chemokine receptor and UL147 is putative CXC chemokine. While the remaining genes have no associated function; UL25 is a tegument phosphoprotein. UL27 is the locus to maribavir resistance. UL31 is a member of the DURP family. All of these genes appeared to have no affect on CD112 or MHC-1.

6.4. RAd-HCMV-ORF library screening results: MHC-1

There are four HCMV proteins US2, US3, US6 and US11 have all been shown by various groups to result in the down-regulation of MHC-1 from the cell surface. As expected these four proteins all showed a down-regulation of MHC-1 from the cell surface in RAd infections expressing these genes, figure 6.36. No other HCMV gene appeared have any effect of the cell surface levels of MHC-1. US3 and US6 resulted in a further down-regulation than US2 and US11. All had no affect on the cell surface expression of CD155.

6.5. RAd-HCMV-ORF library screening results: US2 appeared to be involved in CD112 down-regulation

Of the entire RAd-HMCV-ORF library there was only one gene that resulted in a further down-regulation of CD112 when co-infected with UL141, compared to RAd-UL141 infection alone, and that was US2, Figure 6.37. Although the shift was small, the result was highly reproducible and due to the robust CD112 staining seen in all negative results, this shift was deemed to be a positive hit. Both US2 and UL141 worked as expected as cell surface CD155 was down-regulated in the presence of UL141 and MHC-1 was down-regulated in the presence of US2. This confirmed not only that both transgenes were expressed, but also that co-infection of two RAd's could be successfully achieved. RAd-US2 had no affect on cell surface CD112 when expressed in the absence of RAd-UL141.

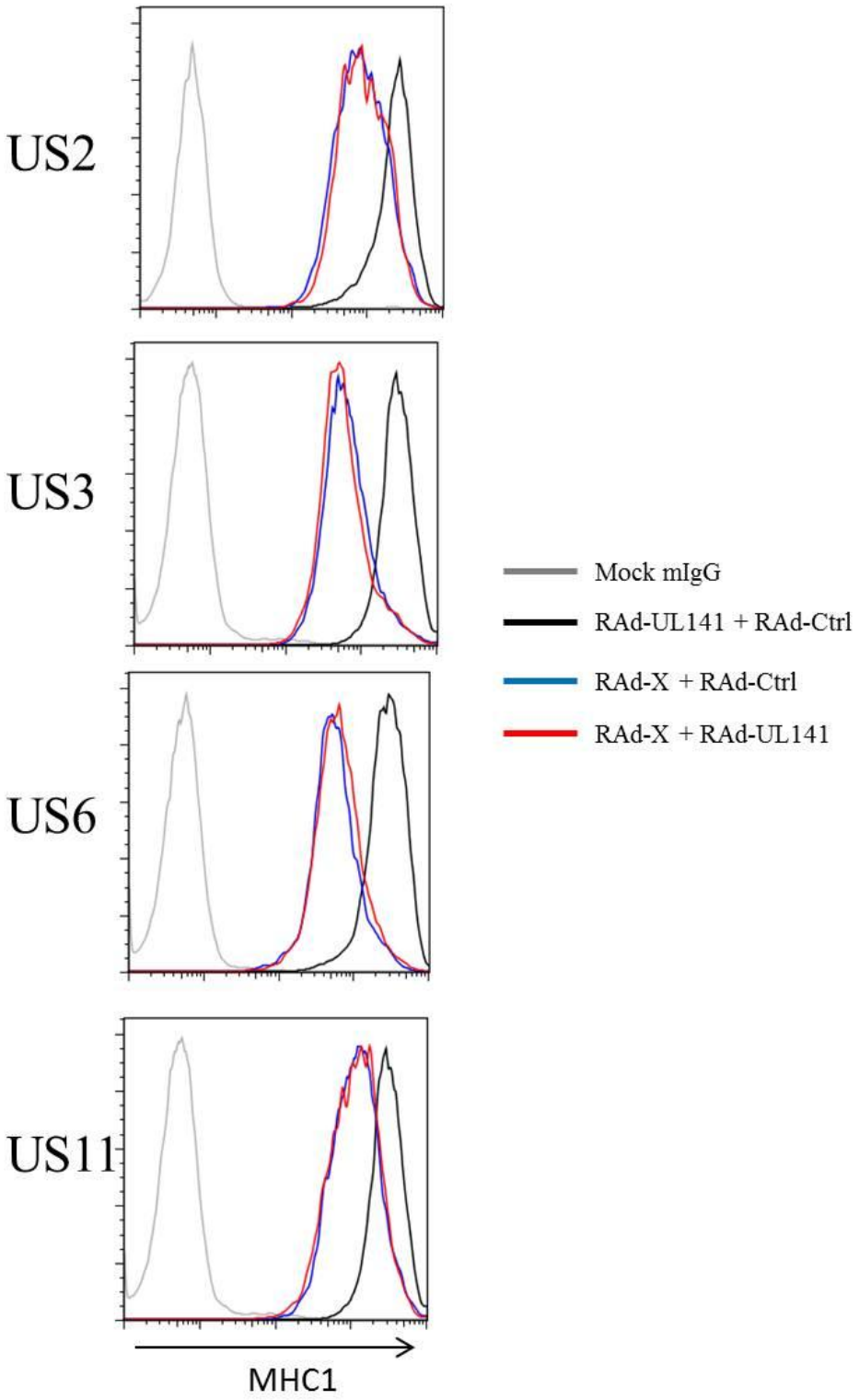


Figure 6.36. US2, US3, US6 and US11 downregulate MHC-1.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10), RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of MHC-1 was analysed 3 days p.i. by flow cytometry.

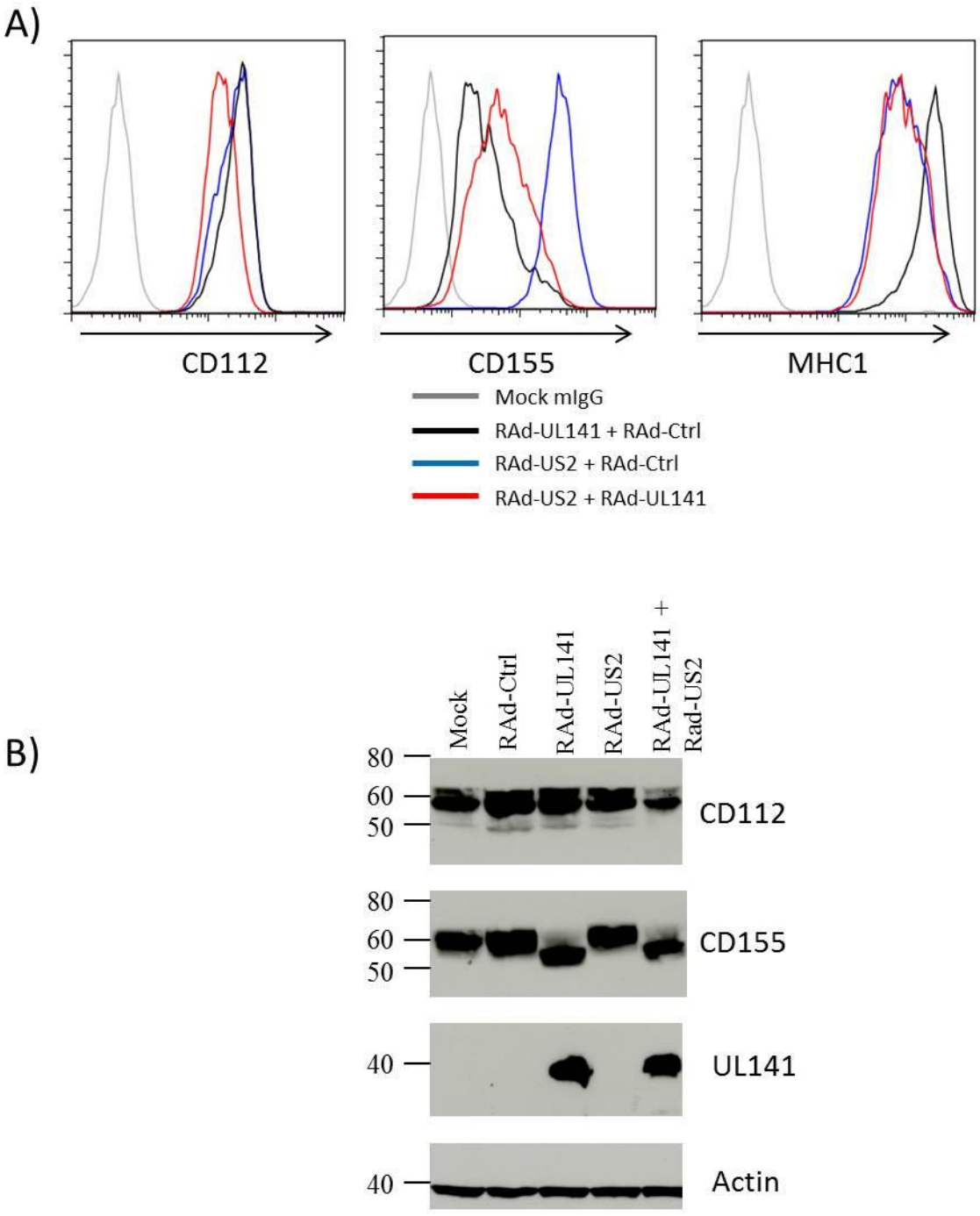


Figure 6.37. The coinfection of RAd-US2 and RAd-UL141 resulted in the downregulation of CD112.

HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10),** **RAd-US2 (MOI10) + RAd-Ctrl (MOI10)** or **RAd-US2 (MOI10) + RAd-UL141 (MOI10)** A) Infections were analysed by flow cytometry for the cell surface expression of CD112, CD155 and MHC-1. B) Infections were harvested and underwent partial purification using TritonX-114 before being analysed by immunoblot for CD112, CD155, UL141 and actin.

However, CD155 was not down-regulated to the same extent in RAd-UL141+RAd-US2 infection as compared to RAd-UL141 + RAd-Ctrl.

Experiments were also harvested and underwent partial purification using TritonX-114 fractionation. CD155 was found in a lower molecular weight form in all infections that contained UL141. During co-infection of US2 and UL141, CD112 appeared to be found at a reduced concentration compared to control samples. In particular the upper band of the two CD112 bands appeared to be markedly reduced compared to control samples. This correlated with observations seen in flow cytometry screening.

6.6. Conclusion

The cell surface down-regulation of CD112 by HCMV strain Merlin was confirmed in the in HFFF-hCAR cell line. This cell line was then used for the screening of the RAd-HCMV-ORF library to identify genes involved in CD112 down-regulation by flow cytometry. Screening involved the co-infection of UL141 with a RAd from the RAd-HCMV-ORF library in an attempt to reproduce the Merlin phenotype. Results identified US2 to be the only protein that resulted in a further down-regulation of CD112 when co-infected with UL141. Importantly both RAd-US2 and RAd-UL141 had no affect on CD112 when expressed individually, indicating they acted in concert. The shift in CD112 was small but was highly reproducible.

7.0. INVESTIGATION INTO THE US1-11 REGION OF HCMV FOR INVOLVEMENT IN CD112 DOWN-REGULATION.

Screening of the RAd-HCMV-ORF library identified US2 as a candidate for involvement in the cell surface down-regulation of CD112 during HCMV infection. In order to further evaluate whether US2 potentially played a role in CD112 down-regulation during HCMV infection, the recombineering technique was used to generate HCMV deletion mutants of US2, US11 and US1-11. It was hypothesised that if US2 was involved in CD112 down-regulation then its deletion would result in a loss of function, similar to HCMVΔUL141, chapter 3.0. US11 is highly homologous to US2 and both US2 and US11 target MHC-I for proteasome mediated degradation. A US11 deletion was also made as a negative control. In addition a deletion virus spanning the US1-11 region was also constructed as an additional negative control.

7.1. Generating HCMV deletion mutants using the technique of Recombineering

Dr R Stanton had previously developed a high efficiency system for the insertion/removal of genetic material from a HCMV BAC that eliminated the need for traditional cloning steps (Stanton, 2008, Stanton et al., 2010). This system utilised recombination mediated genetic engineering (recombineering) and made use of *E.coli* strain SW102 that contained a single copy HCMV BAC. The SW102 strain also contained a defective phage expressing lamda red genes, which were transiently induced by heatshock and mediated homologous recombination between the BAC vector and an oligonucleotide containing short arms of homology.

In order to generate HCMV deletion mutants two rounds of recombineering were required. During the first round, a selection cassette was amplified using 100bp primers. These primers contained 20bp that allowed amplification of a selection cassette and 80bp that allowed homologous recombination with a desired region of the HCMV genome. If the first round of recombineering was successful then the selection cassette was inserted into the desired part of the HCMV genome to be removed. A second round of recombineering

with a 100bp oligonucleotide, with 50bp designed to flank either side of the genomic region to be removed. A successful recombination event removed both the desired genomic material and also the selection cassette. Following plating onto selection plates a colony was indentified and deletion was confirmed by DNA sequence analysis and, where possible, PCR.

7.2. A HCMVΔUS1-11 mutant did not down-regulate CD112 in infected cells.

Two Merlin BACs were chosen to make HCMV deletions, 1111BAC and 1278BAC. The 1111BAC encoded the Merlin BAC genome, while 1278BAC encoded a modified BAC that contained deletions of UL16 and UL18 and GFP-tagged UL32. The 1278BAC was used to construct the HCMV block deletions utilised in chapter 4.0. The RCMVΔUS1-11 virus was therefore constructed in the 1278BAC background in order to integrate the virus into the existing HCMV block deletion library.

Initially the *SacB* selection cassette was chosen, as this was routinely used in the laboratory. The *SacB* selection cassette was ~4.5kb and contained, *AmpR* that conferred resistance to ampicillin, *SacB* that conferred sensitivity to sucrose and *LacZ* that encoded a β-galactosidase that turned blue when galactose is metabolised and allowed for blue/white colour selection.

During the first round of recombineering, the *SacB* selection cassette was correctly inserted into the US1-11 region, as evident by the presence of Blue colonies that contained the selection cassette, Figure 7.1A**.** During the second round of recombination the selection cassette was successfully removed. White colonies were picked, grown overnight, minipreped and analysed for the correct deletion of US1-11. A BamH1 restriction digest of genomic DNA showed that there was no gross genomic alteration compared to the parent RCMV1278 genome, Figure 7.1B. The minipreped BAC DNA was also sequenced to confirm the removal of the US1-11 region using the in-house CBS sequencing facility. The RCMVΔUS1-1 mutant later underwent deep sequencing of the entire genome carried out by Dr Andrew Davidson, Glasgow University, who confirmed not only that the US1-11 region was successfully deleted but that there was no other genetic mutation in the RCMVΔUS1-11 genome.

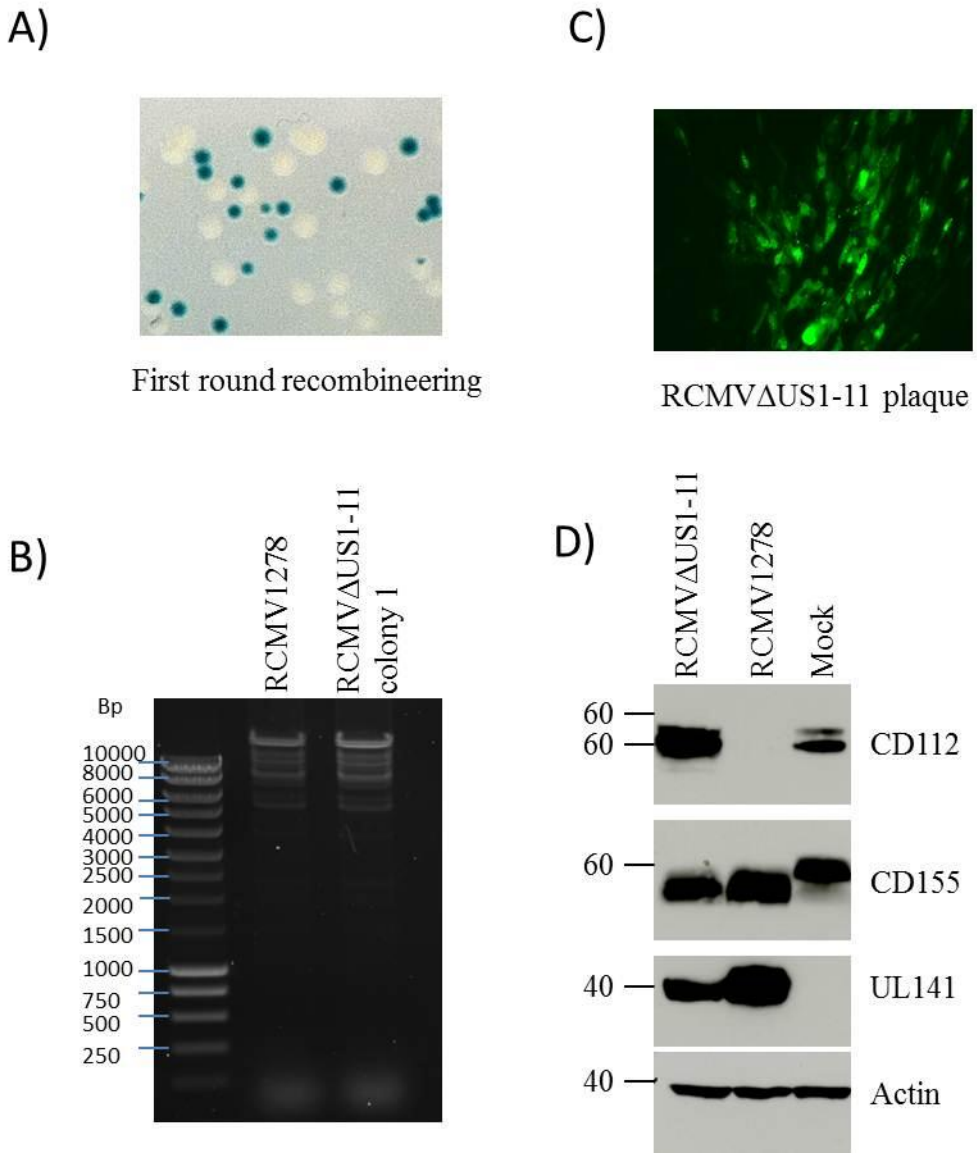


Figure 7.1. The construction of RCMV ΔUS1-11 using the recombineering technique and the *AmpR/SacB/LacZ* selection cassette.
A) Following the first round of recombineering, bacteria were plated onto selection plates. Successful insertion of the *AmpR/SacB/LacZ* selection cassette into the US1-11 region resulted in blue colonies. B) Minipred BAC DNA from RCMV1278 and RCMVΔUS1-11 colony1 were digested with BAMH1 restriction enzyme and digested sample ran on an agarose gel. C) Cells transfected with RCMVΔUS1-11 BAC and plaques visualised by fluorescent microscopy. D) HFFF-hTerts infected RCMVΔUS1-11 , RCMV1278 or uninfected (mock) were harvested 3 days p.i. and samples were prepared by tritonX-114 fractionation and the detergent fraction was analysed by immunblot for the detection of CD112, CD155, UL141 and Actin.

Once sequencing had confirmed the correct deletion, RCMVΔUS1-11 BAC DNA was maxipreped and transfected into HFFF-hTert's. As the parental BAC contained GFP-tagged UL32, plaques appeared green when visualised with fluorescence microscopy, Figure 7.1C. Once plaques were observed, infections were progressed and a viral stock generated.

Following the generation of a HCMVΔUS1-11 stock, HFFF-hTert's were infected at MOI10 and analysed by immunoblot for the detection of CD112, CD155, UL141 and actin, Figure 7.1D. Infected cells were also analysed by flow cytometry for the cell surface detection of CD112, CD155, MHC-1, MICA/B and MICB, Figure 7.2.

Immunoblot detection of CD112 showed that CD112 was clearly detectable in uninfected HFFF-hTert's, while in RCMV1278 (MerlinUL32GFPΔUL16ΔUL18) infected cells, CD112 was no longer detectable, Figure 7.1D. In HFFF-hTert's infected with RCMVΔUS1-11, CD112 was readily detectable and was detected at a higher concentration than found in uninfected HFFF-hTERt's. This indicated that a gene in the US1-11 region was essential for HCMV mediated down-regulation of CD112 as its deletion resulted in a loss of function.

CD155 was found in a lower molecular weight form as expected in both RCMV1278 (Merlin) and RCMVΔUS1-11 compared to uninfected cells. UL141 was also detected in HCMV infected but not uninfected cells as expected, Figure 7.1D.

Flow cytometry data confirmed the observations seen in immunoblot experiments, Figure 7.2. HFFF-hTert's were infected with RCMV1278 or RCMVΔUS1-11 and three days p.i. the cell surface expression of mIgG, CD112, CD155, MHC-1, MICA/B and MICB were analysed on the GFP high population, as per protocol developed for screening HCMV block deletions, chapter 4.0.

The cell surface expression of CD112 was down-regulated in RCMV1278 infected cells compared to uninfected HFFF-hTert's, Figure 7.2. This confirmed the observation that the US1-11 region was essential for HCMV mediated down-regulation of CD112 and deletion of US1-11 resulted in a loss of function.

MHC-1 was down-regulated in RCMV1278 infected cells but was not down-regulated in RCMVΔUS1-11 infected cells, Figure 7.2. This was as expected as all four HCMV genes involved in MHC-1 down-regulation (US2, US3, US6 and US11) were deleted in this

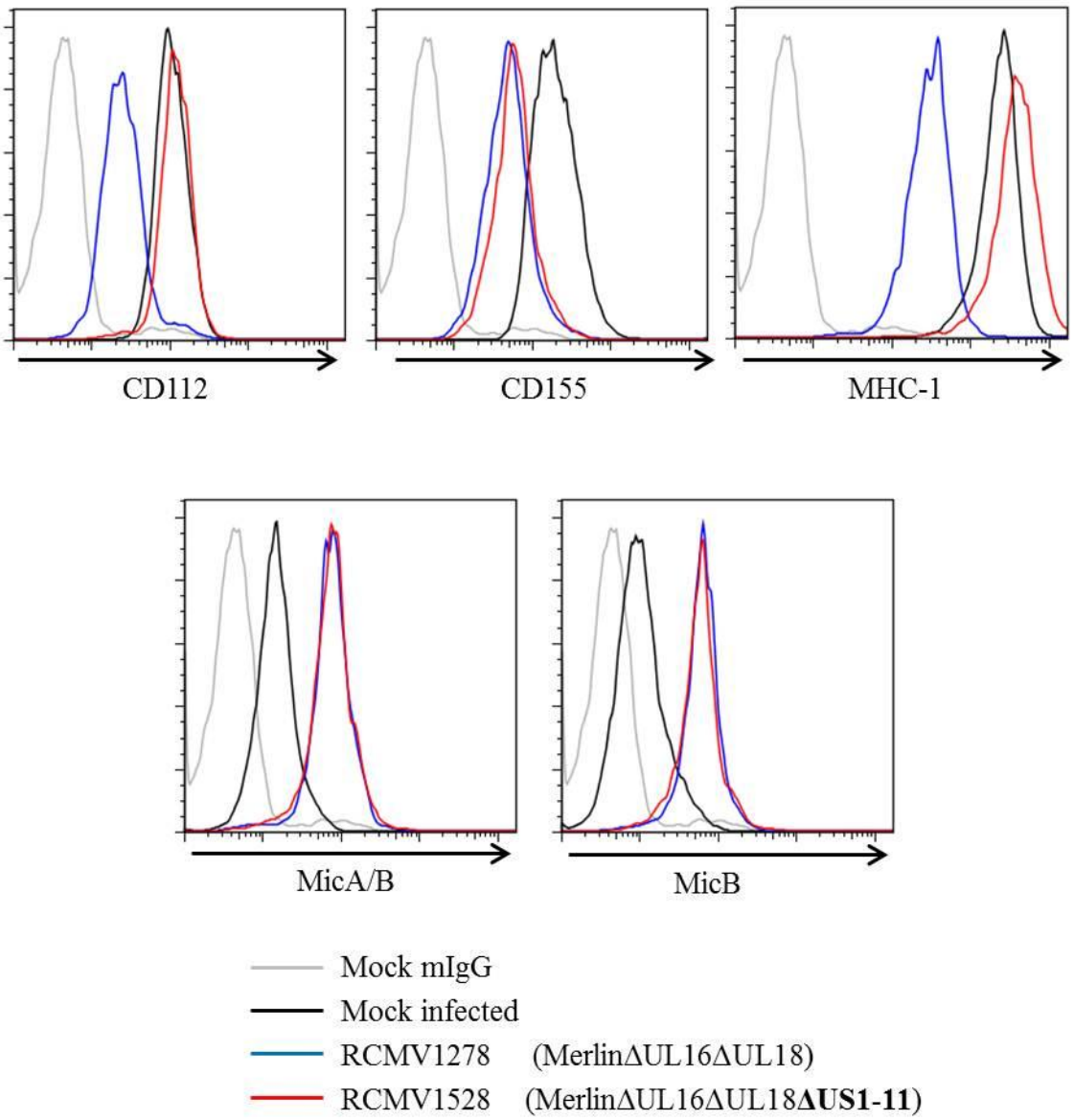


Figure 7.2. RCMV ΔUS1-11 does not downregulate CD112 or MHC-1.
1 x 10^6 HFFF-hTert's were infected with MOI of 10 of RCMV1278 (blue), RCMVΔUS1-11 (RCMV1528) (Red) or mock infected (black). 3 days p.i. Cells were harvested and analysed for the cell surface detection of CD112, CD155, MHC-1, MicA/B and MicB by flow cytometry.

RCMV mutant. The cell surface staining of MHC-1 therefore served as an additional control confirming the successful deletion of the US1-11 region.

During HCMV infection, the HCMV gene UL16 successfully sequesters several proteins, including MicB, in infected cells thus preventing their cell surface expression. As RCMV1278 and RCMVΔUS1-11 both contained UL16 deletions, then the MicB ligand would not be retained. The cell surface expression of MicB was analysed using antibodies that either recognised both MicA and MicB (MicA/B) or just MicB. Infection with either RCMV1278 or RCMVΔUS1-11 resulted in the up-regulation of MicB at the infected cells surface, compared to uninfected HFFF-hTert's, Figure 7.2.This observation served as an additional staining control confirming the functional deletion of UL16.

CD155 was down-regulated in both RCMV1278 and RCMVΔUS1-11 infected cells due to the presence of UL141 in these viruses.

7.3. Recombineering using the GALK selection cassette generated HCMVΔUS11 but not HCMVΔUS2.

Primers designed to create US2 and US11 deletions, successfully amplified the *SacB* selection cassette (data not shown). However, the first round of recombineering was unsuccessful and the *SacB* selection cassette was not inserted into US2 or US11. Another selection cassette was chosen that allowed for more stringent selection.

The *E.coli* galactose operon consists of four genes, *GalE*, *GalT*, *GalK* and *GalM*, that are necessary for the growth and the metabolism of galactose, when galactose is the only carbon source. The *GalK* product, galactokinase, catalyses the first step in the galactose degradation. The galactokinase also catalyses the phosphorylation of a glactose analog, 2-deoxygalactose-1-phosphate (DOG). The product of this reaction cannot be further metabolised, leading to a toxic build up. Thus both positive and negative selection is conferred by *GalK*, which is absent in the *E.coli* SW102 strain.

The *GalK* selection cassette was successfully amplified and inserted into US2 and US11 as evident by white colonies (data not shown). White colonies were picked and spread onto MacConkey Agar plates, where colonies that contained the *GalK* cassette fermented galactose present in the agar plates and appeared pink, Figure 7.3A. A functional *GalK*

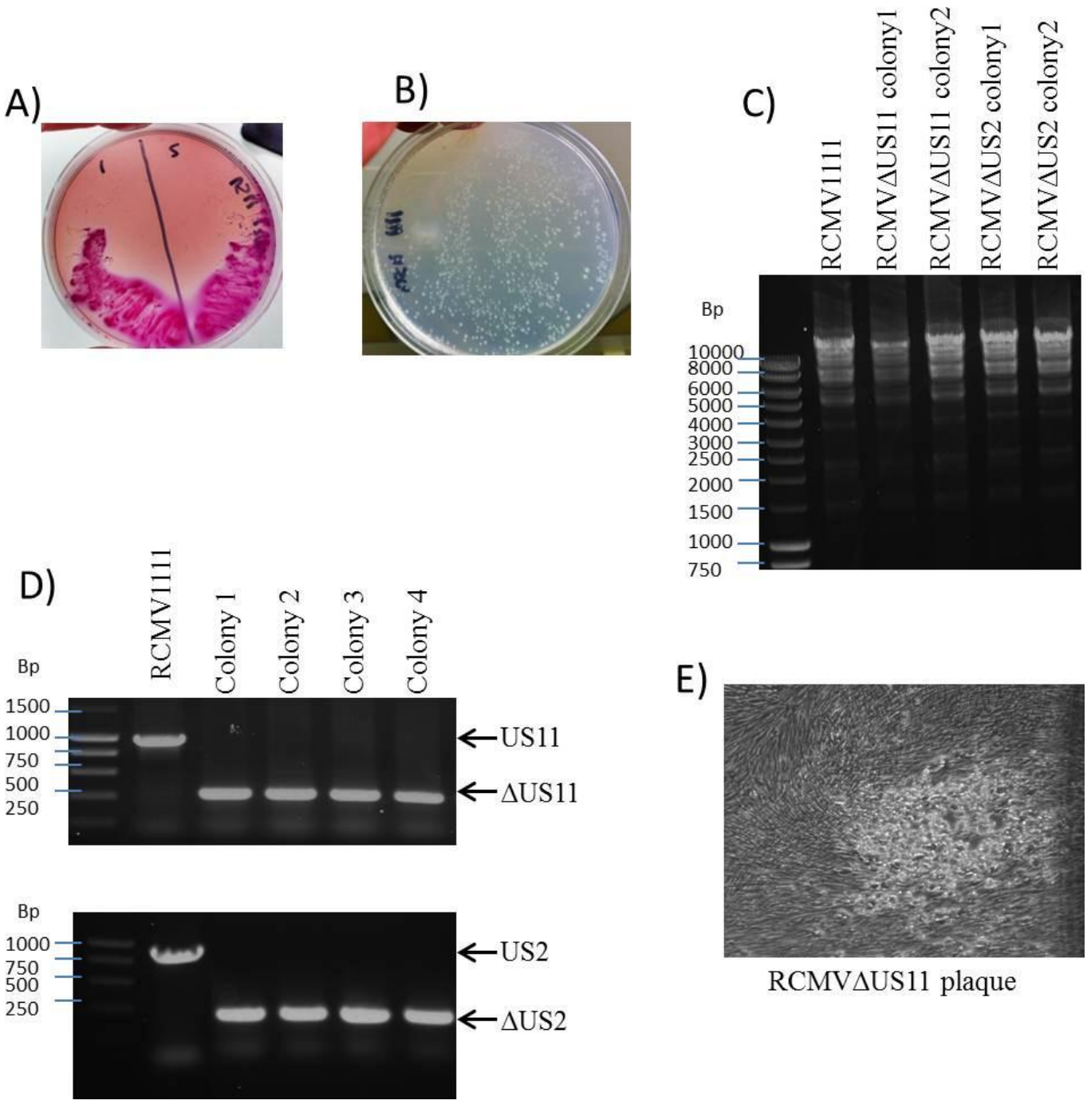


Figure 7.3. The construction of RCMV ΔUS11 using the recombineering technique and the GalK selection cassette.

A) Following the first round of recombineering, bacteria were plated onto selection plates. A white colony was then streaked onto MacConkey indicator plates to test for Functional GalK. B) Following the second round of recombineering,recovered bacteria were plated onto DOG plates. C) Minipred BAC DNA from RCMV1111 and two colonies representing RCMVΔUS11 and two colonies representing RCMVΔUS2 were digested with BAMH1 restriction enzyme and digested sample ran on an agarose gel. D) PCR reaction was carried out on minipreped BAC DNA from 4 colonies representing RCMVΔUS11 and 4 colonies representing RCMVΔUS2 and RCMV1111 as a control. PCR reactions were run on a 0.7% agarose gel. E) Cells transfected with RCMVΔUS11 BAC and plaques visualised by phase contrast microscopy.

was also confirmed by negative selection, where pink colonies were streaked onto DOG plates and did not grow (data not shown). The *GalK* selection cassette was then removed during the second round of recombineering, as evident by white colonies**,** Figure7.3B.

Colonies were picked and sequenced to confirm the correct deletion. A BamH1 restriction digest of two colonies of RCMVΔUS11 and two colonies of RCMVΔUS2 purified BAC showed that there were no gross genomic alterations, Figure7.3C. A PCR reaction of US11 showed that US11 had been successfully deleted in 4 colonies, Figure 7.3D. A PCR product of ~1042bp was expected if US11 was present and a PCR product of ~ 300bp if US11 was successfully deleted. A band of approximately 1kb was amplified from control RCMV1111BAC, a PCR product near the 250bp marker band was amplified from all 4 colonies, corresponding to the deletion of US11. A PCR reaction of US2 also showed that US2 had been successfully deleted in 4 colonies, Figure 7.3D. A PCR product of ~ 870bp was expected if US2 was present and ~147bp if US2 had been successfully deleted. A band in between the 750bp and 1kb marker was amplified from control RCMV1111 BAC DNA, a PCR product of less than 250bp was amplified from all 4 colonies, corresponding to the deletion of US2. Once sequencing confirmed a successful deletion, a colony was picked, BAC DNA maxipreped, transfected into HFFF-hTert's and observed for the development of HCMV plaques. After approximately 3weeks post transfection, the RCMVΔUS11 successfully generated plaques, Figure 7.3E, and a RCMVΔUS11 stock was generated. HFFF-hTert's transfected with RCMVΔUS2 BAC DNA did not produce plaques and a virus stock could not be generated.

Following the generation of a RCMVΔUS11 stock, HFFF-hTert's were infected with RCMV1111 (Merlin) and RCMVΔUS11 and three days p.i. the cell surface expression of mIgG, CD112, CD155 and MHC-1, were analysed by flow cytometry, Figure 7.4.

CD155 was down-regulated from the cell surface in both RCMV1111 (Merlin) and RCMVΔUS1-11 infections due to the presence of UL141. The cell surface of MHC-1 was also down-regulated to a similar degree by both RCMV111 and RCMVΔUS1-11. It appeared that the deletion of US11 had no effect on the ability of HCMVΔUS1-11 to downregulate MHC-1 from the cell surface of infected cells at 3 days p.i.

CD112 was down-regulated on both RCMV1111 (Merlin) infected cells and also RCMVΔUS11 infected cells. As there was no loss of function, and CD112 was still successfully down-regulated during RCMVΔUS11 infection, it was concluded that US11

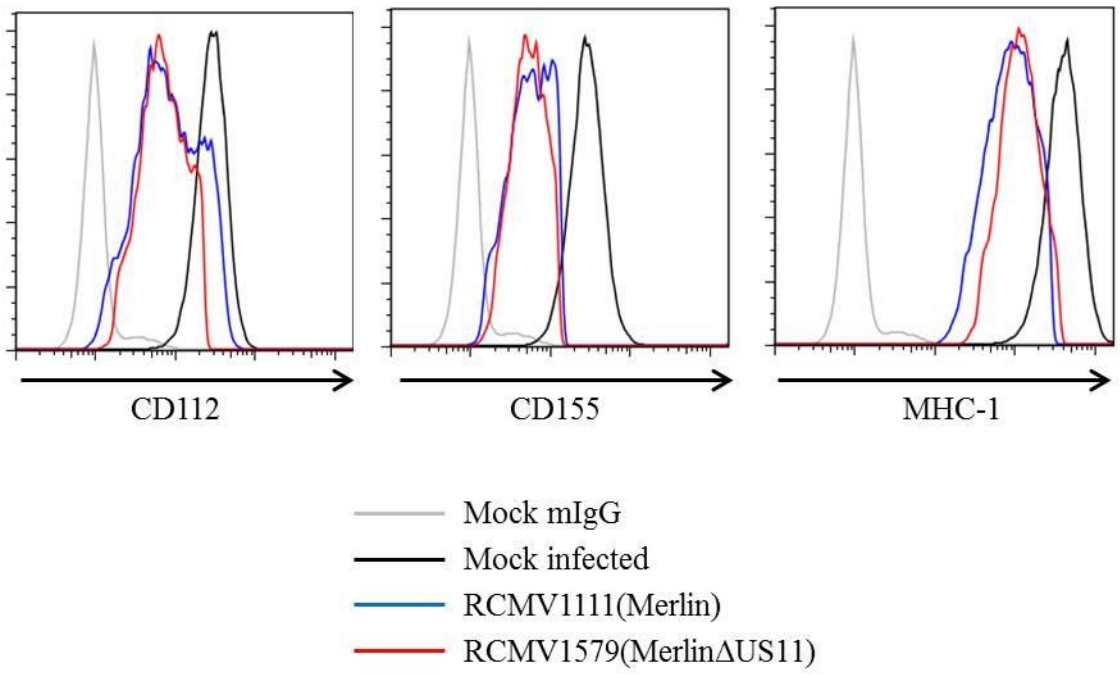


Figure 7.4. RCMV ΔUS11 is still capable of downregulating CD112.
1 x 10^6 HFFF-hTert's were infected with MOI of 10 of RCMV1111 (blue), RCMVΔUS11 (RCMV1579) (Red) or mock infected (black). 3 days p.i. Cells were harvested and analysed for the cell surface detection of CD112, CD155, MHC-1, MicA/B and MicB by flow cytometry.

did not play an essential role in the down-regulation of CD112 during HCMV infection and could be eliminated from future studies.

7.4. Recombineering using the Strep selection cassette did not generate HCMVΔUS2.

Although PCR and sequencing confirmed US2 had been deleted using the *GalK* selection system, Figure 7.3D, and a BAMH1 digest indicated that there were no gross genomic alterations, Figure 7.3C. A successful HCMVΔUS2 virus could not be generated. A third selection system was therefore tried. The *rpsL-Neo-LacZ* system takes advantage of the fact that the S12 ribosomal protein is the target of streptomycin. Most commonly used *E.coli* strains, including SW102, carry a mutation in the *rpsL* gene that confers streptomycin resistance. If the wild type *rpsL* gene is introduced then the strain will become streptomycin sensitive. The bacterial neomycin-kanamycin phosphotransferase, type II enzyme is encoded by the *Neo* gene and conferred resistance to kanamycin. When the *rpsL-Neo-LacZ* cassette is inserted colonies will be sensitive to streptomycin and resistant to Kanamycin. When the *rpsL-Neo-LacZ* cassette is lost, colonies will be resistant to streptomycin but sensitive to kanamycin. The presence of *LacZ* in the selection cassette allowed for blue/white screening of colonies.

The *rpsL-Neo-LacZ* selection cassette was successfully amplified (data not shown) and successfully inserted into US2 during the first round of recombineering, as evident by blue colonies, Figure 7.5A.The selection cassette was successfully removed during the second round of recombineering as evident by white colonies, Figure 7.5B. Colonies were picked, minipred and BAC DNA analysed for the correct deletion of US2.

A PCR reaction of US2 showed that US2 had been successfully deleted in 3/4 colonies, Figure 7.5D. A PCR product of ~897bp was expected is US2 was present and ~ 147bp if US2 was successfully deleted. A PCR product amplified from control RCMV1111 BAC DNA produced a product in between the 750bp and 1kb marker. A PCR product amplified from BAC DNA from 6/7 colonies, colony 1 and colonies 3-7, produced a product just less than the 250bp marker which corresponded to the deletion of US2. Colony 2 did not produce the expected PCR product and was eliminated from future work. Sequencing confirmed the successful

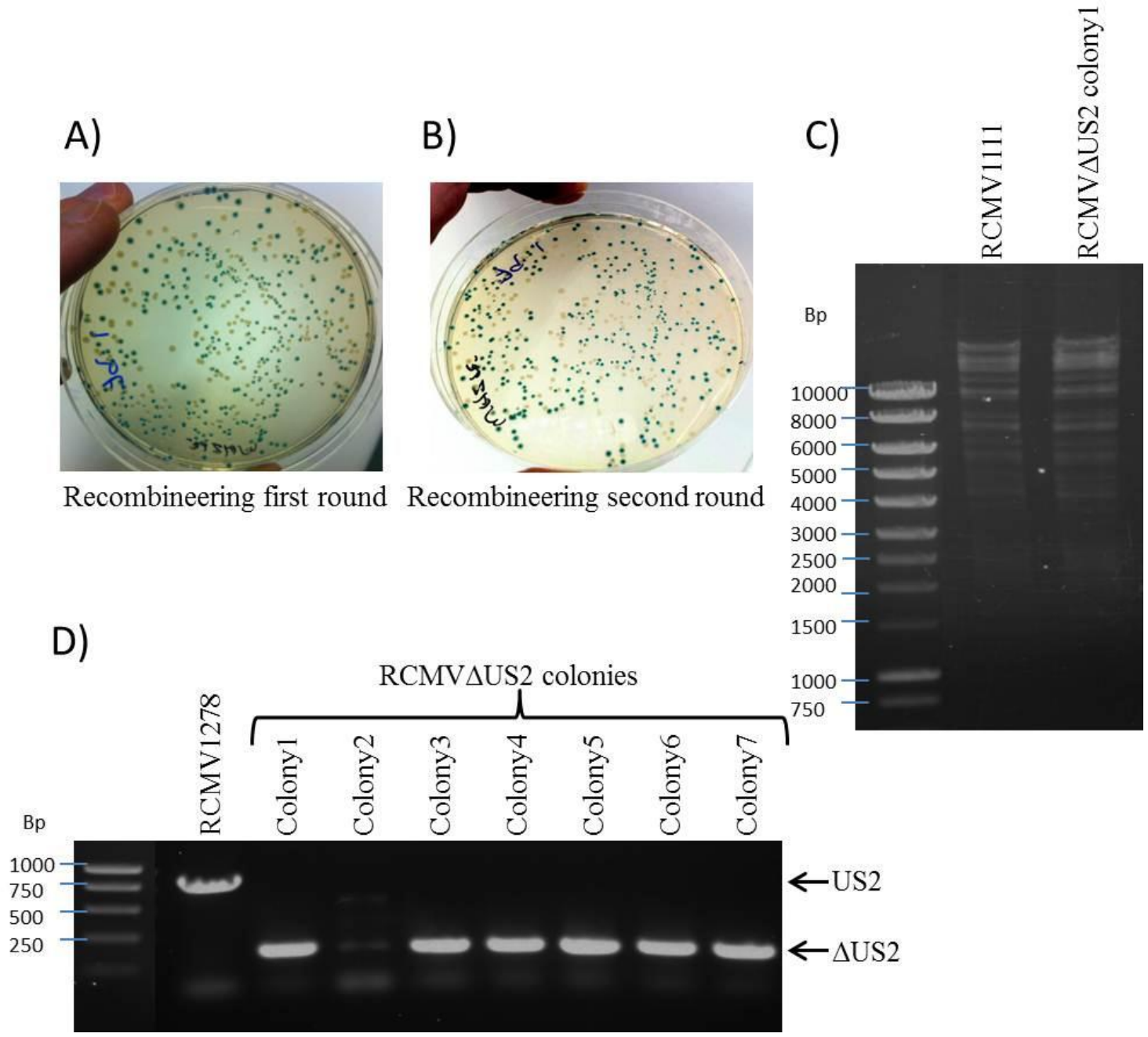


Figure 7.5. RCMV ΔUS2 could not be generated using the recombineering technique and the *RpsL-Neo-LacZ* selection cassette.

A) Following the first round of recombineering, bacteria were plated onto kanamycin selection plates. B) A blue colony from the first round of recombineering underwent a second round and once recovered was plated onto Streptomycin selection plates. C) Minipred BAC DNA from RCMV1111 and RCMVΔUS colony 1 were digested with BAMH1 restriction enzyme and digested sample ran on an agarose gel. D) PCR reaction was carried out on minipreped BAC DNA from 7 colonies representing RCMVΔUS2 following second round of recombination and plating onto streptomycin plates. RCMV1111 was used as a control. PCR reactions were ran on 0.5% agarose gel.

deletion of US2 (date not shown). A BAMH1 digest of colony 1 showed that there were no gross genomic alterations in the BAC DNA compared to RCMV1111, Figure7.5C.

Once sequencing confirmed a successful deletion, BAC DNA was maxipreped, transfected into HFFF-hTert's and observed for the development of HCMV plaques. However, HFFF-hTert's transfected with RCMVΔUS2 BAC DNA failed to produce plaques and a virus stock could not be generated.

7.5. Co-infection of RCMVΔUS1-11 with RAd expressing one of the US1-11 genes did not rescue the Merlin phenotype of CD112 down-regulation.

The Merlin phenotype of CD112 down-regulation could be rescued by co-infection of Merlin ΔUL141 with RAd-UL141, chapter 3.0, Figure 3.5. A similar result was expected when RMCVΔUS1-11 was co-infected with a RAd expressing one of the US1-11 ORFs.

Results are shown for US2, US3, US6 and US11, Figure 7.6. MHC-1 was down-regulated in all RAd infections (black line), compared to uninfected cells (purple line). However, when co-infected with RCMVΔUS1-11, MHC-1 is no longer efficiently down-regulated in cells infected with RAd-US2, RAd-US3, RAd-US6 or RAd-US11 (red line). As expected there was no down-regulation in cells infected with MerlinΔUS1-11 (blue line). The fact that MHC-1 is not down-regulated in the presence of one of these proteins perhaps explains why HCMV has 4 proteins targeting MHC-1 using different mechanisms. Or it is possible that co-infection of RAd and HCMV did not occur.

CD155 was down-regulated in all HCMV infections due to the presence of UL141. The Merlin phenotype of CD112 down-regulation could not be restored by co-infection of MerlinΔUS1-11 with any of the US1-11 RAd's. The cell surface expression of CD112 was equivalent between MerlinΔUS1-11 and MerlinΔUS1-11 + RAd-X, Figure 7.6.

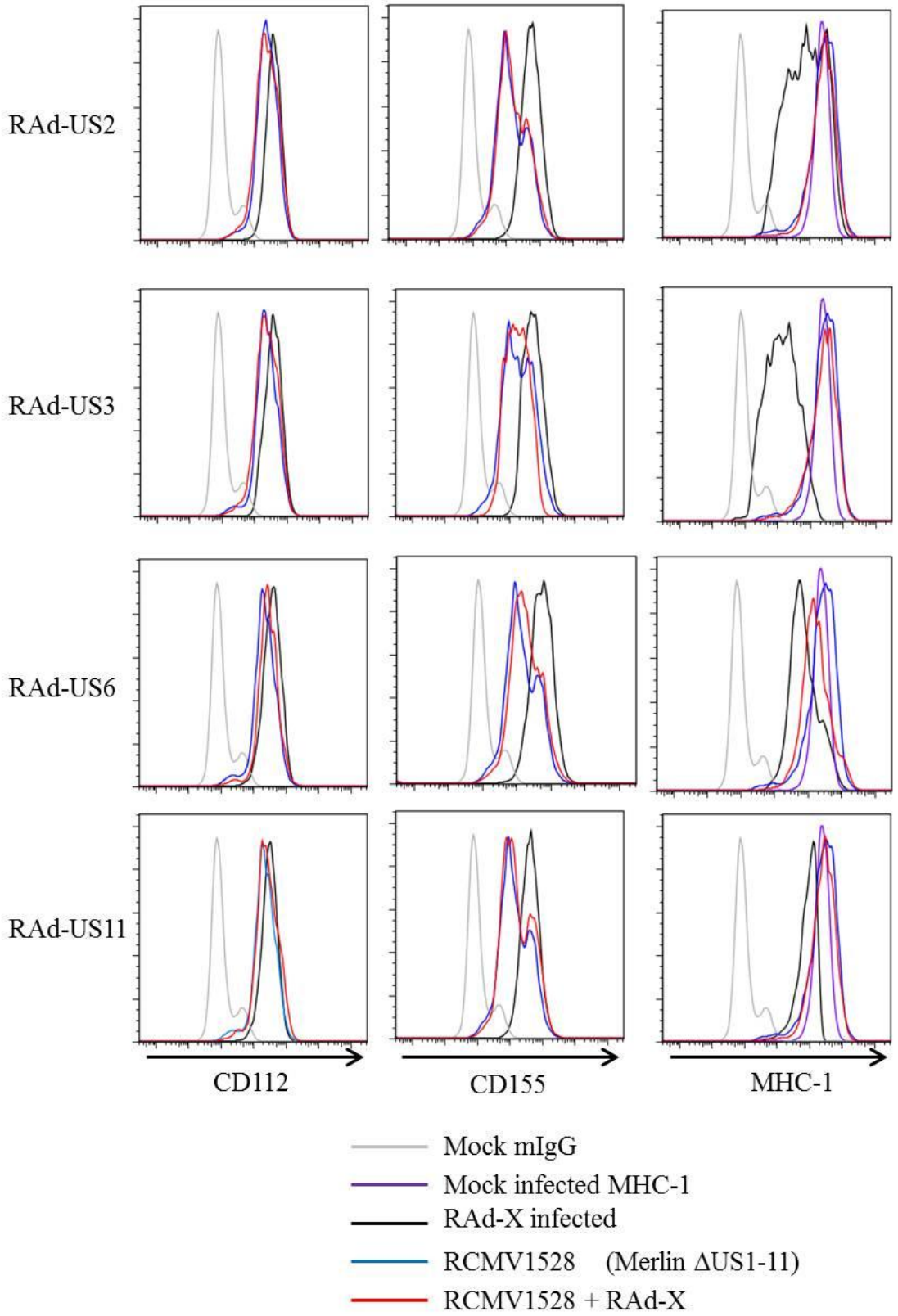


Figure 7.6. Coinfection of RAd with RCMVΔUS1-11 did not rescue the Merlin phenotype.

1 x 10^6 HFFF-hTert's were seeded into a 25cm^2 tissue culture flask and the following day infected with MOI of 10 of RCMV and/or MOI10 RAd. Infections consisted of RAd-X (**black**), where X denoted the RAd expressing genes labelled in the figure. RCMV1528 (RCMVΔUS1-11) (**blue**), RCMVΔUS1-11 + RAd-X (**Red**) or mock infected (**purple**). 3 days p.i. Cells were harvested and analysed for the cell surface detection of CD112, CD155 and MHC-1 by flow cytometry.

7.6. Infecting with multiple RAd's in the US1-11 region did not reproduce the Merlin phenotype.

The rescue function experiment whereby RCMVΔUS1-11 was co-infected with RAd expressing one of the US1-11 genes failed to reproduce the HCMV phenotype of CD112 down-regulation. It was hypothesised that more than one gene in the US1-11 region might be involved in this process. Triple RAd infections of UL141 + US2 + one of RAdUS1-11, denoted RAd-X in the figure legend, were carried out but there was no further down-regulation of CD112, compared to UL141 + US2 infection, Figure 7.7. CD155 was down-regulated as expected in all infections containing UL141, Figure 7.8. MHC-1 was also down-regulated in all conditions where US2 was present as expected, Figure 7.9. For those genes known to be involved in MHC-1 down-regulation (US3, US6 and US11) there was a further down-regulation of MHC-1 when co-infected with UL141 and US2 proving that three RAd's could be successfully co-infected into HFFF-hCAR's

7.7. CD112 was down-regulated in RAd-UL141 infected HELA-US2 cells, but not -UL141 infected Hela-US11.

The cell surface expression of CD112 was down-regulated in RAd-UL141 + RAd-US2 infected HFFF-hCAR's. To confirm this observation the cell surface expression of CD112 was analysed on a stable Hela cell lines expressing either US2 or US11, which were infected with RAd-UL141. A titration using a RAd-GFP identified an MOI100 was required to provide 100% infection, while infection at MOI200+ proved to be toxic to HELA cells, which resulted in cells becoming detached from the tissue culture flask (data not shown).

Hela cell lines were infected with RAd-Ctrl, RAd-UL14 or RAd-UL141 and 3 days p.i. the cell surface of mIgG, CD112, CD155 and MHC-1 was analysed by flow cytometry. UL14 was chosen as a control as UL14 and UL141 form the UL14 family, but importantly, RAd-UL14 had shown no effect on cell surface expression of CD112 during screening of the RAd-HCMV-ORF library, chapter 6.0. In Hela and Hela-US11 cells there was no effect on cell surface expression levels of CD112 when infected with RAd-Ctrl or RAd-UL14, Figure 7.10. There was a slight shift down in cell surface expression level of CD112 in

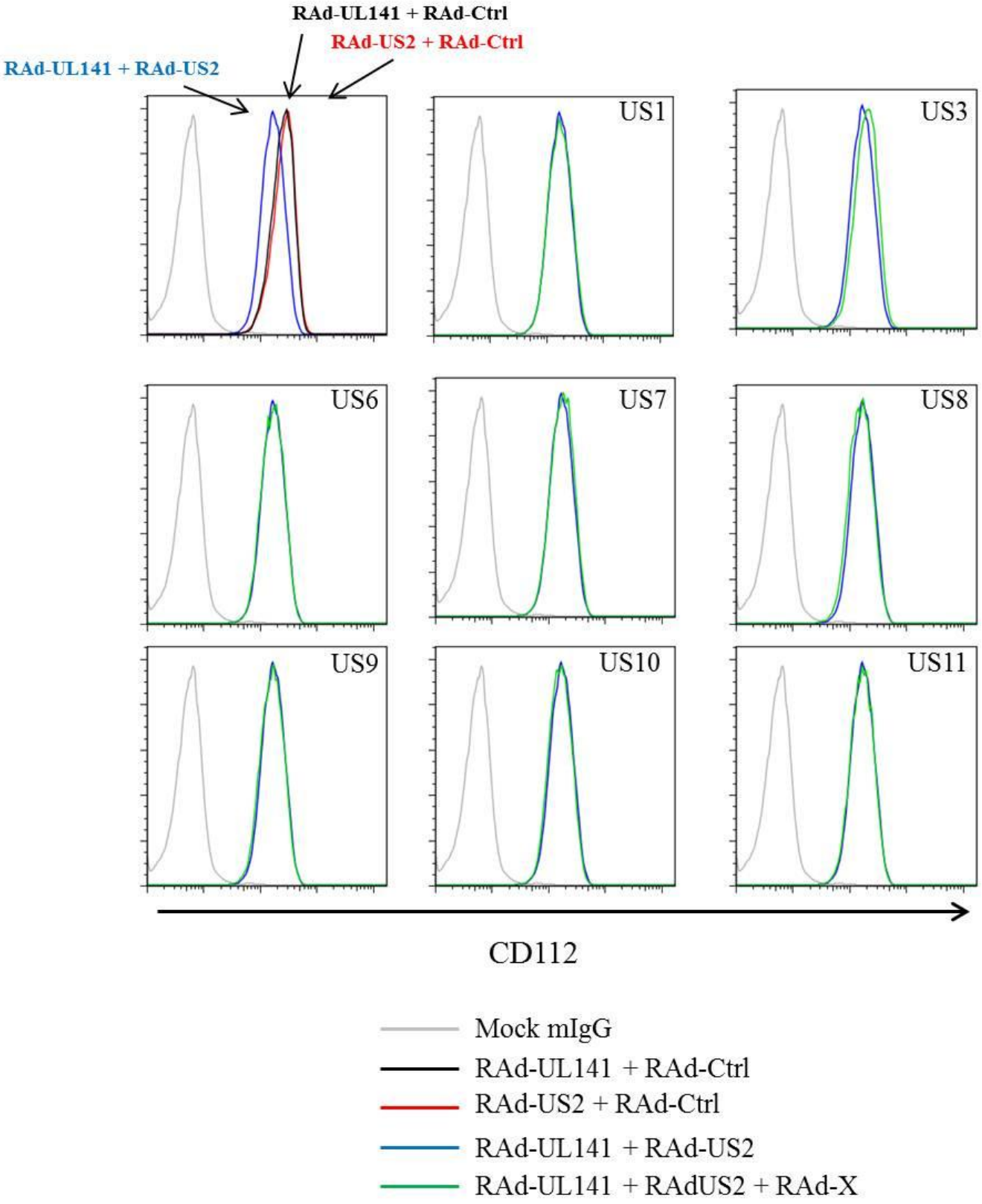


Figure 7.7. Flow cytometry detection of CD112 in HFFF-CARs infected with three RAds: US2, UL141 and one RAd covering the US1-11 region

1 x 10^6 HFFF-hTert's were seeded into a 25cm^2 tissue culture flask and the following day infected with RAd with a total MOI 15. Control conditions were RAd-UL141 (MOI5) + RAd-Ctrl (MOI10) (black), RAd-US2 (MOI5) + RAd-Ctrl (MOI10) (**Red**) and RAd-UL141 (MOI5) + RAd-US2 (MOI5) + RAd-Ctrl (MOI5) (**Blue**). Experimental condition consisted of RAd-UL141 (MOI5) + RAd-US2 (MOI5) + RAd- X (MOI5) (**Green**), where X denoted a RAd expressing one of the HCMV US1-11 ORFs as denoted in the figure. 3 days p.i. Cells were harvested and analysed for the cell surface detection of CD112 by flow cytometry.

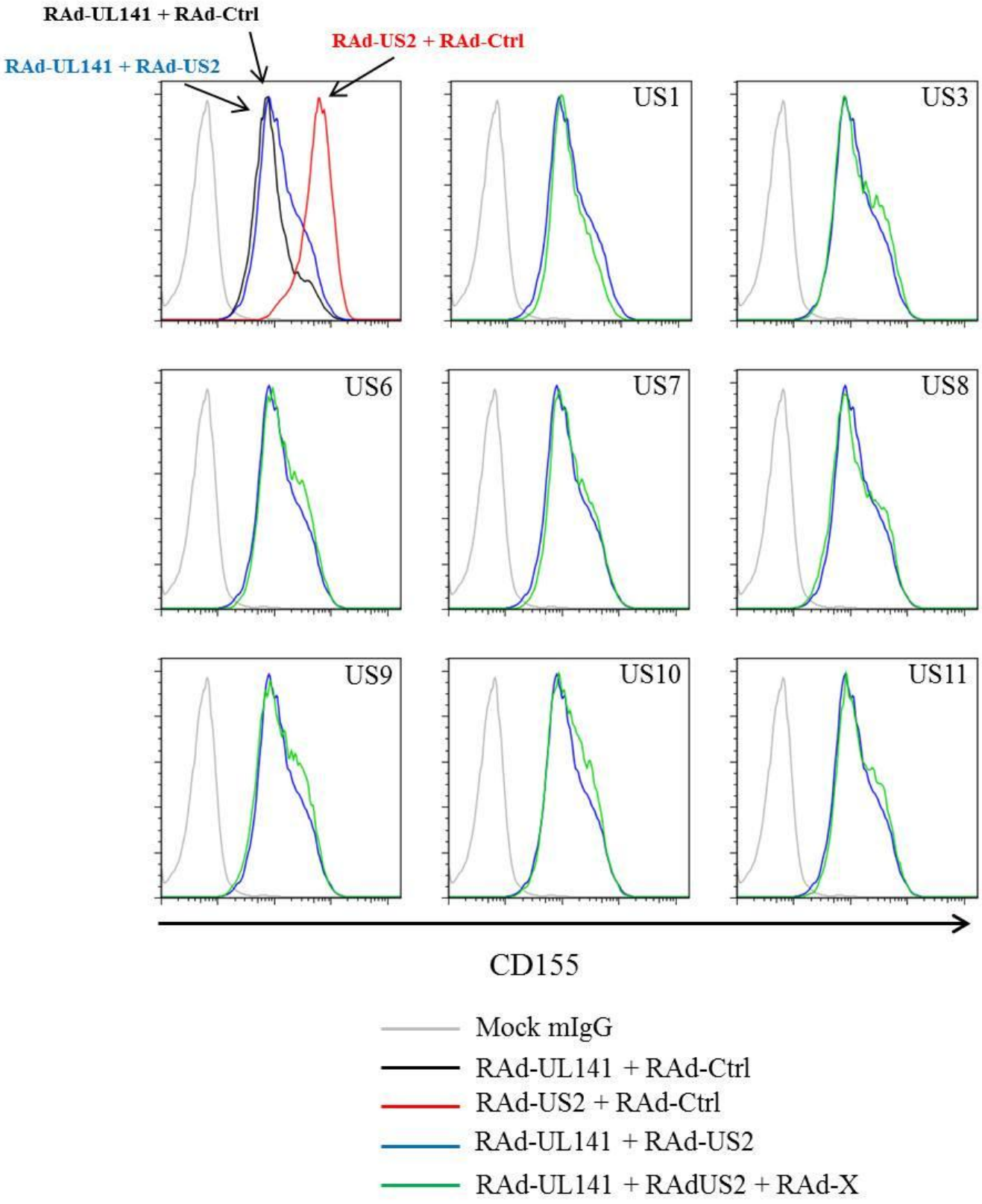


Figure 7.8. Flow cytometry detection of CD155 in HFFF-CARs infected with three RAds: US2, UL141 and one RAd covering the US1-11 region

1 x 10^6 HFFF-hTert's were seeded into a 25cm^2 tissue culture flask and the following day infected with RAd with a total MOI 15. Control conditions were RAd-UL141 (MOI5) + RAd-Ctrl (MOI10) (**black**), RAd-US2 (MOI5) + RAd-Ctrl (MOI10) (**Red**) and RAd-UL141 (MOI5) + RAd-US2 (MOI5) + RAd-Ctrl (MOI5) (**Blue**). Experimental condition consisted of RAd-UL141 (MOI5) + RAd-US2 (MOI5) + RAd- X (MOI5) (**Green**), where X denoted a RAd expressing one of the HCMV US1-11 ORFs as denoted in the figure. 3 days p.i. Cells were harvested and analysed for the cell surface detection of CD155 by flow cytometry.

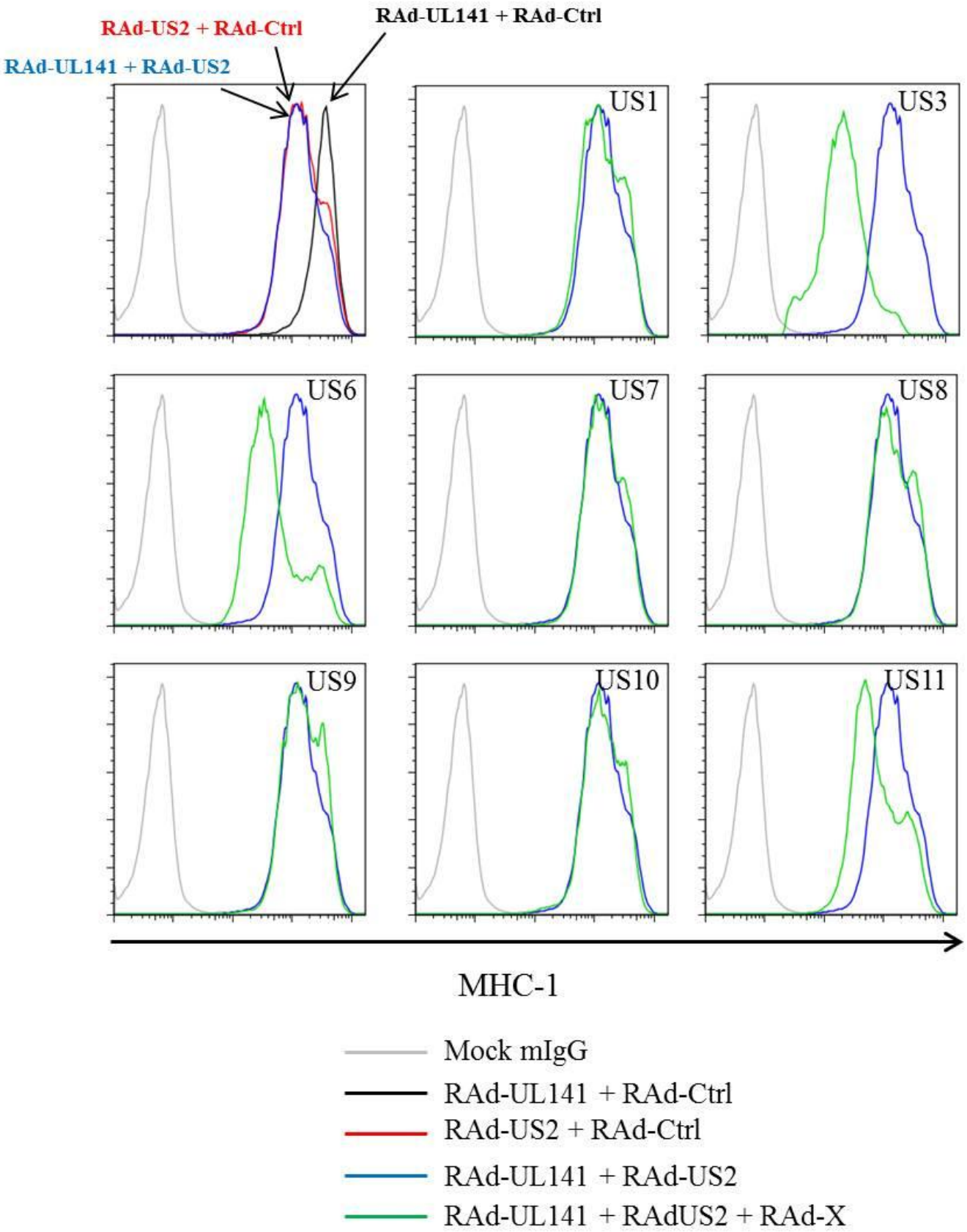


Figure 7.9. Flow cytometry detection of MHC-1 in HFFF-CARs infected with three RAds: US2, UL141 and one RAd covering the US1-11 region

1 x 10^6 HFFF-hTert's were seeded into a 25cm^2 tissue culture flask and the following day infected with RAd with a total MOI 15. Control conditions were RAd-UL141 (MOI5) + RAd-Ctrl (MOI10) (black), RAd-US2 (MOI5) + RAd-Ctrl (MOI10) (**Red**) and RAd-UL141 (MOI5) + RAd-US2 (MOI5) + RAd-Ctrl (MOI5) (**Blue**). Experimental condition consisted of RAd-UL141 (MOI5) + RAd-US2 (MOI5) + RAd- X (MOI5) (**Green**), where X denoted a RAd expressing one of the HCMV US1-11 ORFs as denoted in the figure. 3 days p.i. Cells were harvested and analysed for the cell surface detection of MHC-1 by flow cytometry.

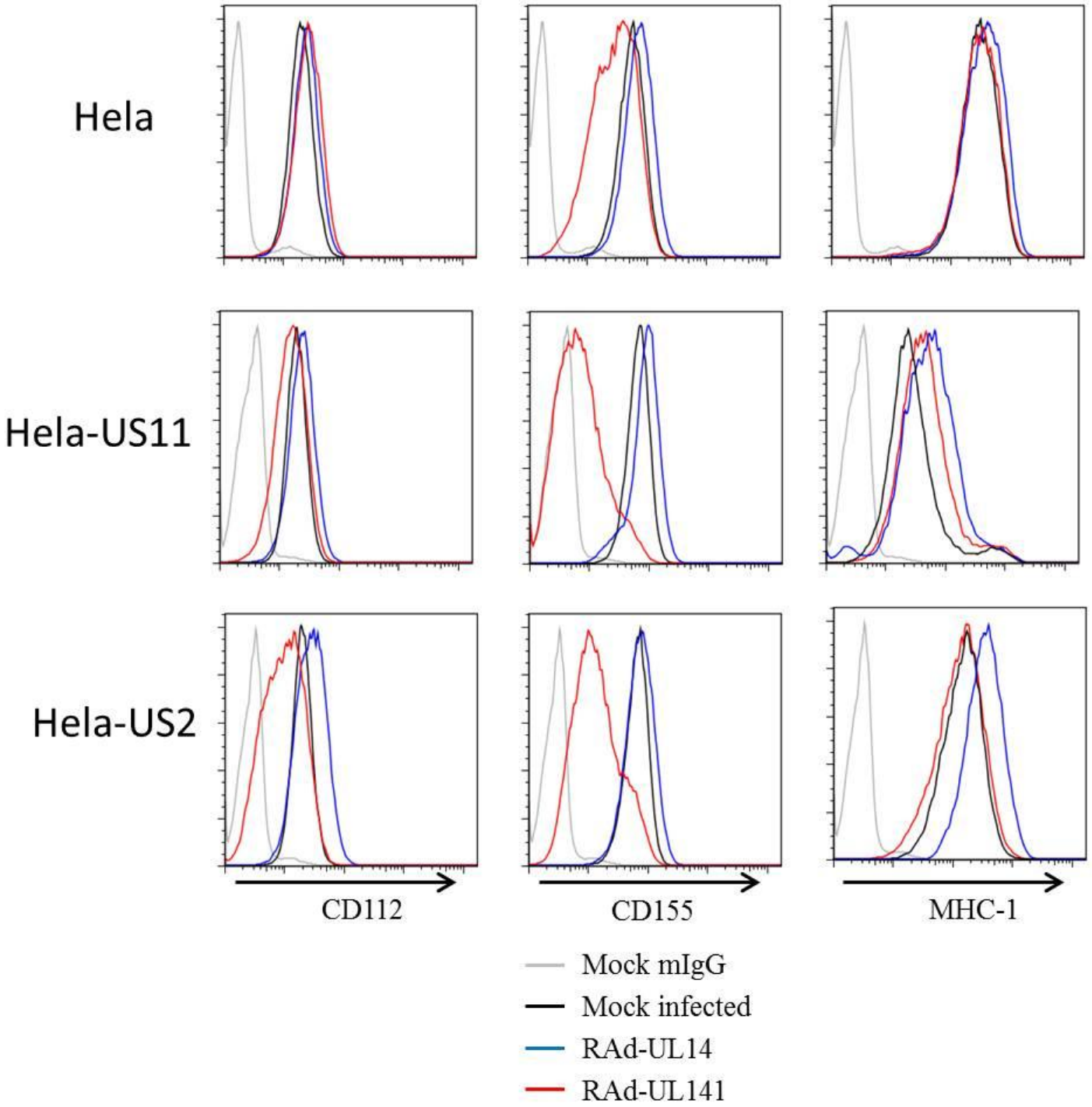


Figure 7.10. CD112 is downregulated in RAd-UL141 infected Hela-US2 cells.
2 x 10^6 Hela, HelaUS11 of Hela-US2 cells were seeded into a 25cm^2 tissue culture flask and the following day infected with RAd-UL14 (**Blue**), RAd-UL141 (**Red)** on Rad-Ctrl (**black**) at MOI100. 3 days p.i. Cells were harvested and analysed for the cell surface detection of CD112, CD155 and MHC-1 by flow cytometry.

Hela-US11 cell lines infected with UL141, but not in Hela cells. Previous data, Figure 6.34 and Figure 7.4., had already discounted US11 from being involved in CD112 cell surface down-regulation. Therefore this small shift was classified as background. In Hela-US2 cells infected with UL141, CD112 was notably down regulated from the cell surface of infected cells. This result confirmed previous observations that the co-expression of US2 and UL141 resulted in a down-regulation of cell surface CD112.

The cell surface expression of CD155 was equivalent in all three cell lines and CD155 was down-regulated in cells infected with RAd-UL141 but not RAd-UL14 or RAd-Ctrl.

The cell surface expression of MHC-1 was down regulated in HELA-US2 and HELA-US11 cell lines compared to HELA cells. MHC-1 was not affected in Hela cells infected with RAd-UL141 or RAd-UL14. MHC-1 was up-regulated in the Hela-US2 cell line when infected with RAd-UL14, but not RAd-UL141 or RAd-Ctrl. MHC-1 was up-regulated in Hela-US11 cells infected with either RAd-UL141 or RAd-UL14.

7.8. CD112 is degraded via the proteasome.

Results from screening RAd-HCMV-ORF library resulted in the identification of US2 as potentially being involved in HCMV mediated down-regulation of CD112. US2 is already known to be involved in MHC-I degradation via the proteasome. To determine whether CD112 was degraded via the proteasome HCMV infected HFFF-hTert's were treated with proteasome inhibitor MG132 overnight before harvesting at 72hours p.i. Harvested infections were processed by tritonX-114 fractionation and visualised by immunoblot, Figure 7.11.

CD112 could be detected in uninfected but not RCMV1111 (Merlin) infected cells. In RCMV1111 (Merlin) infections treated with proteasome inhibitor MG132, CD112 detection was partially restored. Restoration of CD112 appeared to be in a concentration dependant manner with a higher proteasome inhibitor concentration resulting in a larger amount of CD112 being detected. The proteasome inhibitor appeared to have no affect on CD112 in uninfected HFFF-hTert's.

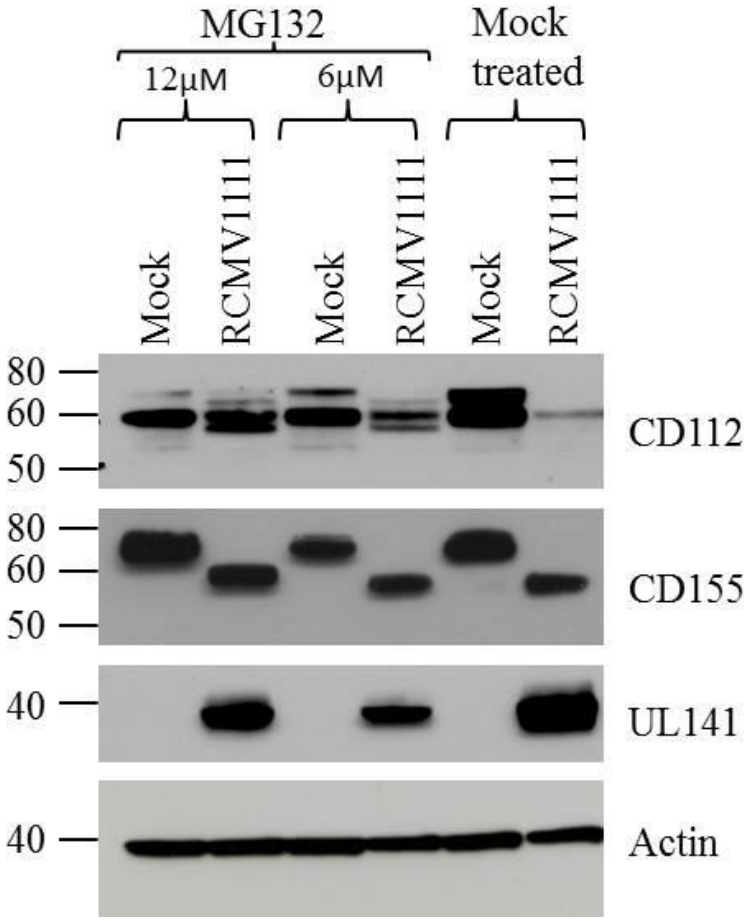


Figure 7.11. Proteasome inhibitors prevent the degradation of CD112 in HCMV infection of HFFF's.
HFFF were infected (MOI20) for 48 h with RCMV1111 (Merlin) or mock-infected, then treated for 12 h with proteasome inhibitors MG132 as indicated and analysed by immunoblot using antibodies to: CD112, CD155, UL141 and actin.

CD155 was found in a lower molecular weight form in RCMV1111 (Merlin) infected cells compared to uninfected cells. UL141 was clearly detectable in RCMV1111 (Merlin) infected cells but not in uninfected cells. The proteasome inhibitor appeared to have no affect on CD155 of UL141 uninfected or RCMV1111 infected HFFF-hTert's.

7.9 Conclusion

Using the recombineering technique RCMVΔUS1-11 and RCMVΔUS11 mutants were generated. A RCMVΔUS2 mutant could not be generated, despite utilising three different selection cassettes. The US2 gene was clearly deleted using both *GalK* and *RpsL-Neo-LacZ* selection cassettes as evident by both correct PCR products and sequencing results. However, a viral stock of RCMVΔUS2could not be generated when transfected into HFFF-hTerts, and no plaques were formed. The successful generation of a RCMVΔUS1-11 mutant showed that US2 is not an essential gene, as US2 was absent in this construct. It was hypothesised that the oligonucleotide used in the second round of recombineering, to simultaneously remove the selection cassette and delete US2, might also be causing genomic alteration elsewhere in the genome rendering the virus unviable. Sequencing of the entire BAC would reveal if this was the case, while new primers would need to be designed if recombineering were to be retried.

Deletions of US1-11 in RCMVΔUS1-11 resulted in loss of function and CD112 was unable to be down-regulated, identifying a gene in the US1-11 region to be involved in the down-regulation of CD112. US11 was unlikely to be essential in CD112 down-regulation as a US11 deletion mutant, RCMVΔUS11, retained the ability to down-regulate the cell surface expression of CD112. Additionally, infection of Hela –US11 with RAd-UL141 showed no notable CD112 down-regulation. However, CD112 was down-regulated in a Hela-US2 cell line infected with RAd-UL141.

Preventing proteasome degradation during HCMV infection by treating with proteasome inhibitors restored the ability to detect CD112 expression by western blot. US2 is known to degrade MHC-1 via the proteasome, and while this data does not prove the involvement of US2 in this process it adds weight to the hypothesis that US2 is involved.

It therefore appears that US2 and UL141 cooperatively act to degrade CD112 via the proteasome to prevent CD112 cell surface expression.

8.0. DISCUSSION

CD112 is involved in a variety of functions including cell adhesion, contact inhibition and NK-cell activation. Specifically, CD112 signals via CD226 (DNAM-1) activating receptor to modulate NK-cell cytotoxicity. Since the CD226-activating ligand, CD155, was down-regulated from the cell surface during HCMV infection (Tomasec, 2005) it was important to address whether CD112 was also down-regulated from the cell surface during productive HCMV infection.

Prior to commencement of this project, preliminary screening of the cell surface proteome of HCMV-infected cells performed in this laboratory identified that CD112 was down-regulated compared to uninfected cells. This initial observation was confirmed and the deletion of UL141 from the HCMV genome was able to restore CD112 cell surface expression in HCMVΔUL141-infected cells, as demonstrated in Chapter 3.0. The expression of UL141 on its own had no effect on CD112 cell surface expression (Tomasec et al 2005 and chapter 3.0.). Since UL141 appeared necessary but not sufficient to modulate CD112 expression, this thesis focused on a hypothesis whereby UL141 was acting in a conjunction with another HCMV protein. Two complementary approaches were initially projected to identify HCMV genes essential in CD112 down-regulation:

The first approach was to screen a library of HCMV block deletion mutants for a loss of the ability to down-regulate CD112, which anticipated that a second HCMV gene acting with UL141 played an essential role in CD112 down-regulation and its deletion would restore CD112 cell surface expression. If a result scored then this approach would identify a block of genes potentially involved in CD112 down-regulation, narrowing down potential gene candidates for future studies. This approach was the focus of chapter 4.0

The second approach was the screening of a library of RAd vectors encoding individual HCMV ORFs by co-infecting with RAd-UL141 for a gain of the ability to down-regulate CD112. This approach anticipated that a combination of two specific HCMV genes would be sufficient for this effect. This approach would specifically identify a second essential HCMV gene and was the focus of chapter 6.0.

Both approaches were predicted to independently identify gene(s) involved in CD112 down-regulation. By employing two complementary approaches, the probability of a

successful outcome was increased along with confidence in the result if both approaches delivered the same result.

Screening of a library of a HCMV-block deletion mutants did not identify any potential candidates. Results from chapter 4.0 showed that all RCMVs down-regulated the cell surface expression of CD112 to a similar degree as Merlin infected cells. As a result, the 46 genes deleted in these mutants were screened as a low priority in further analysis. The remaining genes were screened using a RAd-HCMV-ORF library, chapter 6.0. The HCMV genes were screened both individually and also in conjunction with UL141. The co-infection of RAd-UL141 and RAd-US2 resulted in a single, clear, positive hit in the final screening process. Although the shift in cell surface down-regulation in RAdUL141/US2 infected cells was not of the same magnitude as during HCMV infection it was robust and highly reproducible. A down-regulation of CD112 was also seen immunoblots where RAd-UL141 and RAd-US2 were co-infected but not in RAd-UL141 or RAd-US2 infected HFFF-hCAR's, chapter 6.0. A Hela-US2 cell line that stably expressed US2 also down-regulated CD112 when infected with RAd-UL141, but not RAd-UL14 or RAd-Ctrl, chapter 7.0. However, the possibility still exists that more than 2 HCMV genes and/or an additional HCMV-upregulated cellular function is required to realise full CD112 down-regulation seen in productive HCMV infection.

In order to further these observations, HCMV mutants with deletions in US2, US11 or US1-11 were constructed. Despite multiple attempts, using three different recombineering strategies with selection cassettes (*AmpR/SacB/LacZ*, *GalK* and *RpsL/Neo/LacZ*) a HCMVΔUS2 mutant could not be generated. However RCMVΔUS11 and RCMVΔUS1-11 mutants were successfully constructed, chapter 7.0.

Deletions in the US1-11 region in RCMVΔUS1-11 resulted in a loss of function and CD112 was unable to be fully down-regulated from the cell surface of RCMVΔUS1-11 infected cells. This phenotype indicated that a gene in the US1-11 region was essential for the down-regulation of cell surface CD112. A RCMVΔUS11 was successfully generated and crucially was still capable of down-regulating the cell surface expression of CD112 during productive infection, Figure 7.4. RAd-US11 also scores negatively in the screening of the RAd-HCMV-ORF library when co-infected with RAd-UL141, Figure 6.34. As a consequence, US11 was excluded as a candidate for involvement in CD112 down-regulation.

The addition of proteasome inhibitors to HCMV infections resulted in the partial recovery of CD112 in a concentration dependant manner, chapter 7.0. This result suggested some obvious routes for further investigation. It is well documented that both US2 and US11 degrade MHC class I via the proteasome. Provided that CD112 and MHC-I are degraded via the same mechanism then the knockdown of essential proteins known to be involved in MHC-I degradation pathways could be investigated. Protein disulphide isomerise (PDI) is involved and is essential in US2 mediated MHC-I down-regulation during HCMV infection (Lee et al., 2010). Derlin-1 is an ER membrane protein essential in US11 dependant degradation of MHC-1 during HCMV infection (Lilley and Ploegh, 2004). Therefore, through RNA interference, siRNAs could be used to knock down PDI and Derlin-1 to see if these pathways are involved in the degradation of CD112.

It is worth noting that UL141 is involved in the down-regulation of both CD155 via ER sequestration and CD112 through proteasome mediate degradation. Why CD155 is retained, while CD112 is degraded remains unanswered to date. One possibility is that sequestered CD155 still has some signalling ability which is preferential for HCMV. The fact that Ul141 both sequesters and is involved in targeted degradation suggests that UL141 is a multifunctional protein. Indeed, UL141 has also recently been reported to be involved in the down-regulation of TRAILR1 and TRAILR2 (Smith et al., Under submission). The fact that both DNAM-1 activating ligands are removed from the surface of infected cells again demonstrates the importance of this activating receptor in the context of NK cytotoxicity during HCMV infection. However these experiments become complicated when looked at in the context of HCMV infection as using a HCMVΔUL141 deletion virus would affect CD155, CD112, TRAILR1 and TRAILR2. While expressing UL141 in isolation from RAd-UL141 would affect CD155, TRAILR1 and TRAILR2 but not CD112. Due to the effect of UL141 on a range of NK cell activating ligands it will be essential to dissect these functions apart in order to study the importance of CD112 down-regulation in functional NK assays. Functional domain mapping using a series of UL141 deletions and modifications might identify domains/residues that are essential for one, or multiple UL141 functions and is something to be considered if this work is to be taken further.

8.1. Use of RAd-HCMV-ORF library in screening for HCMV function.

This project was the first to utilise the entire RAd-HCMV-ORF library to screen for function. The RAd-HCMV-ORF library clearly provided an extremely powerful tool for the screening of HCMV gene function as results were highly repeatable and robust. During optimisation it was important to take practical steps to reduce signs of toxicity, chapter 6.0., to reduce false positive hits. Some toxic genes fell into regions deleted in the RCMV block deletion mutants. As all RCMV block deletions were capable of down-regulating CD112, it was unlikely that these genes played an essential role in CD112 down-regulation. For the remaining genes that demonstrated signs of toxicity, results were excluded from analysis due to the likelihood of a false positive result. However, potentially one of these genes could be play a role in CD112 down-regulation. HCMV deletions would need to be generated to definitively discount these genes from having any involvement in CD112 down-regulation.

Immunofluorescent detection of the RAd-HCMV-ORF- library showed that the majority of HMCV proteins were a) detectable and b) found in their expected cellular location when compared to the observations of others. However, there were examples of proteins that were in disagreement with the literature and were not expressed in the expected location. For example, in this study UL86 was found to be expressed in the cytoplasm but in the literature UL86 was described as being present in the vRC in the nucleus during HCMV infection, due to the NLS of UL80 (Plafker and Gibson, 1998). It was therefore possible that some genes expressed from RAd vector require a localisation signal from another interacting protein in order to locate to the correct cellular compartment to carry out function. There are many other reasons hypothesised as to why transgenes may not found in the correct location summarised in chapter 5.0.

Transgene detection could not be achieved for all RAd constructs. A handful of genes were found to be undetectable by immunofluorescence. As reagents used in this study were raised against the V5 tag, then it could be an inability of V5 detection rather than a lack of transgene expression. Indeed, while transgene detection proves expression, the lack of transgene detection does not necessarily preclude expression. In order to definitively prove whether transgenes are expressed from RAd vectors, antibodies would have to be raised against these specific proteins.

A further consideration is that the RAd-HCMV-ORF-library uses only one ORF sequence. However, these ORFs may, or may not, be alternatively spliced during HCMV infection to produce a different protein. Altered transcriptional profile during HCMV is evident from E, IE and L gene expression and there is evidence that some genes are alternatively spliced during HCMV infection. This is true for IE1 and IE2, which are differentially spliced variants from the MIE locus along with IE1p38, IE2p55 and IE2p18 (Kerry et al., 1995). These differentially spliced products are expressed at different timepoints throughout infection, or only in a particular cell type. Alternative splicing during HCMV infection could result in the production of a protein, which would not be picked up using the RAd-HCMV-ORF library.

8.2. Building further screening tools

Due to the complexity of HCMV infection perhaps the next stage in building tools for screening for HCMV function would be to create a library of HCMV single gene deletions, similar to the HCMV block deletion library. Deletions of UL141 and US1-11 in viruses HCMVΔUL141 and HCMVΔUS1-11 respectively, resulted in a loss of function. These viruses were unable to down-regulate cell surface CD112 during infection. To further this study, individual gene deletions in US1, US2, US3, US6, US7, US8, US9 and US10 would need to be generated to identify the specific gene in the US1-11 region involved in CD112 down-regulation (US11 has already been discounted, chapter 7.0).

Some genes in HCMV are essential for growth/replication and cannot be deleted. For essential genes, conditional knockouts could be created. By fusing a destabilising domain of the FK506-binding protein to essential cytomegalovirus proteins, Glab et al (2009) generated virus mutants that allowed for the conditional and reversible disruption of fusion proteins regulated by the synthetic ligand shield. This method allowed the study of essential herpesvirus proteins. A HCMV-UL51-ddFKBP mutant was constructed that encoded fusions of ddFKBP to the N terminus of UL51, along with an influenza viral haemagglutinin derived epitope (HA), inserted at an ectopic genomic location ΔUL1-10 of BAC-cloned AD169. In the absence of shield-1 the fusion proteins were knocked down but in the presence of shield-1, pUL51 showed a nuclear location (GlaB et al., 2009). This methodology could be employed to create a library of HMCV mutants with conditional knockdown of essential genes.

8.3. Potential future work: CD112 in NK and DC cell interaction

Work in this thesis has involved HCMV infection of fibroblasts. However, there are other cell types permissive to HCMV infection. Cells of the myeloid lineage are considered to be sites of HCMV latency, and differentiation of myeloid progenitors to dendritic cells may reactivate HCMV (Sinclair, 2008). Indeed, dendritic cells are also capable of being infected with HCMV. Monocyte-derived DC (moDCs) appeared susceptible to *in-vitro* infection and HCMV infection of moDCs by endothelial cell adapted strains, resulted in inhibited cell surface expression of MHC I and II, costimulatory molecules and chemokine receptors (CCR1 and CCR5) (Riegler et al., 2000, Kessler et al., 2008, Moutaftsi et al., 2002, Varani et al., 2005). In addition, a second DC subtype, plasmacytoid DCs (pDCs), which are found in secondary lymphoid tissue are also permissive to HMCV infection (Schneider et al., 2008, Varani et al., 2007).

Dendritic cells may cross talk with NK cells and reciprocally regulate their functions both in inflamed peripheral tissues and in secondary lymphoid compartments (Zitvogel, 2002). Myeloid DCs can induce NK cell functions and activated NK cells may alternatively kill immature DCs or promote their maturation. Different receptor-ligand interactions and cytokine secretion characterise this cross talk (Degli-Esposti and Smyth, 2005). These interactions allow NK cells to discriminate between iDCs (that typically underexpress HLA class I molecules) and mDCs that, after Ag uptake, up-regulate HLA class I expression (Della Chiesa et al., 2003). The process by which NK cells eliminate iDCs has been shown to initiate with the engagement of NKp30 and DNAM-1 activating receptors with their cognate ligands (Pende et al., 2006). In particular, during the maturation process, CD112 expression was up-regulated on iDCs, as compared with monocytes, and reached maximal surface density on mDCs, whereas up-regulation of CD155 was confined to mDCs (Pende et al., 2006). Blocking of NKp30 or DNAM-1 by specific mAbs inhibited NK mediated killing of both iDCs and mDCs (Pende et al., 2006). I therefore hypothesise that a reduction of the ligands for these receptors (such as down-regulation of CD155 and CD112 in HCMV infection) would also inhibit NK mediated killing of DCs during the maturation process, in particular DCs with low MHC class I expression due to HCMV infection.

The DNAM-1 ligands have been shown to play a role in the NK cell mediated ‘quality control’ of DC maturation, a process whereby the most-fitting DC’s, characterised by high

expression of HLA class I molecules and of costimulatory ligands, are optimised for the ability to prime T-cells, polarising T-cells into the relevant subset required for the particular immune response (Della Chiesa et al., 2005, Moretta, 2005, Pende et al., 2006). One subset of circulating $CD4^+$ T-cells are $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cells (Treg) which have suppressive properties (Sakaguchi, 2000, Shevach, 2000). Human Treg cells have been identified and characterised in peripheral blood and the thymus (Baecher-Allan et al., 2001, Jonuleit et al., 2001, Stephens et al., 2001). Treg cells can also be induced in the periphery after antigen activation and are termed adaptive Treg (Bluestone and Abbas, 2003). These adaptive Treg cells can be induced *in-vitro* by cytokine priming and co-culture with iDCs (Jonuleit et al., 2000, Yamagiwa et al., 2001). In addition to suppressing auto- and alloreactive T-cells maintaining self-tolerance, Treg cells can also suppress immune responses to human tumours and to bacterial and acute viral infections in animal models (Belkaid et al., 2002, Suvas et al., 2003, Woo et al., 2002). It is therefore hypothesised that HCMV infection of DC's results in the down-regulation of DNAM-1 activatory ligands, in particular CD112, allowing inefficient, virally infected DCs which possess low HLA class I surface expression to mature, or alter lineage commitment to a more tolerant DC phenotype. These DCs are also implicated in T-cell polarisation and induce tolerance through the presentation of antigen with inadequate costimulation and cytokine production for effector T-cell activation (Sotomayor et al., 2001). The result would hypothetically be the tolerisation of the human immune response to HCMV antigen, in a similar way to tolerisation of the immune system to self antigen. Therefore HCMV proteins could effectively be recognised as 'self' and therefore would not induce a potent enough immune response capable of clearing the infection. This mimics the theory behind TolDC therapy, whereby an immune suppressive environment is sought to suppress immune response to self antigen. Injection of *ex-vivo* modified TolDCs has proven to be beneficial in models of autoimmune disease, including collagen-induced arthritis, diabetes and experimental autoimmune encephalo-myelitis (Popov et al., 2006, Chorny et al., 2005, Adorini, 2003). In addition, DC's that express low MHC-1 have been indicated in tolerisation of antigen in therapeutic cancer vaccines, limiting the potency of an antigen-specific T-cell response (Fiore et al., 2005, Camporeale et al., 2003). This could have an important implication for HCMV vaccine research, which has thus far failed to deliver a vaccine that is capable of preventing infection, or treatments that are capable of clearing infection. Such questions remain to be answered and provide many avenues for future research into HCMV down-regulation of DNAM-1 ligands.

8.4. Conclusion

The HCMV UL141 protein was identified as having an essential role in HCMV mediated down-regulation of CD112 during HCMV infection. However, UL141 was unable to down-regulate the cell surface expression of RAd-UL141 infected cells. Since UL141 appeared necessary but not sufficient to modulate CD112 expression, this thesis focused on a hypothesis whereby UL141 was acting in a conjunction with another HCMV protein. Screening strategies were implemented and this project was the first to utilise the entire RAd-HCMV-ORF library to screen for function. The RAd-HCMV-ORF library clearly provided an extremely powerful tool for the screening of HCMV gene function as results were highly repeatable and robust. The co-infection of RAd-UL141 and RAd-US2 resulted in a single, clear, positive hit in the final screening process. Although the shift in cell surface down-regulation in RAdUL141/US2 infected cells was not of the same magnitude as during HCMV infection it was robust and highly reproducible. A down-regulation of CD112 was also seen immunoblots where RAd-UL141 and RAd-US2 were co-infected but not in RAd-UL141 or RAd-US2 singly infected HFFF-hCAR's, while a Hela-US2 cell line that stably expressed US2 also down-regulated CD112 when infected with RAd-UL141. US2 is known to target MHC-1 for proteasome mediated degradation. The addition of proteasome inhibitors was able to partially restore CD112 expression in HCMV infected cells. It therefore appeared that US2 and UL141 act to degrade CD112 via the proteasome during HCMV infection. This may have the potential to prevent DNAM-1:CD112 interaction between HCMV infected targets and effector cells of the immune system.

9.0. REFERENCES

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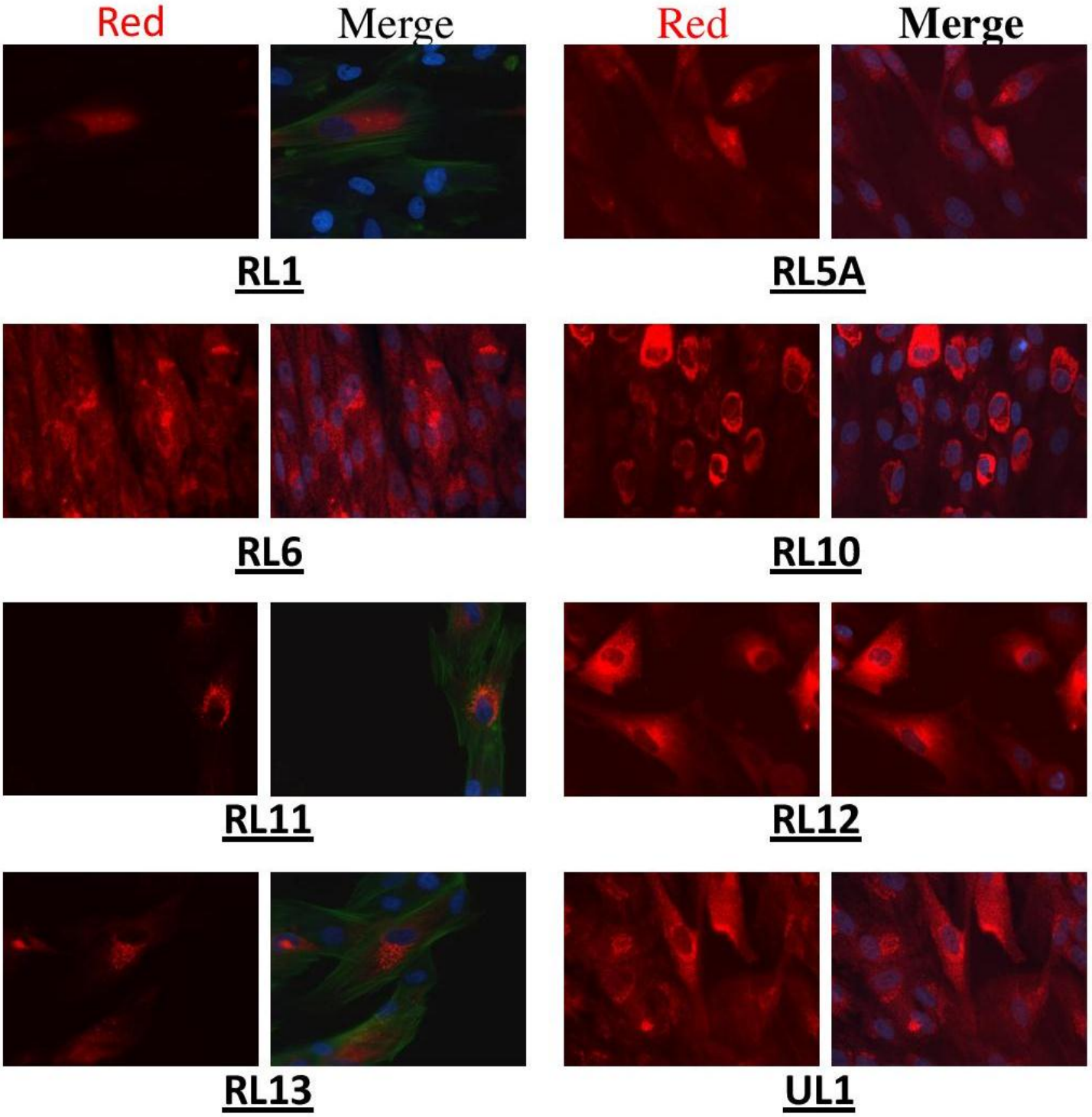
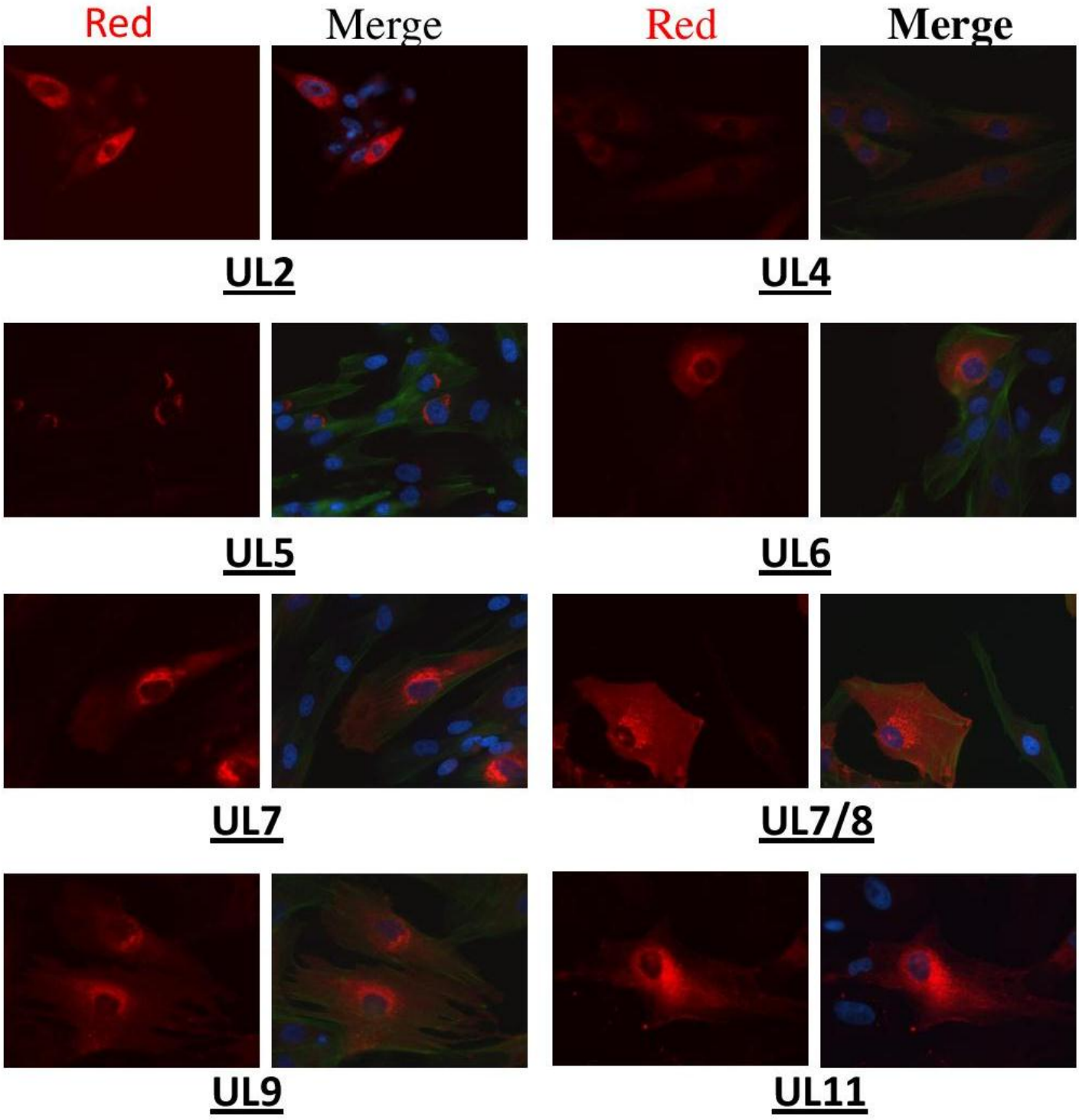
10.0 APPENDIX I

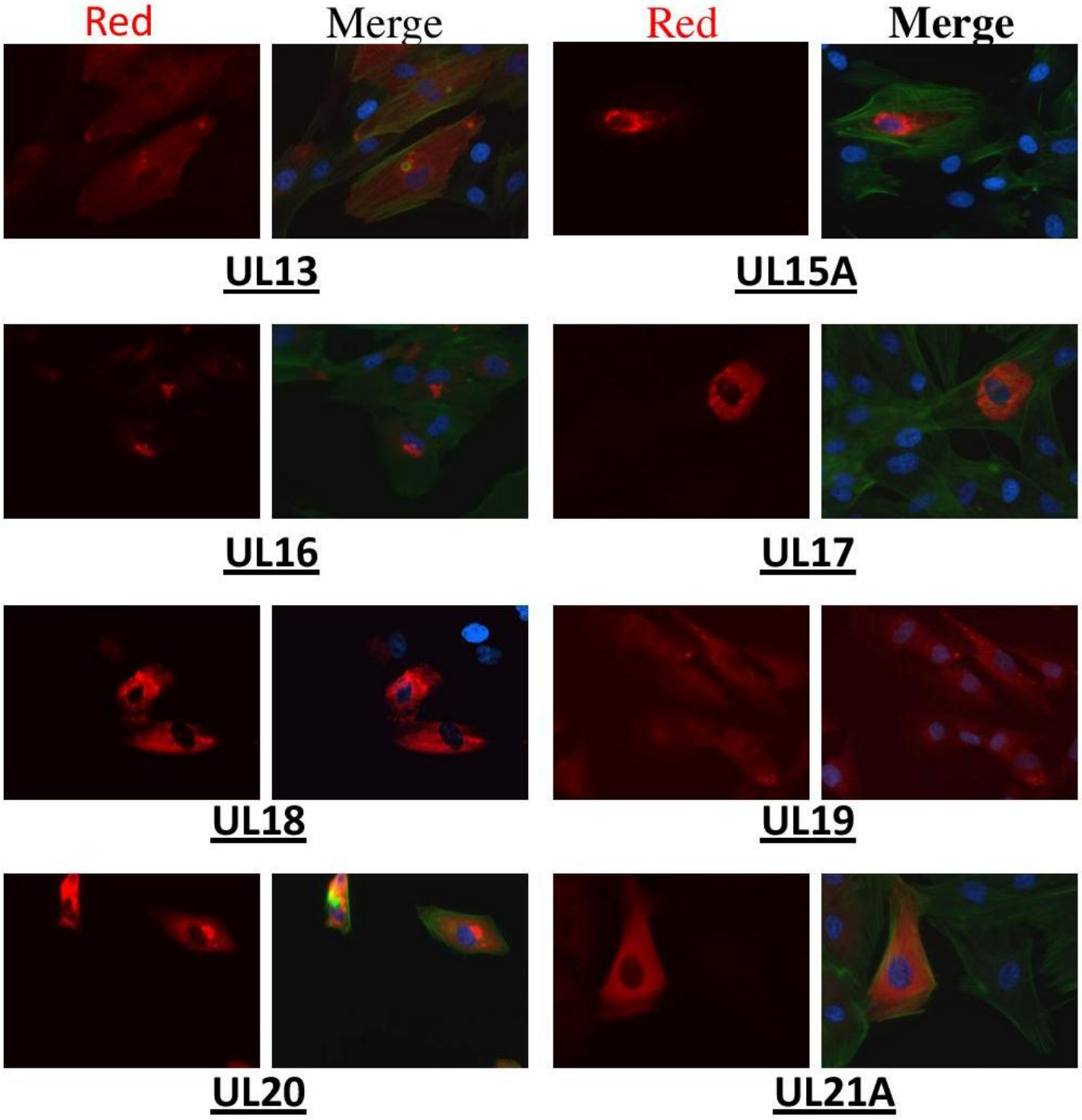
Immunofluorescent detection of RL1-UL40 and UL111A-UL150 and IRS, carried out by Mr Sepehr Seiferian.



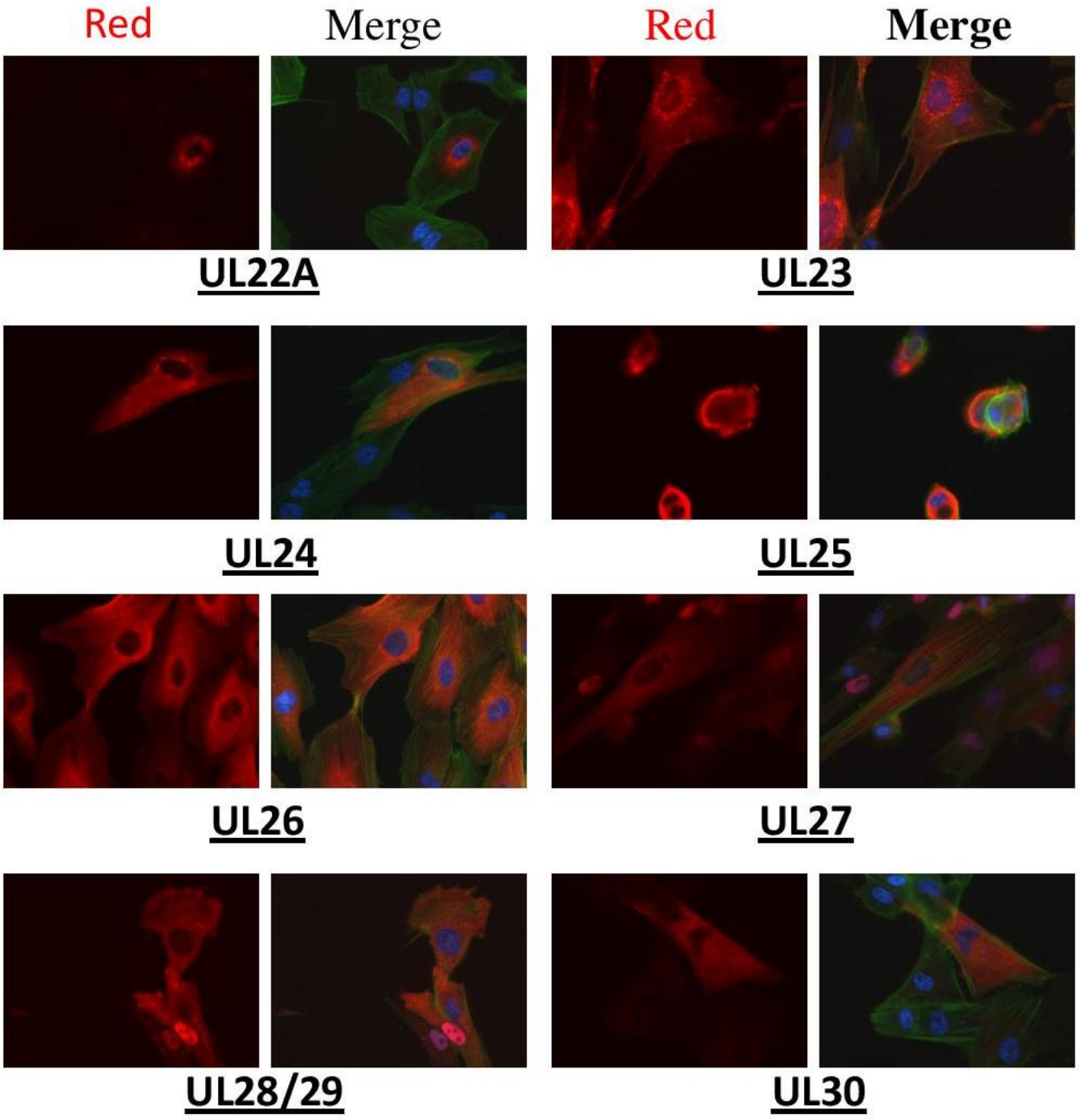
Appendix.I.A. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: RL1-UL1.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).



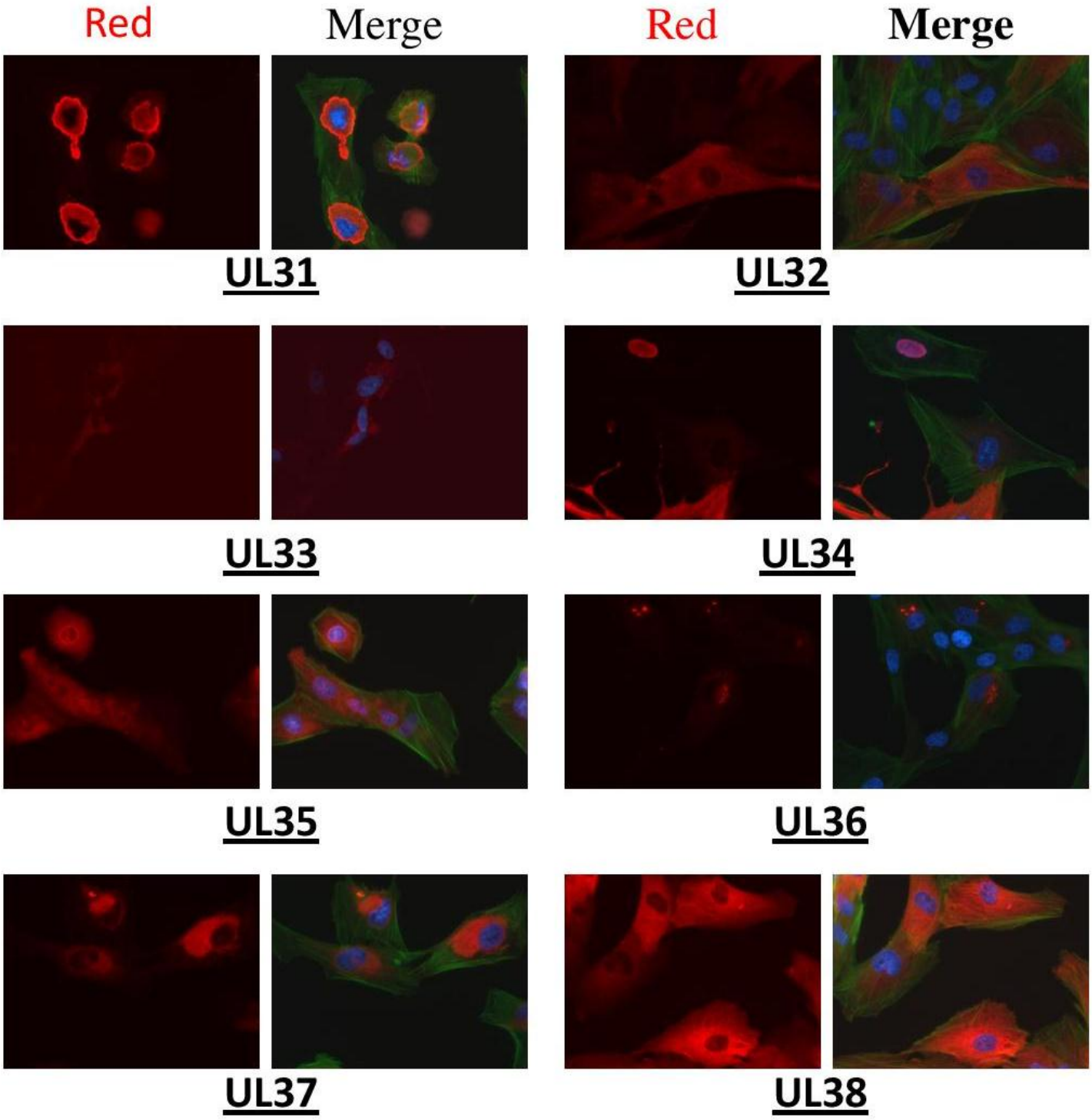
Appendix.I.B. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL2-UL11.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37^{oC}, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).



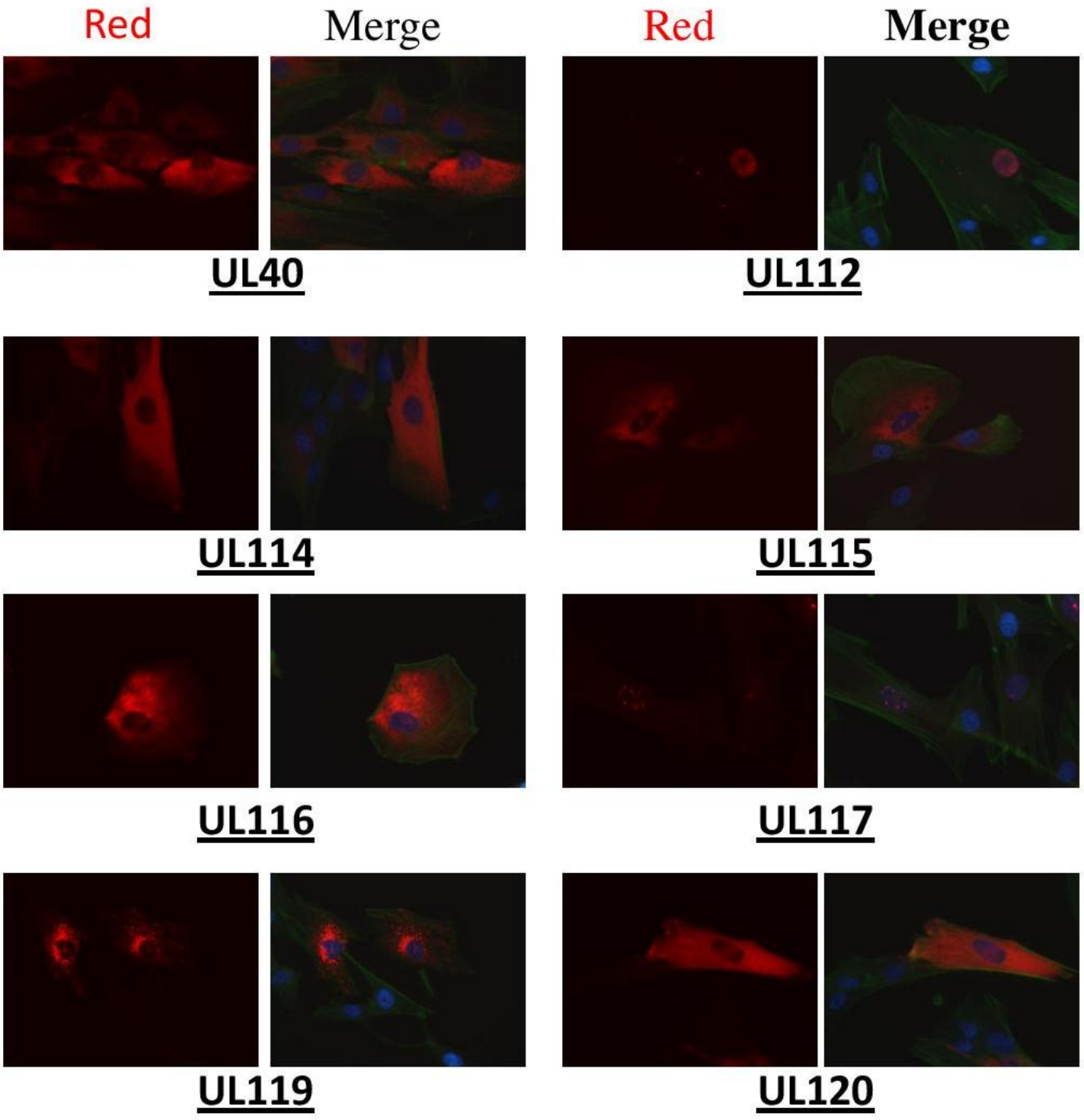
Appendix.I.C. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL13-UL21A.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).



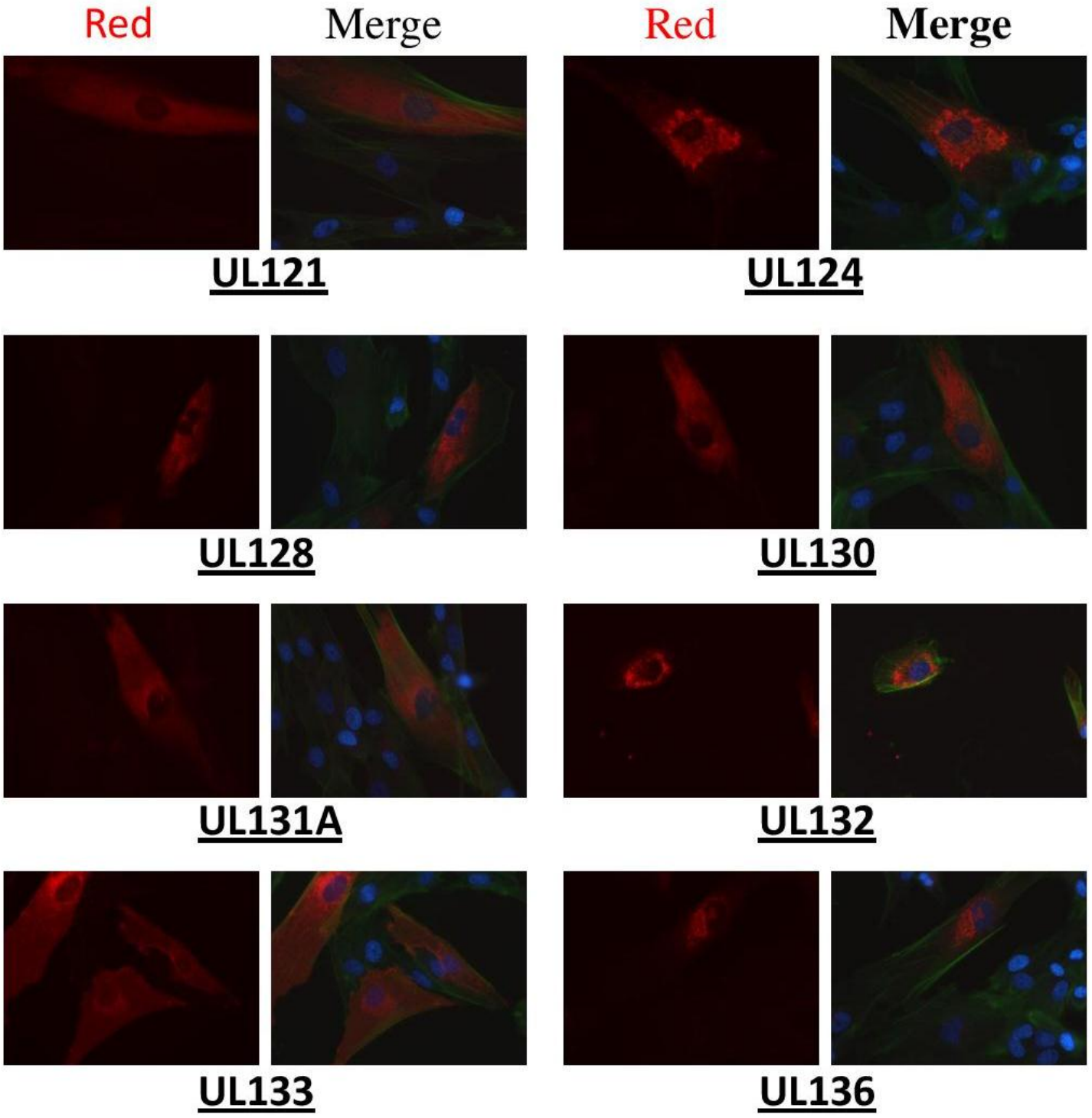
Appendix.I.D. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL22A-UL30.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).



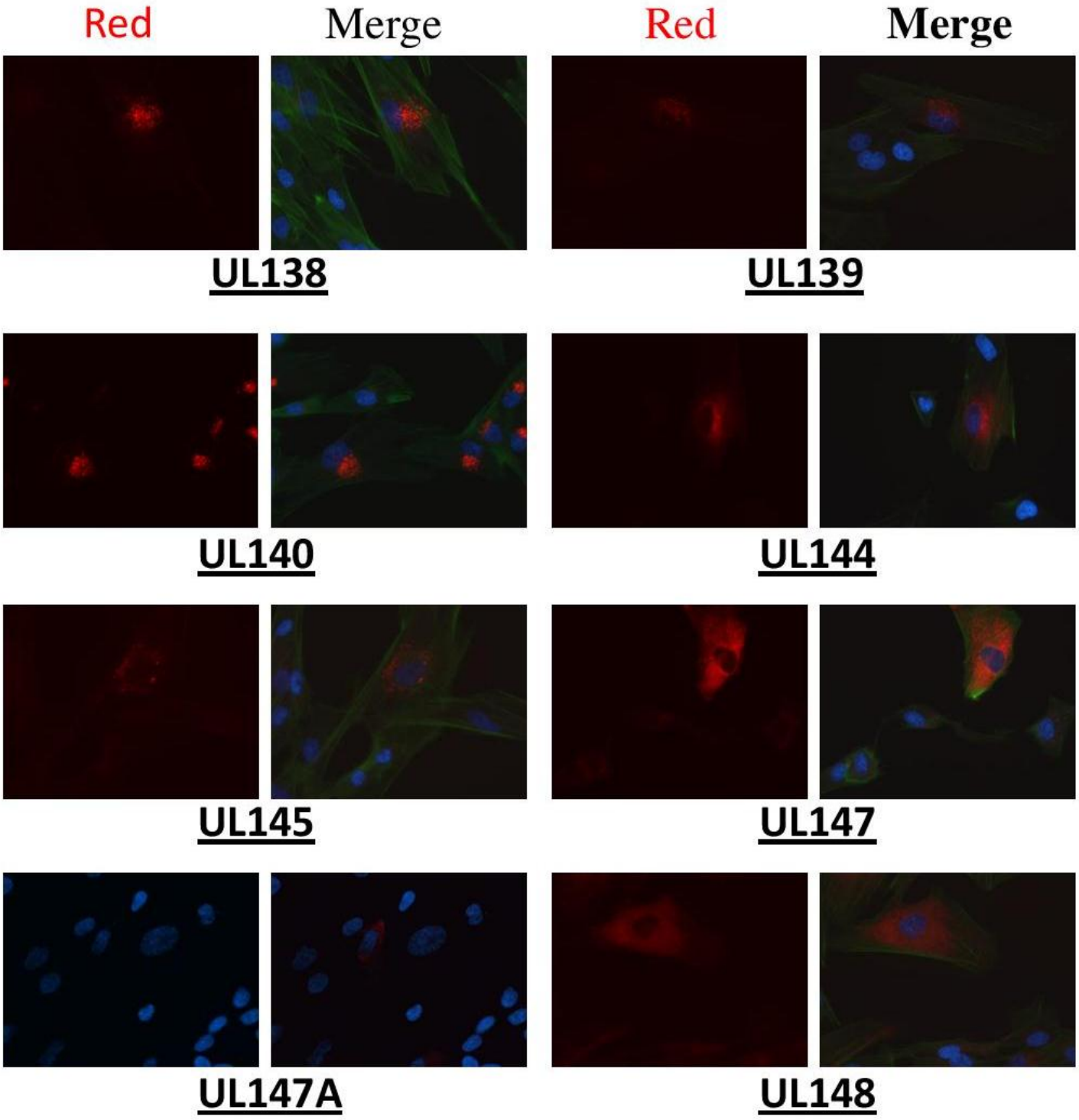
Appendix.I.E. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL31-UL38.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).



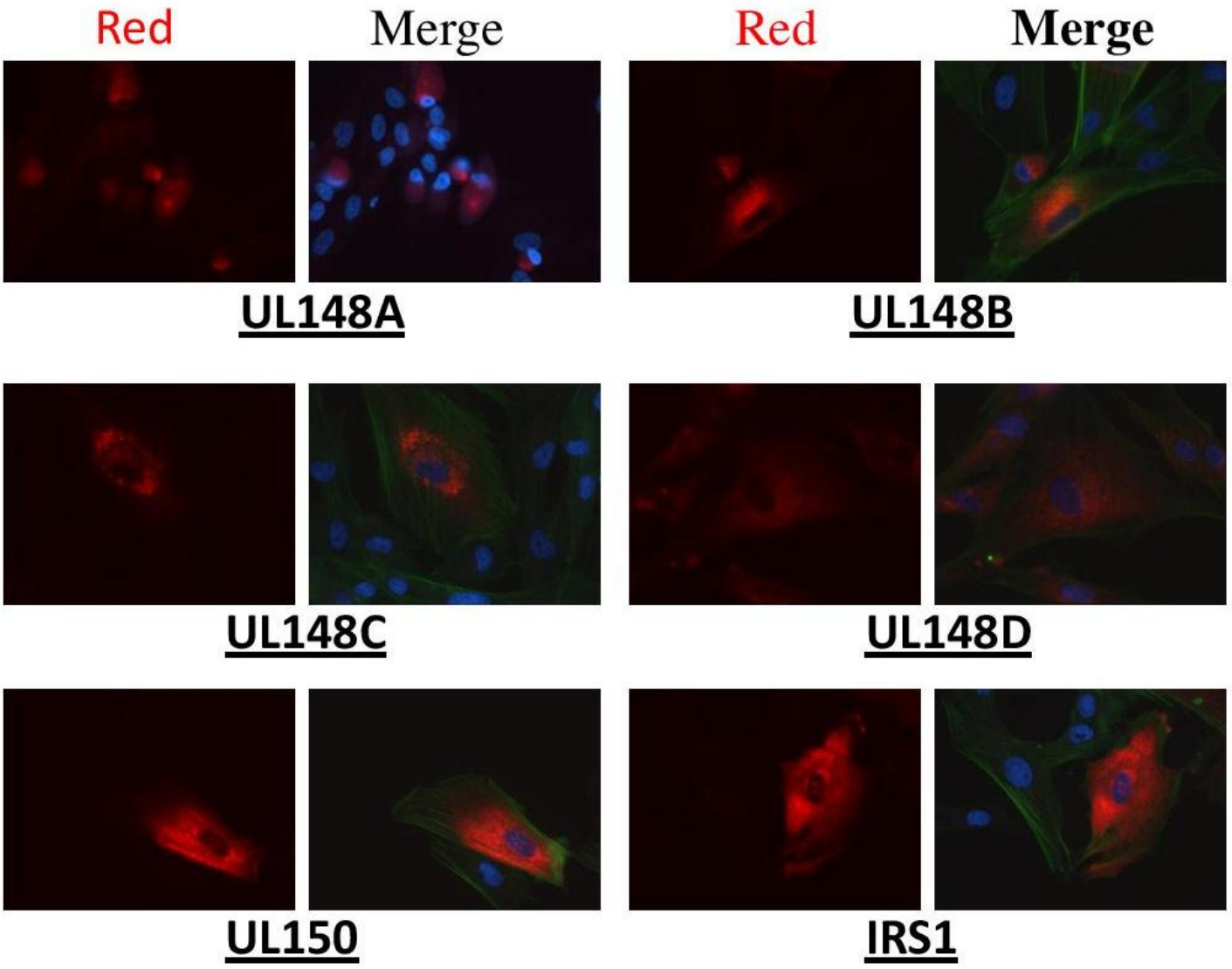
Appendix.I.F. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL40-UL120.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).



Appendix.I.G. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL121-UL136.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).



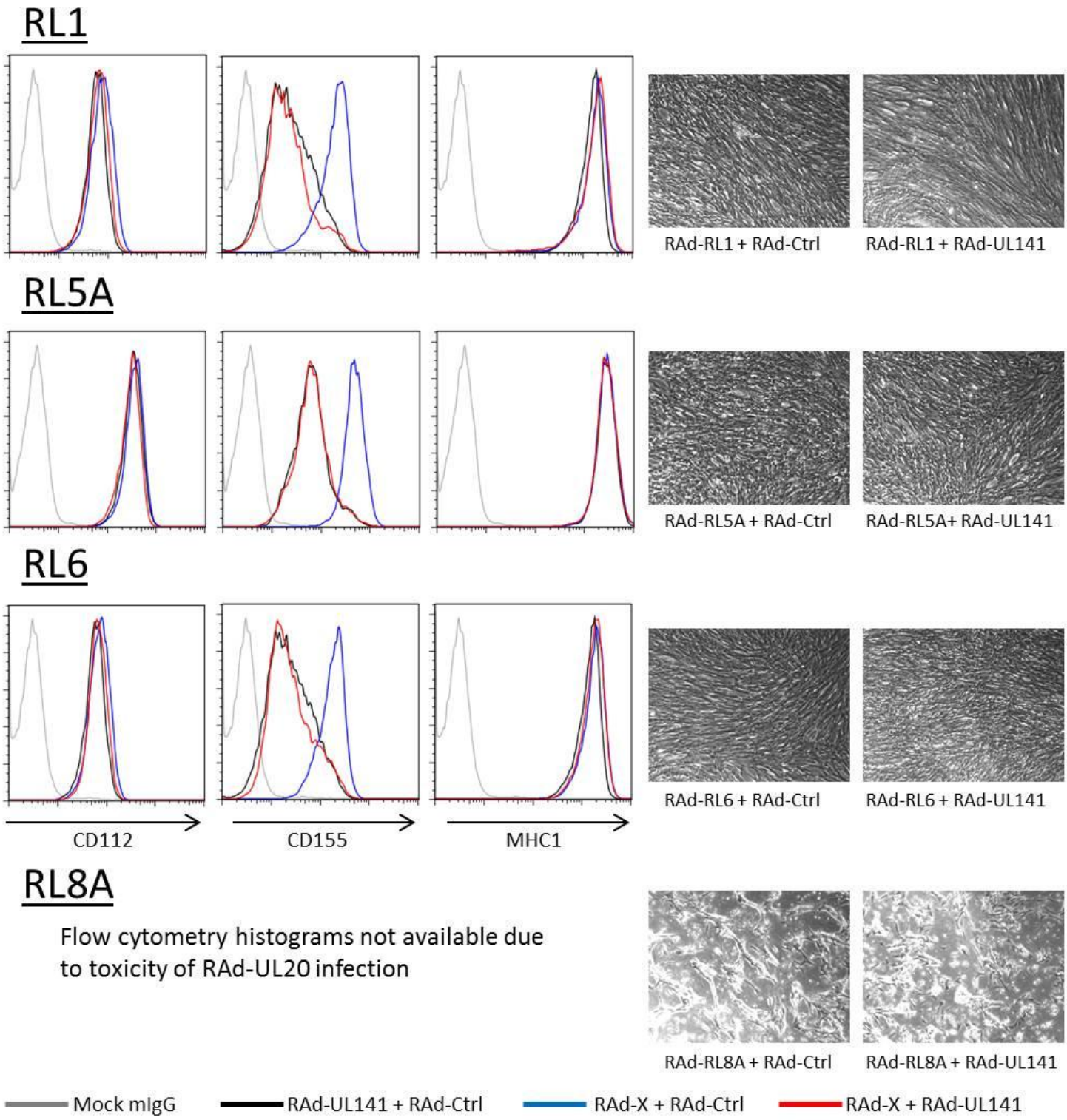
Appendix.I.H. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL138-UL148.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°^{C}, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).



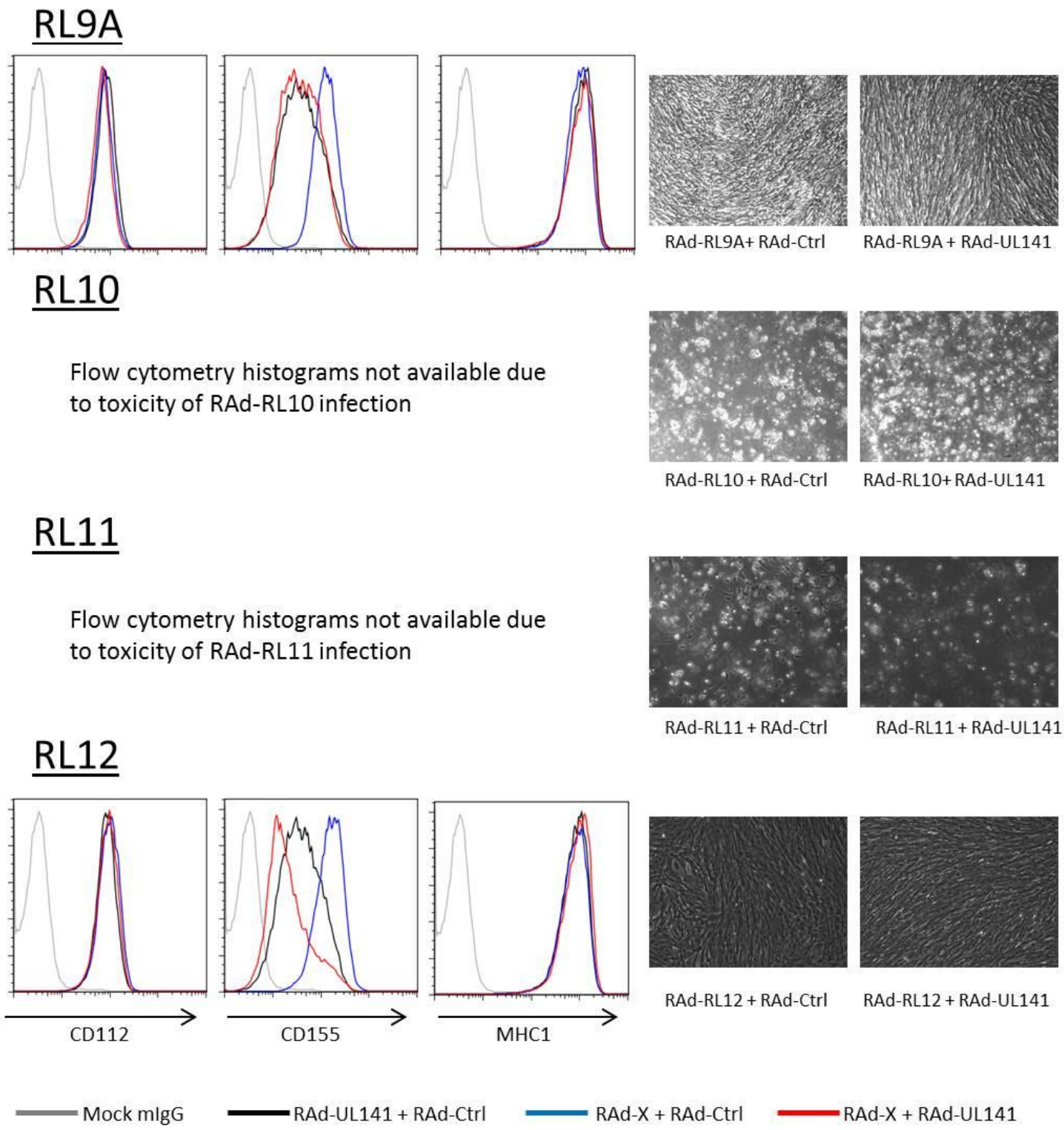
Appendix.I.I. Immunofluorescent staining of cells infected with a RAd from the RAd-HCMV-ORF library to determine transgene expression and subcellular location: UL148A-IRS1.
HFFF-hCARs were seeded at a concentration of $5x10^5$ in a glass bottomed 96-well plate. The following day HFFF-hCAR's were infected with a RAd from the RAd-HCMV-ORF library, as indicated, at MOI10 for 2hrs in an incubator 37°C, 5%CO_2 on an oscilating platform at 5 osc.min. After 2hours, media was removed and replaced with fresh DMEM-10 and incubated for 3days. After incubation cells were washed in PBS, fixed in 2% paraformaldehyde and underwent immunofluorescence staining using Saponin based IF-buffer. Antibodies were used as follows: Primary mouse anti-V5 was used 1 in 2,000, phalloining-488 1 in 50, DAPI 1 in 10,000 and a secondary α-mouse IgG(FAb') AlexaFluor- 594 conjugate (red channel) was used to detect anti-V5, used 1 in 500. Cells were fixed and imaged using fluorescent microscopy using a x40 oil objective. Two images are shown for each transgene. The first is individual **transgene expression** as detected with anti-V5 primary (red channel). The second is a merged image consisting of **transgene expression** (red channel), **phalloidin-488** (green channel) and **DAPI** (Blue channel).

11.0 APPENDIX II

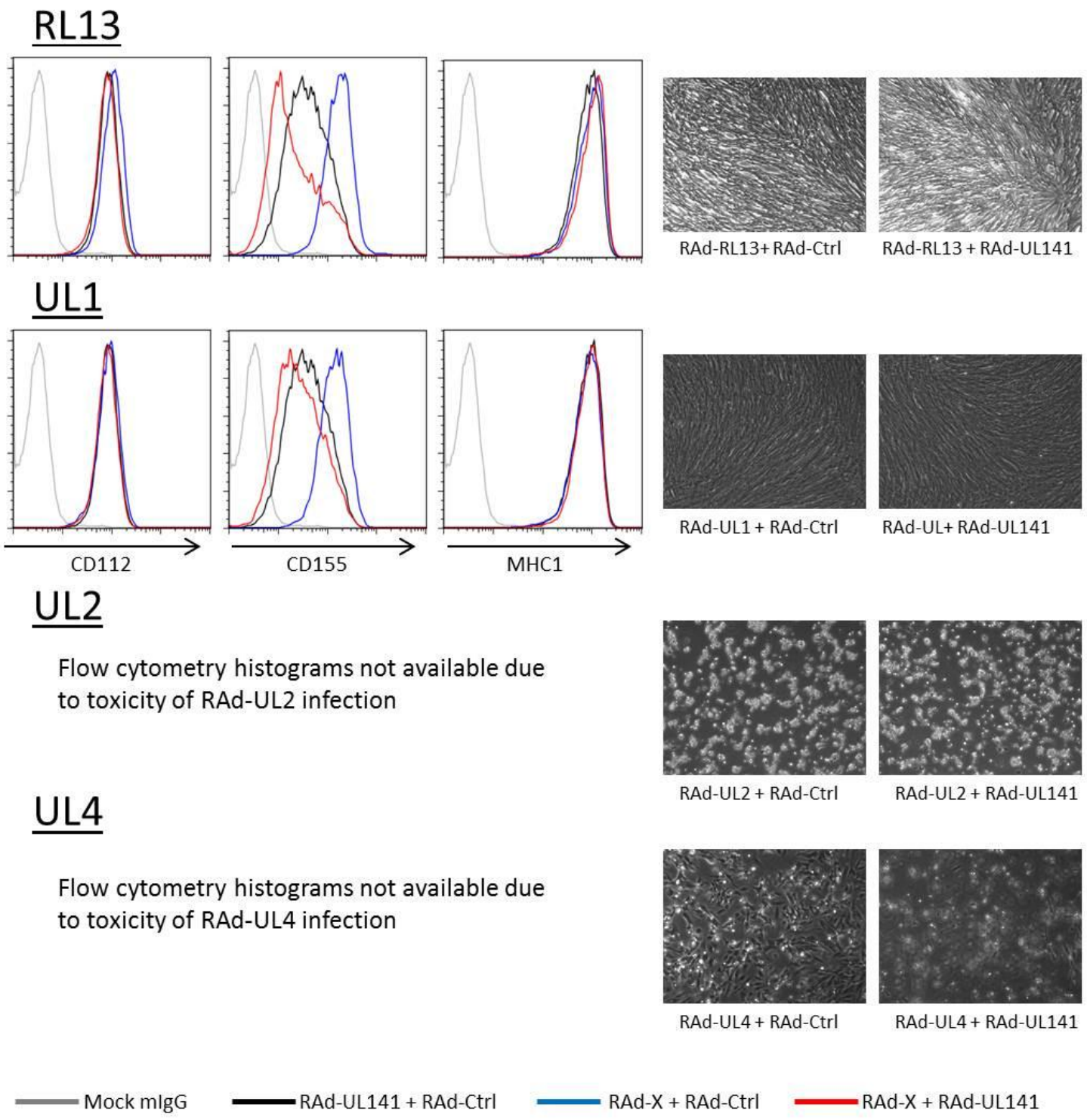
Screening RAd-HCMV-ORF library for CD112 down-regulation: Genes covered by RCMV block deletion mutants



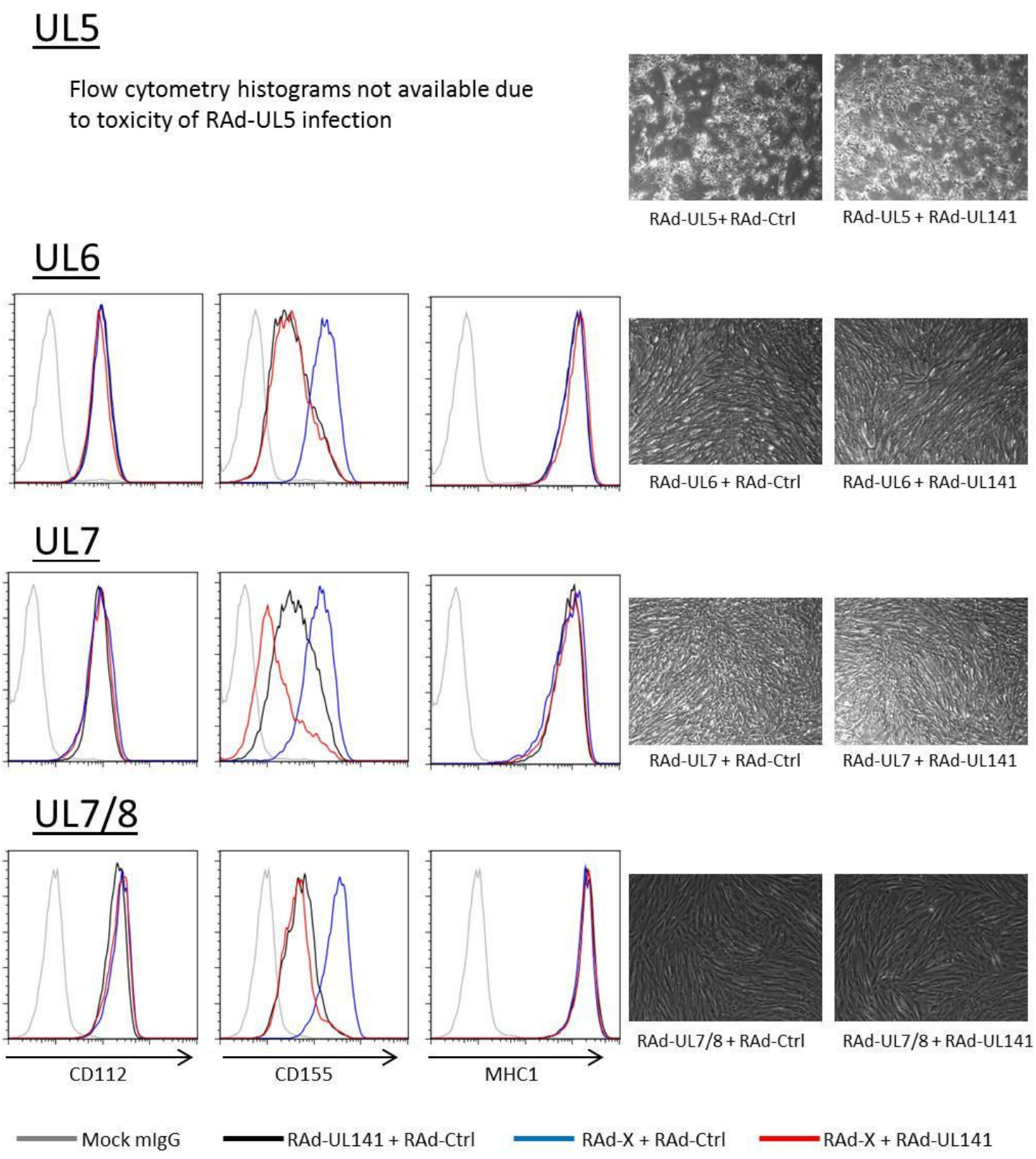
Appendix II.A. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-RL1, RAd-RL5A, RAd-RL6 and RAd-RL8A.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10**), where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



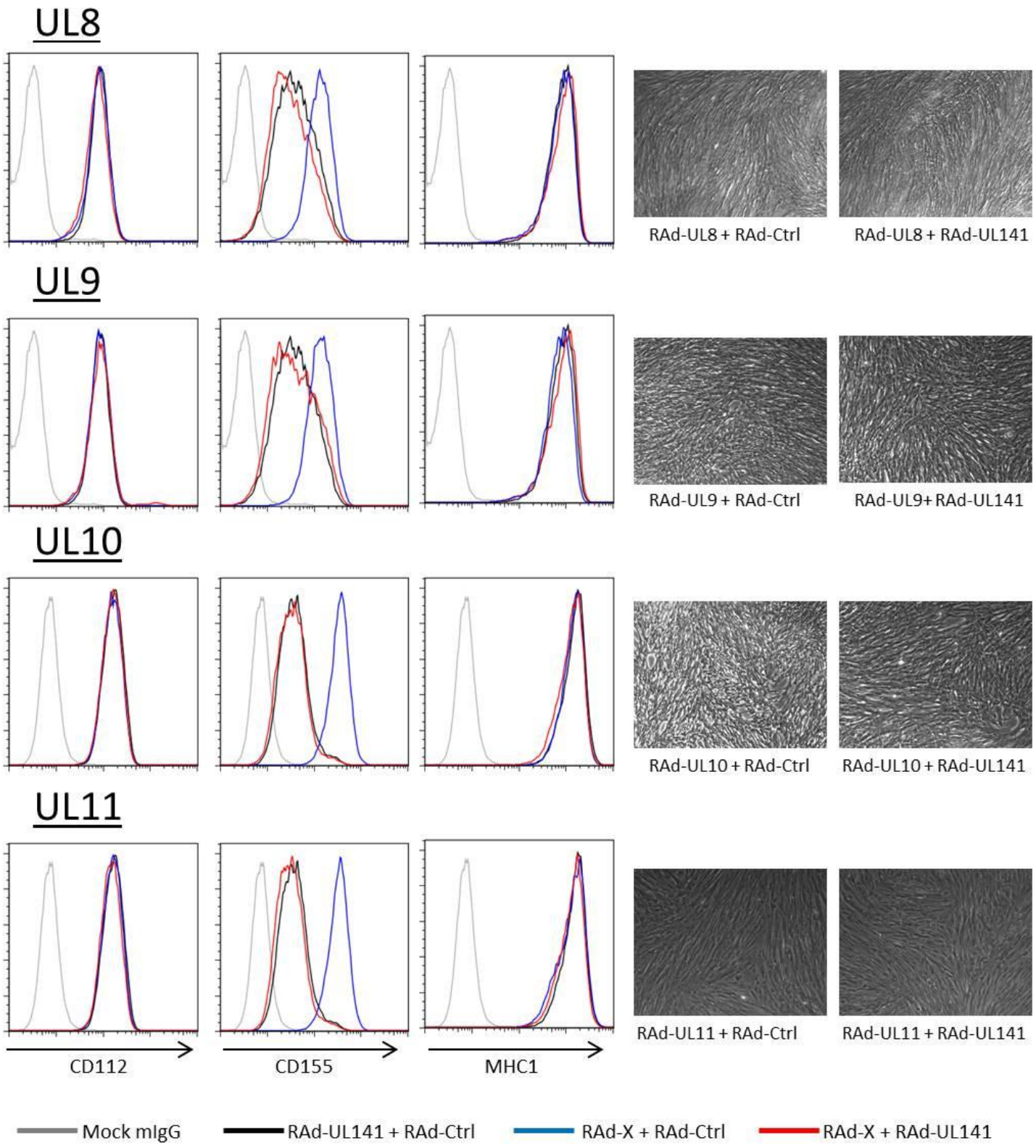
Appendix II.B. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-RL9A, RAd-RL10, RAd-RL11 and RAd-RL12.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10**), where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



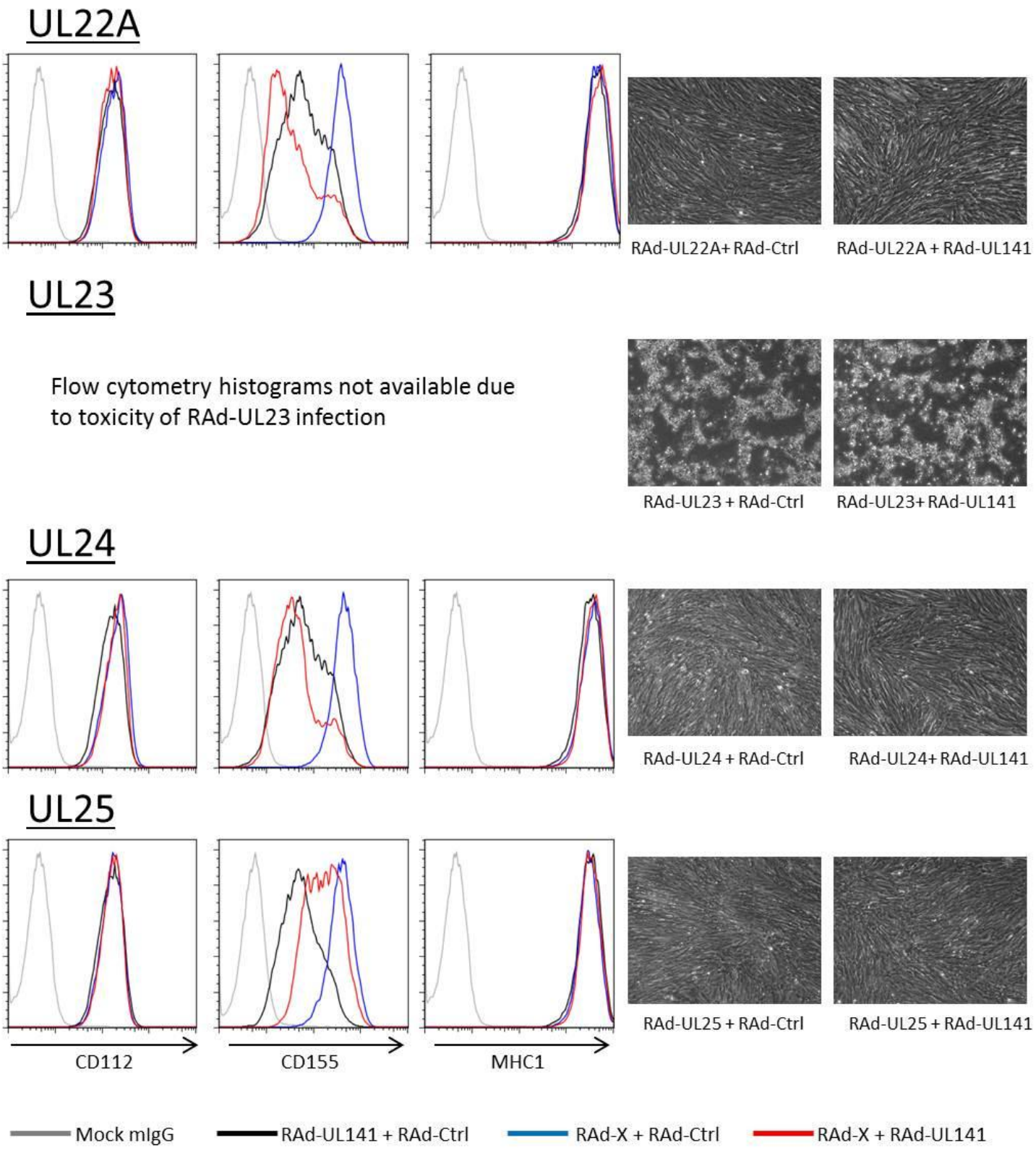
Appendix II.C. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-RL13, RAd-UL1, RAd-UL2 and RAd-UL4.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



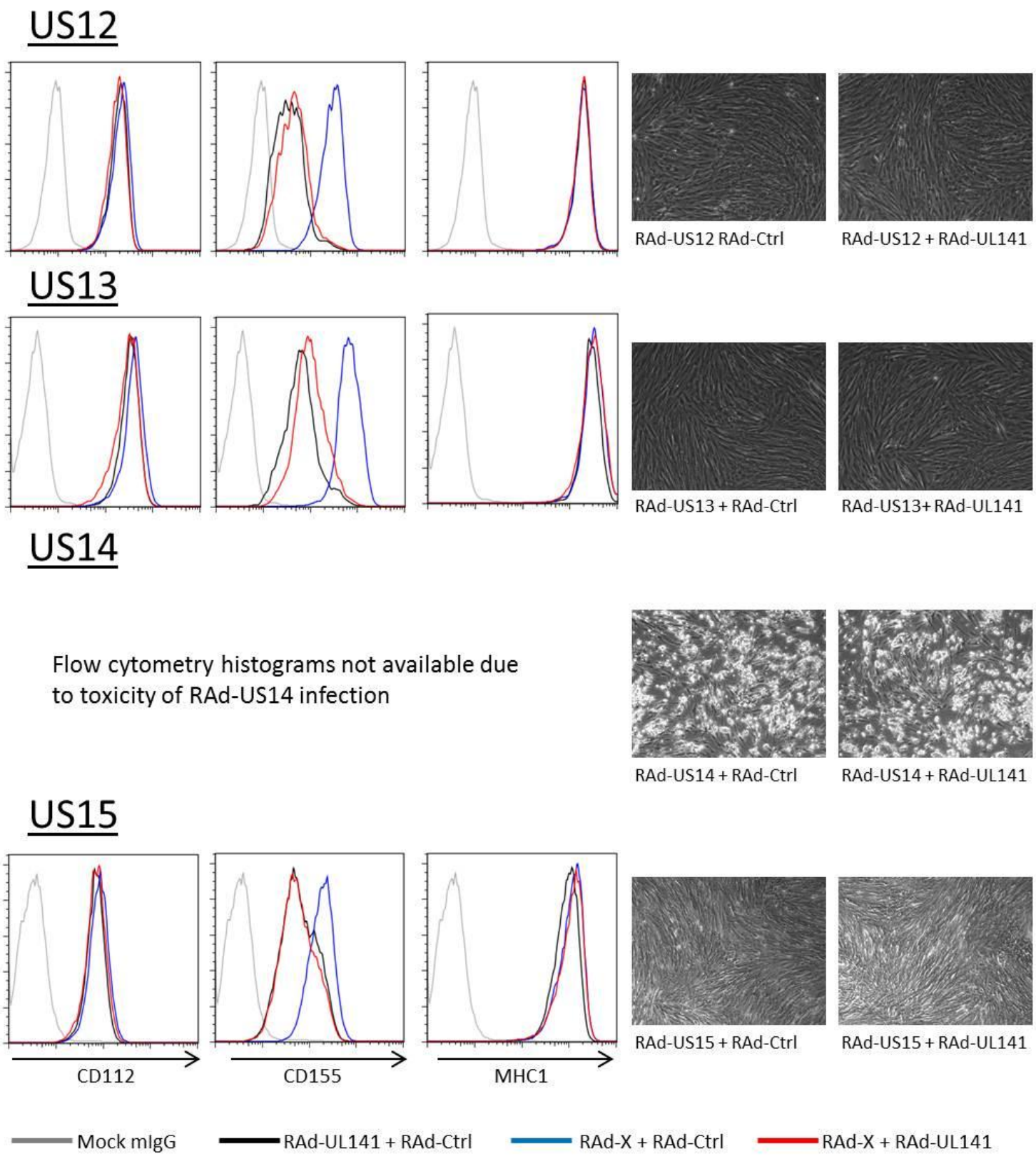
Appendix II.D. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL5, RAd-UL6, RAd-UL7 and RAd-UL7/8.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10**), where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



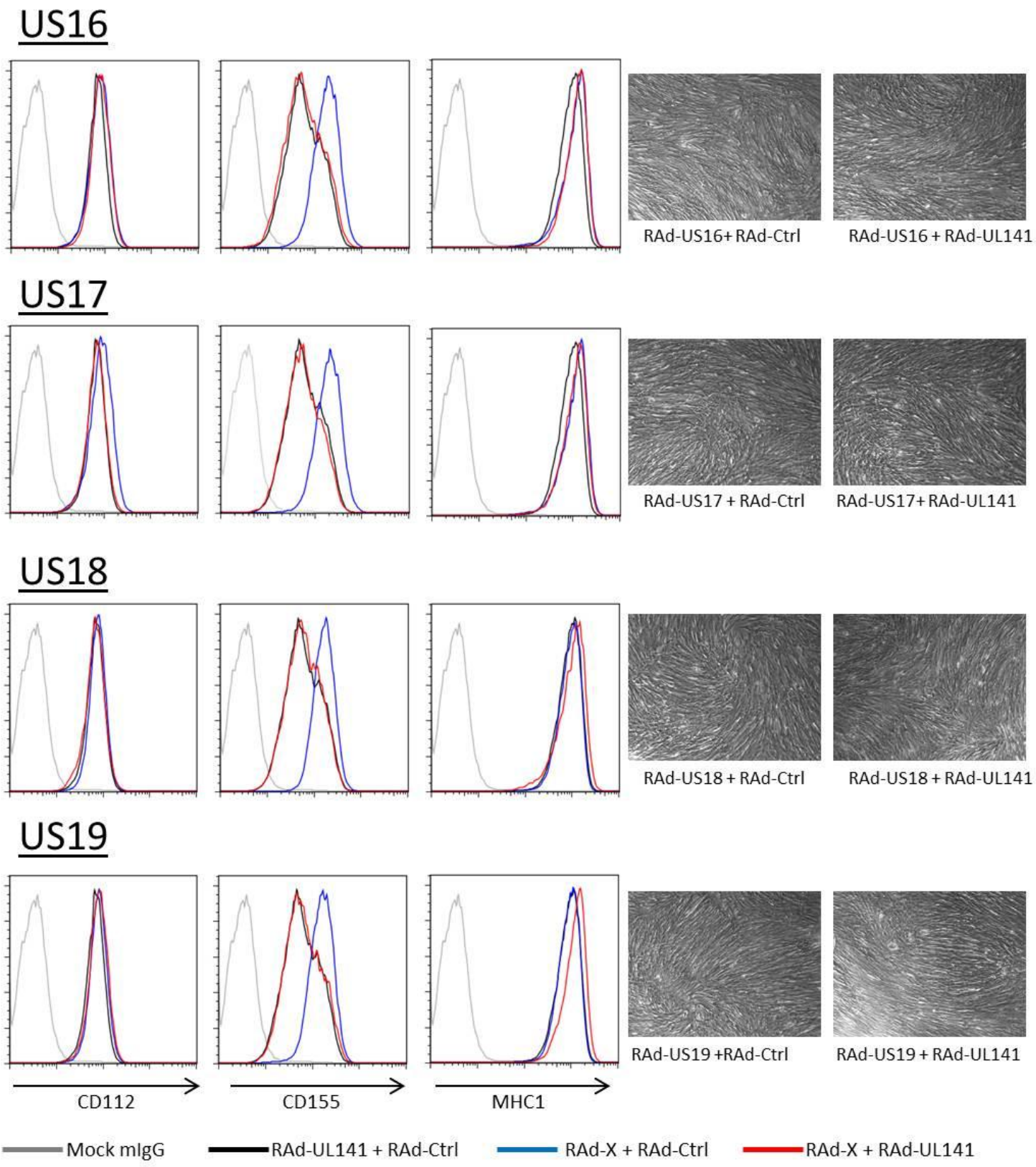
Appendix II.E. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL8, RAd-UL9, RAd-UL10 and RAd-UL11.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10**), where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



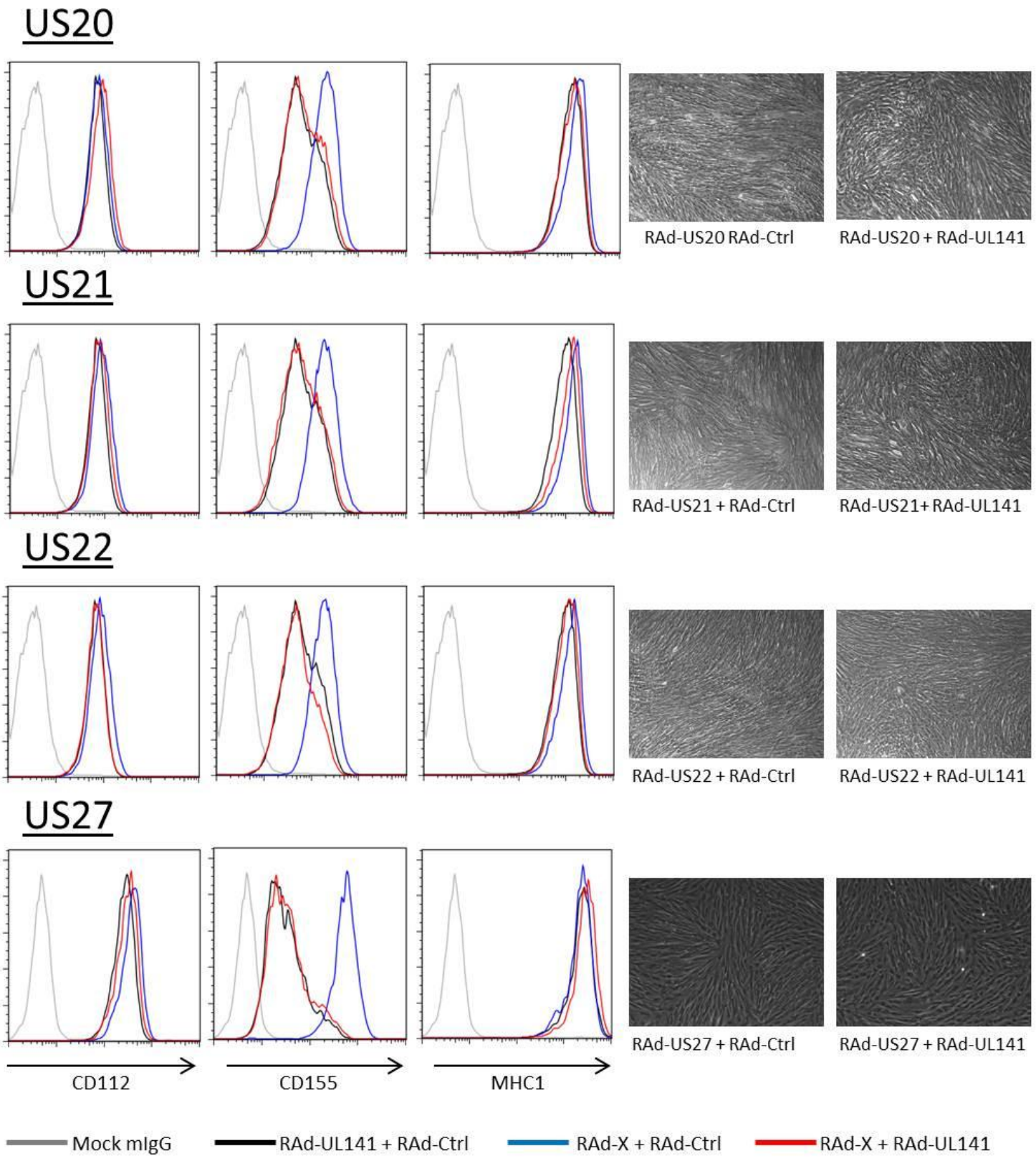
Appendix II.F. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-UL22A, RAd-UL23, RAd-UL24 and RAd-UL25.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



Appendix II.G. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-US12, RAd-US13, RAd-US14 and RAd-US15.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10**), where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



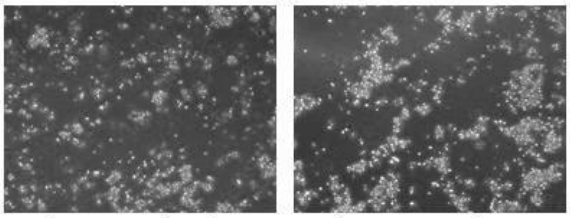
Appendix II.H. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-US16, RAd-US17, RAd-US18 and RAd-US19.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10**), where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



Appendix II.I. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-US20, RAd-US21, RAd-US22 and RAd-US27.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10**), where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.

US28

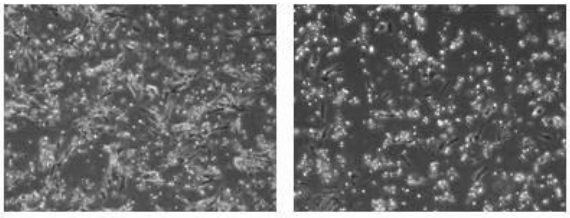
Flow cytometry histograms not available due to toxicity of RAd-UL28 infection



RAd-US28 RAd-Ctrl RAd-US28 + RAd-UL141

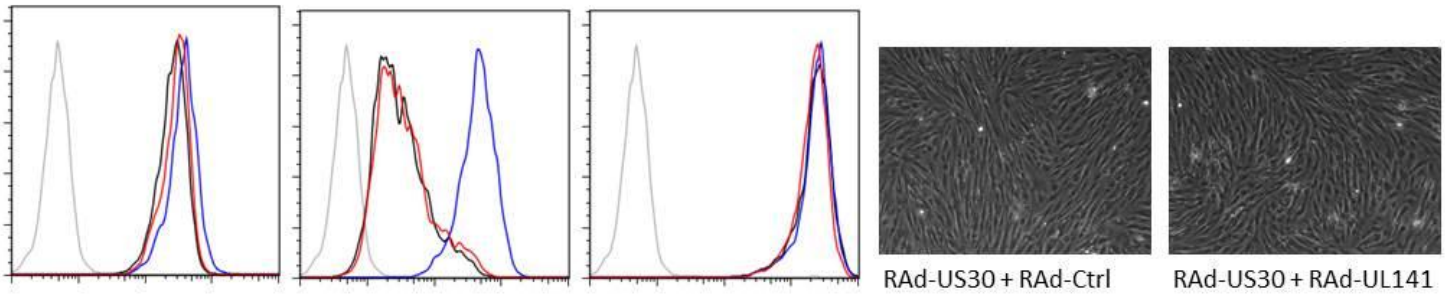
US29

Flow cytometry histograms not available due to toxicity of RAd-UL29 infection



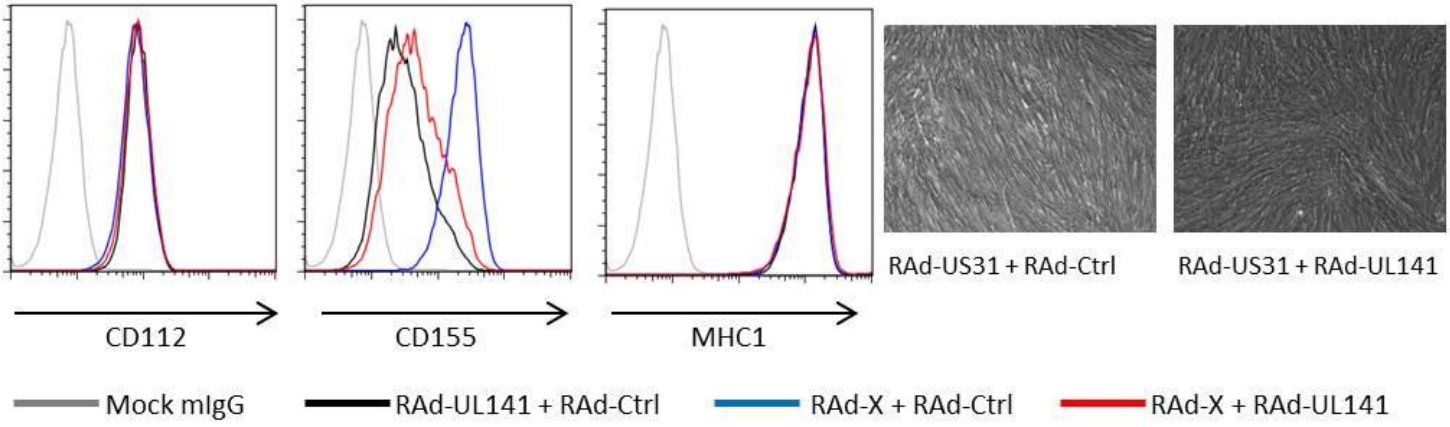
RAd-US29 + RAd-Ctrl RAd-US29+ RAd-UL141

US30



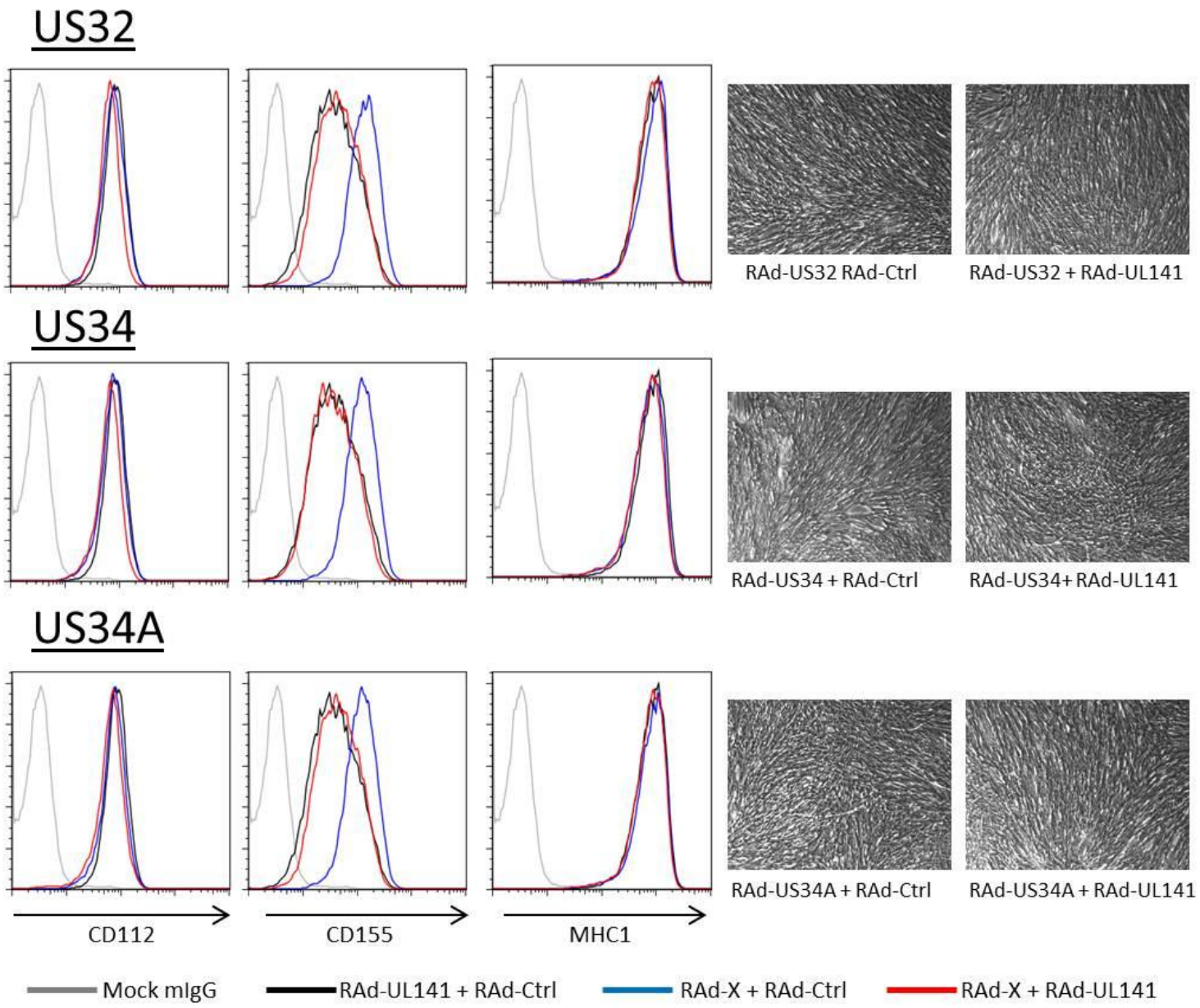
RAd-US30 + RAd-Ctrl RAd-US30 + RAd-UL141

US31



RAd-US31 + RAd-Ctrl RAd-US31 + RAd-UL141

Appendix II.J. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-US28, RAd-US29, RAd-US30 and RAd-US31.
HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10)**, where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.



Appendix II.K.. Screening RAd-HCMV-ORF library for CD112 downregulation: RAd-US32, RAd-US34, RAd-US34A.

HFFF-hCARs were infected with **RAd-UL141 (MOI10) + RAd-Ctrl (MOI10)**, **RAd-X (MOI10) + RAd-Ctrl (MOI10)** or **RAd-X (MOI10) + RAd-UL141 (MOI10**), where X denotes a gene denoted in the figure. The cell surface expression of CD112, CD155 and MHC-1 was analysed 3 days p.i. by flow cytometry. Infections were also monitored by phase contrast microscopy. Flow cytometry histograms were unavailable for RAd-RL8A due to toxicity.

12.0 PUBLICATIONS

Short Communication

Human cytomegalovirus UL141 promotes efficient downregulation of the natural killer cell activating ligand CD112

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Human cytomegalovirus (HCMV) UL141 induces protection against natural killer cell-mediated cytolysis by downregulating cell surface expression of CD155 (nectin-like molecule 5; poliovirus receptor), a ligand for the activating receptor DNAM-1 (CD226). However, DNAM-1 is also recognized to bind a second ligand, CD112 (nectin-2). We now show that HCMV targets CD112 for proteasome-mediated degradation by 48 h post-infection, thus removing both activating ligands for DNAM-1 from the cell surface during productive infection. Significantly, cell surface expression of both CD112 and CD155 was restored when UL141 was deleted from the HCMV genome. While gpUL141 alone is sufficient to mediate retention of CD155 in the endoplasmic reticulum, UL141 requires assistance from additional HCMV-encoded functions to suppress expression of CD112.

Human cytomegalovirus (HCMV), the prototype species of the subfamily *Betaherpesvirinae*, has a high prevalence in populations worldwide. Although HCMV is recognized to be an important human pathogen, particularly in immunocompromised individuals or following congenital infection, the vast majority of primary infections are subclinical and accompanied by asymptomatic lifelong carriage. HCMV encodes highly effective systems to provide for latency, persistent reactivation and transmission; as part of this process the virus acquired an impressive array of genes that act both to evade and redirect the host immune response (Wilkinson *et al.*, 2008). The fact that individuals with genetic defects in their natural killer (NK) cell response are particularly susceptible to severe HCMV disease (Biron *et al.*, 1989; Gazit *et al.*, 2004) provided a rationale to focus attention on this arm of the immune response.

NK cells are composed of heterogeneous populations expressing a 'mosaic' of different activating and inhibitory receptors, the function of each cell being regulated by integration of signals received from ligands presented on potential target cells (Lanier, 2008). Inhibitory signals received mainly from autologous MHC class-I molecules normally dominate, to maintain NK cells in a resting state. However, HCMV not only efficiently downregulates MHC-I (Ahn *et al.*, 1997; Furman *et al.*, 2002; Jones *et al.*, 1996; Trgovcich *et al.*, 2006; Wiertz *et al.*, 1996a, b), but also stimulates the expression of recognized NK cell activating ligands, e.g. MHC-I-related chains (MIC) A and B, UL16-binding proteins (ULBP) 1–3, retinoic acid early transcripts (RAET)1E/ULBP4, RAET1G/ULBP5, RAET1L/ULBP6 and CD155 (Bacon *et al.*, 2004; Bahram *et al.*, 1994; Bauer *et al.*, 1999; Chalupny *et al.*, 2003; Cosman *et al.*, 2001; Eagle *et al.*, 2009; Groh *et al.*, 2001; Tomasec *et al.*, 2005). Despite this, HCMV-infected cells actually prove to be highly resistant to NK cells in functional assays (Cerboni *et al.*, 2000; Tomasec *et al.*, 2005). This resilience can be attributed to a substantial proportion of HCMV genome being directed towards evading the NK cell response.

Although HCMV downregulates endogenous MHC-I, the virus also encodes its own MHC-I homologue (gpUL18) that binds the inhibitory receptor LIR-1 (ILT-2) with high affinity (Beck & Barrell, 1988; Chapman *et al.*, 1999; Prod'homme *et al.*, 2007) and a peptide in the UL40 leader sequence that acts to promote cell surface expression of the non-classical MHC-I molecule HLA-E, the ligand for the inhibitory receptor CD94/NKG2A (Tomasec *et al.*, 2000; Ulbrecht *et al.*, 2000; Wang *et al.*, 2002). The activating receptor NKG2D is remarkable in recognizing eight ligands. To combat their activation UL16 retains MICB, ULBP1 and ULBP2 in the endoplasmic reticulum (ER); miR-UL112 targets the MICB transcript, while UL142 downregulates MICA (Chalupny *et al.*, 2006; Cosman *et al.*,

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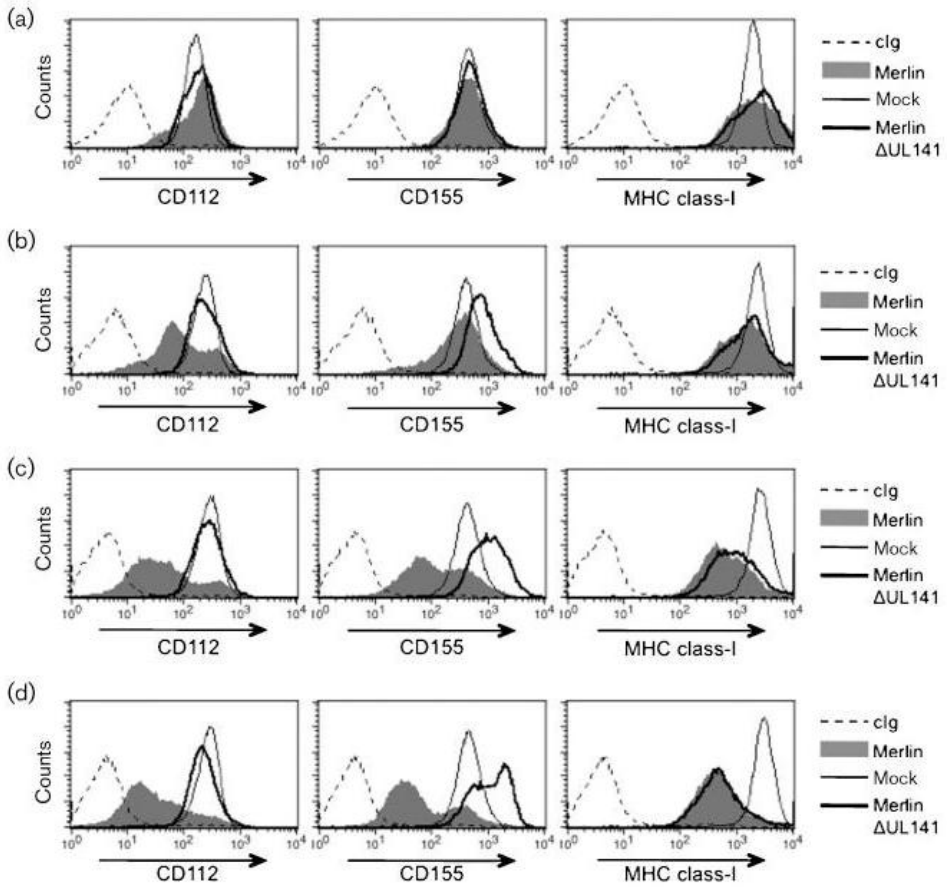

Fig. 1. HFFF were infected (m.o.i.=25) for (a) 24 h (b) 48 h (c) 72 h or (d) 96 h with HCMV strain Merlin, MerlinΔUL141 or mock-infected and cell surface expression of CD112 (Santa Cruz, sc-65333) was analysed by flow cytometry. For reference, expression levels of CD155 (Abcam, ab-3142) and MHC class-I (W632; ATCC) were also monitored, alongside control Ig (cIg).

2001; Stern-Ginossar *et al.*, 2007; Wills *et al.*, 2005). The NK cell activating receptor DNAM-1 (CD226) recognizes both CD155 and CD112 (Bottino *et al.*, 2003; Fuchs *et al.*, 2004). We previously demonstrated that UL141 elicits efficient protection against NK cell-mediated cytolysis by sequestering CD155 in the ER yet, in isolation, had no effect on CD112 (Tomasec *et al.*, 2005).

CD155 is the poliovirus receptor (PVR) or nectin-like molecule-5 (necl-5), while CD112 is also referred to as nectin-2, herpesvirus entry mediator B (HVEB) or poliovirus receptor-related protein 2 (PRR2). CD112 and CD155 are both structurally and functionally related. Nectins and necls are immunoglobulin-like molecules involved in cell adhesion, movement, proliferation, differentiation, polarization, virus entry and immune recognition (Takai *et al.*, 2008). In view of its important role as an activating ligand for DNAM-1, we sought to analyse CD112 expression in the context of HCMV infection. Initial flow cytometry studies revealed that CD112 was downregulated by the low passage HCMV strain Merlin, but not high passage strain AD169 (not shown). Strain AD169 has a 15 kb deletion encompassing UL132–UL150 that includes the NK cell evasion genes UL141 and UL142. Merlin was derived from a bacterial artificial chromosome (BAC) containing the entire strain Merlin genome (R. J. Stanton, unpublished data). MerlinΔUL141 was generated using technologies developed previously to facilitate manipulation of the adenovirus genome (Stanton *et al.*, 2008). Briefly, a selectable cassette comprising ampicillin resistance, lacZ and SacB was PCR amplified and recombineered into the Merlin BAC in place of nt 184597–185412 (relative to published Merlin sequence GenBank accession no. NC_006273) using primers SacBF-UL141 (5′-caggtagcataggaaacatacggtgaaaatactccaaaatcccaaaaatgccgcgattccccgagtggcccagggagacctgtgacggaagatcacttcg-3′, homology to pAL1111 underlined) and SacBR-UL141 (5′-ccgacgtttgagcggccgacacacggagcaggaacaggcgggcagcgtctctgcgaaaaagggaagaaaagaatcatcctgaggttcttatggctcttg-3′, homology to pAL1111 underlined). In a second recombineering step, the selectable cassette was removed using oligo delUL141 (5′-atactccaaaatcccaaaaatgccgcgattccccgagtggcccagggagagatgattcttttcttccctttttcgcagagacgctgcccgcctgttcctg-3′), leaving behind a seamless deletion of the first 816 bp of the UL141 ORF.

In human fetal foreskin fibroblasts (HFFF) infected with Merlin, cell surface levels of CD155, CD112 and MHC-I were progressively downregulated over the course of infection (Fig. 1), with the change in CD112 being more pronounced at 48 h post-infection (p.i.) (Fig. 1b). In accord with previous observations (Tomasec *et al.*, 2005), cells infected with MerlinΔUL141 had elevated cell surface levels of CD155, while CD112 levels were comparable with the mock-infected HFFF (Fig. 1). Deletion of UL141 therefore ablated downregulation of both CD155 and CD112. This restoration of CD112 expression was unexpected, since UL141 had no overt effect on CD112 when expressed in isolation (Tomasec *et al.*, 2005). Interestingly, a small reproducible decrease in CD112 persisted when MerlinΔUL141-infected and mock-infected

cells were compared at 96 h p.i. (Fig. 1d). Replicate samples from the flow cytometry study were analysed by immunoblot, in order to further assess the fate of the CD112 protein within the cell. Briefly, cells were extracted with Triton X-114 (Bordier, 1981), proteins were separated on NuPAGE gels (Invitrogen) and blots were analysed with two independent polyclonal anti-CD112 antibodies. In Merlin-infected cells, the loss of CD155 from the cell surface (Fig. 1) correlated with the emergence of elevated levels of an immature (endoglycosidase H-sensitive) form of CD155 complexed with gpUL141 in the ER (Cochrane, 2009; Tomasec *et al.*, 2005) (Fig. 2a). In contrast to CD155, the CD112 signal gradually decreased in Merlin-infected cells and was not detected by 72 h p.i. (Fig. 2a).

Quantitative real time-PCR showed CD112 mRNA levels to be marginally increased throughout the infection (not shown), consistent with CD112 expression being regulated post-transcriptionally. To determine whether CD112 was targeted for proteolytic degradation, Merlin-infected cells were incubated in the presence of proteasome inhibitors. Treatment with either MG132 or Epoxomycin (Calbiochem) was able to restore CD112 expression, indicating that HCMV targeted CD112 for proteasome-mediated degradation (Fig. 2b).

UL141 was required for efficient downregulation of both CD112 and CD155 from the cell surface in HCMV-infected cells (Figs 1 and 3a), yet had no effect on CD112 in cells infected with recombinant adenovirus vector encoding UL141 [RAdUL141 (Tomasec *et al.*, 2005); Fig. 3b]. We reasoned that UL141 acted in partnership with an additional HCMV-encoded function(s) to downregulate CD112. Indeed, the residual level of CD112 suppression mediated by the MerlinΔUL141 (Figs 1d, 2a and 3a) could potentially be mediated by this function operating sub-optimally. In cells co-infected with MerlinΔUL141 and RAdUL141, the HCMV deletion mutant was complemented; downregulation of both CD112 and CD155 was restored (Fig. 3c). Similarly, co-infection of strain AD169 with RAdUL141 also resulted in the downregulation of both CD112 and CD155 (Fig. 3d). These data are consistent with UL141 co-operating with additional HCMV-expressed function(s) to efficiently downregulate CD112, and that function also being intact within AD169 strain (thus excluding UL133–150). Through downregulation of CD112, HCMV eliminates from the cell surface an activating ligand for DNAM-1, which presumably contributes to the enhanced killing of HCMV-infected cells observed when UL141 is deleted from the virus (Fig. 3e, f), but not to the protection elicited when UL141 is expressed in isolation (Tomasec *et al.*, 2005). HCMV thus targets both ligands for the NK cell activating receptor DNAM-1. GpUL141 alone is sufficient to sequester CD155 in the ER, while this study predicts that gpUL141 acts in concert with an additional viral function to induce proteasome-mediated degradation of CD112. This additional viral function could either directly co-operate with UL141, or act upon a cellular intermediate.

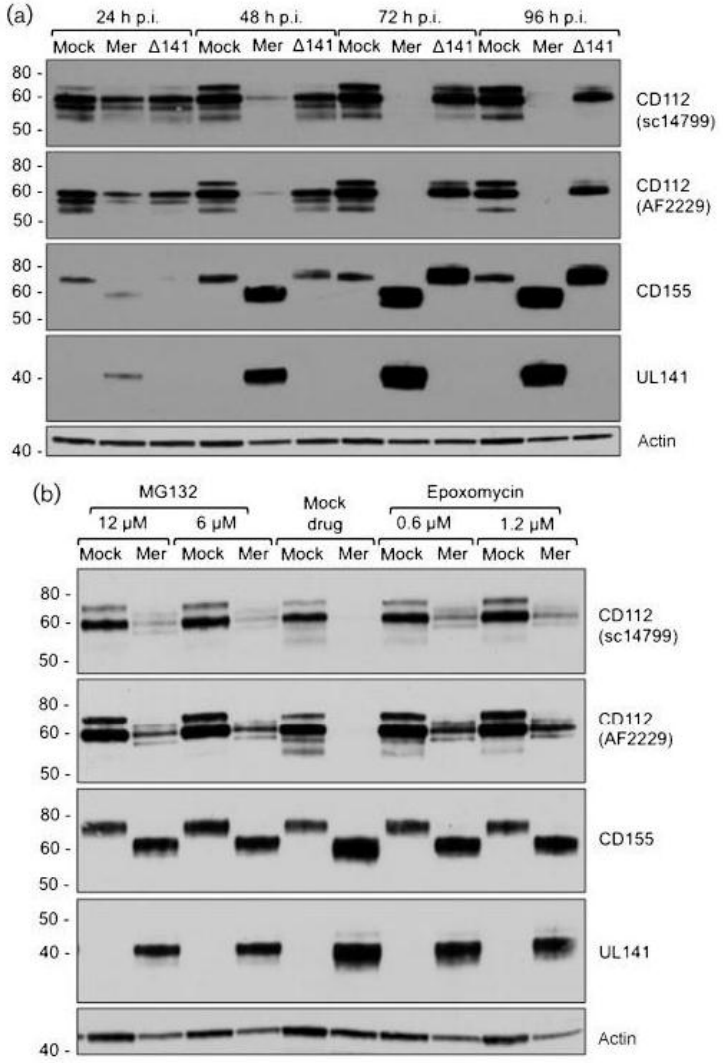

Fig. 2. HFFF were infected (m.o.i.=25) for 24, 48, 72 or 96 h p.i. with HCMV strain Merlin (Mer), MerlinΔUL141 (Δ141) or mock-infected (Mock) and cell extracts were analysed by immunoblot using antibodies to: CD112 (R&D, AF2229; Santa Cruz, sc-14799), CD155 [5D1 (Aoki *et al.*, 1994)], UL141 [M550 (Tomasec *et al.*, 2005)] and actin (A-2066; Sigma). (b) HFFF were infected (m.o.i.=25) for 48 h with HCMV strain Merlin (Mer) or mock-infected, then treated for 12 h with proteasome inhibitors MG132 or Epoxomycin as indicated and analysed by immunoblot as in (a).

DNAM-1 is remarkable in being expressed on all NK cells and plays a major role in regulating their function. HCMV suppression of CD112 and CD155 may have ramifications that extend beyond the regulation of NK cell function. DNAM-1 is also expressed on activated T, NKT, myeloid and mast cells, megakaryocytes, platelets and a subset of B lymphocytes thereby impacting on a wide range of immunological responses and regulating platelet activation (Bachelet *et al.*, 2006; Bottino *et al.*, 2003; Burns *et al.*, 1985; Kojima *et al.*, 2003; Pende *et al.*, 2006; Reymond *et al.*, 2004; Scott *et al.*, 1989; Shibuya *et al.*, 1996, 1999, 2003; Xu & Jin, 2010). For example, the interaction between DNAM-1 and CD112/CD155 has been associated

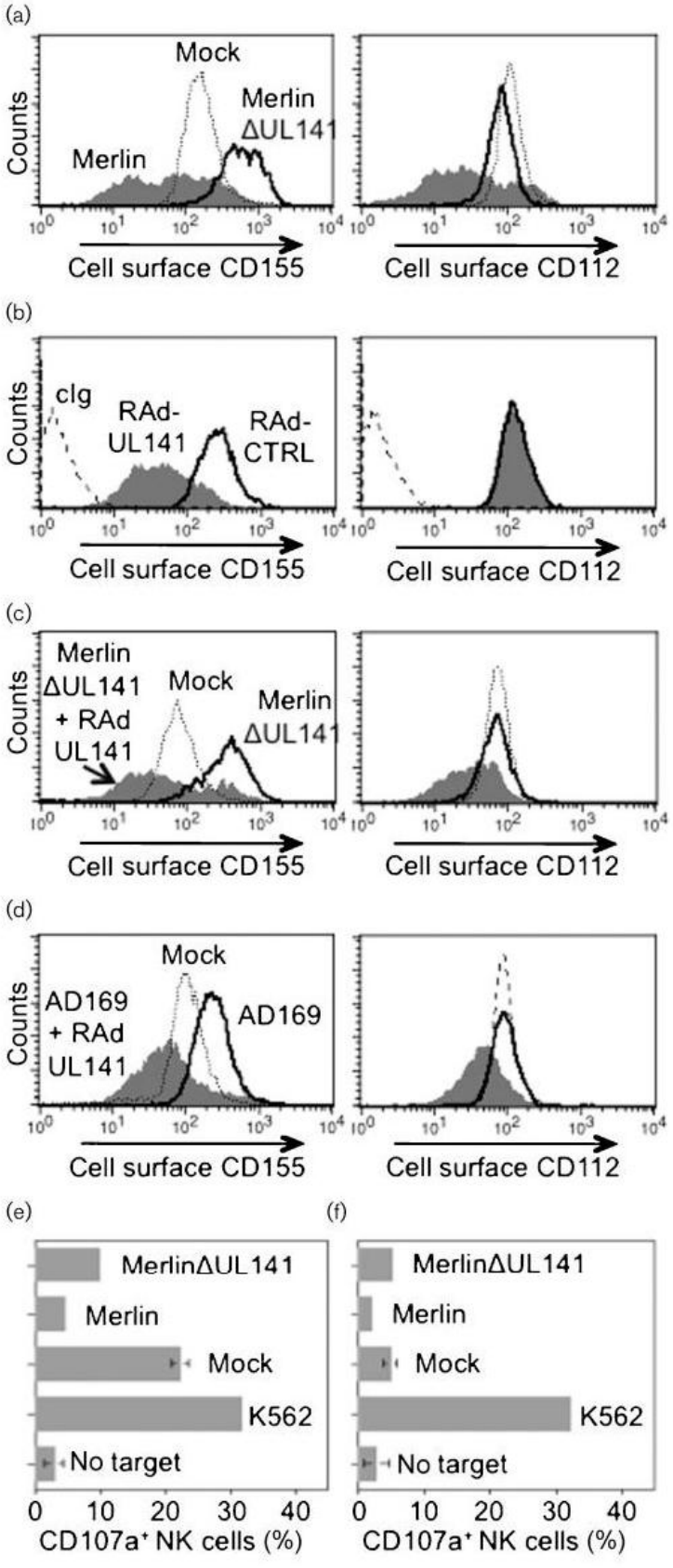

Fig. 3. HFFF were infected for 72 h (m.o.i.=25) with HCMV strain Merlin or MerlinΔUL141, as indicated, and analysed for cell surface expression of CD155 and CD112 by flow cytometry. (b) HFFF were infected for 72 h (m.o.i.=200) with replication-deficient adenovirus vectors encoding HCMV UL141 (RAd-UL141) or equivalent empty RAd (RAd-CTRL) (Tomasec *et al.*, 2005), as indicated, and analysed for cell surface expression of CD155 and CD112 by flow cytometry. (c) HFFF were co-infected for 72 h with MerlinΔUL141+RAd-CTRL or MerlinΔUL141+RAd-UL141, as indicated, and analysed for cell surface expression of CD155 and CD112 by flow cytometry. (d) HFFF were co-infected for 72 h with HCMV strain AD169+RAd-CTRL (AD169) or AD169+RAd-UL141, as indicated, and analysed for cell surface expression of CD155 and CD112 by flow cytometry. Control Ig histograms (clg) were not included in panels (a), (c) and (d) to maintain figure clarity. (e) HFFF were infected for 72 h with HCMV strain Merlin, MerlinΔUL141 or mock infected. Sensitivity to NK cells was measured using alpha interferon (IFN-α) activated PBMC in allogeneic CD107a mobilization assay (Prod'homme *et al.*, 2007) using the following antibodies: anti-CD107a-FITC (553793; BD Biosciences), anti-CD3-PerCP (SK7; BD Biosciences), anti-CD56-APC (N901; Beckman Coulter). PBMC incubated without targets and K562 cells are shown as controls. (f) RS primary skin fibroblasts were infected for 72 h with HCMV strain Merlin, MerlinΔUL141 or mock infected. Sensitivity to NK cells was measured using IFN-α activated RS PBMC in autologous CD107a mobilization assay as described in (e).

with T-cell differentiation, proliferation, cytotoxicity and cytokine secretion (Tahara-Hanaoka *et al.*, 2004). Furthermore, nectins and necls regulate fundamental processes in cell biology including cell adhesion, movement, proliferation, differentiation, survival, polarization and signalling (Takai *et al.*, 2008). HCMV infection is recognized to disrupt focal adhesions and intercellular connections, while inducing cell motility and transendothelial migration (Chan *et al.*, 2009; Stanton *et al.*, 2007). It will be important to determine how the modulation of CD112 and CD115 influences these processes.

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