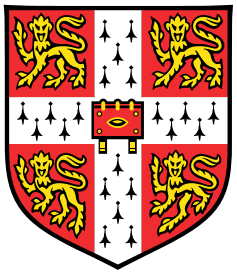
Investigating and exploiting the latency-associated expression of the human cytomegalovirus gene *US28* in early myeloid lineage cells.



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I would like to dedicate this thesis to Mum and Dad, to whom I promise to get a real job, Rachael, who always puts up with me, and the memory of Dr Chris Scanlan, who I hope isn't watching me from heaven.

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements. This dissertation contains fewer than 60,000 words including appendices, figure legends and tables and has fewer than 150 figures.

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Benjamin Krishna, December 2016.

Abstract

Human cytomegalovirus (HCMV) is a betaherpesvirus which establishes a lifelong persistent infection, underpinned by its ability to establish latent infection in early myeloid lineage cells, in the infected host. Although well controlled by a healthy immune system, HCMV causes pathological and life threatening disease in individuals with a compromised or immature immune response, which can come from primary HCMV infection or reactivation of latent infection. Although progress is being made in understanding the mechanisms by which HCMV maintains latency and reactivates, a better understanding is essential towards the aim of targeting and killing latently infected cells.

In this thesis, I will present evidence that the HCMV-encoded chemokine receptor homologue US28, which is expressed during latent infection of CD14+ monocytes, is necessary for maintaining HCMV latency in these monocytes and, in the absence of US28 protein expression, HCMV undergoes lytic infection. US28 expression was found to attenuate cellular signalling pathways in latently infected cells; in particular, MAP kinase and NFκB. Interestingly, deletion of the US28 gene or inhibition of the US28 protein resulted in the expression of lytic antigens which allowed detection of infected monocytes by the immune system. This observation may lead to a potential new immunotherapeutic strategy against latent HCMV.

Having demonstrated that US28 protein is expressed on the surface of latently infected monocytes, I tested whether a new fusion-toxin protein, called F49A-FTP, which binds US28 protein, could be used to target and kill latently infected cells. I developed a protocol for treating latently infected monocytes with F49A-FTP which resulted in a significant reduction in virus reactivation after monocyte differentiation to dendritic cells. I was also able to show that this treatment kills CD34+ progenitor cells, which were experimentally latently infected with HCMV, as well as latently infected monocytes from a healthy, seropositive blood donor.

Finally, during my investigations into the role of US28 during HCMV latency, a mass spectrometry screen was performed to measure changes in cellular protein expression when US28 protein is expressed in isolation, in THP-1 monocyte-like cell line. This identified CTCF, a transcription factor which appears to be modified by US28 in THP-1 cells. I showed that CTCF has a repressive effect on the HCMV MIEP, and that CTCF likely plays a role in HCMV latency.

In summary, this work provides insights into the role of US28 during HCMV latency, and proposes potential novel therapeutic strategies to kill latently infected cells.

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Abbreviations

ACVR1B	Activin A Receptor 1B
ADAM17	A Disintegrin And Metalloproteinase domain 17
ADP	Adenosine Diphosphate
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	one-way Analysis of Variance
BioID	proximity-dependent biotin identification
CTCF	CCCTC-binding factor
CCR	Chemokine Receptor
ChIP	Chromatin Immunoprecipitation
cGAS	Cyclic guanosine monophosphate-AMP synthase
cDNA	Complementary DNA
cIL10	Cellular interleukin 10
COS-7	CV-1 in Origin, and carrying the SV40 genetic material
COX2	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
EBV	Epstein Barr virus
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
eNOS	Endothelial Nitric Oxide Synthase
ERF	ETS domain-containing transcription factor
FACS	Fluorescence Activated Cell Sorting
FAK	Focal Adhesion Kinase
FTP	Fusion Toxin Proteins
gB/gH/gM	Glycoproteins B/H/M
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GBM	Glioblastoma Multiformae
G-CSF	Granulocyte-colony Stimulating Factor
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein

GTP	Guanosine Triphosphate
GKR	G-coupled Receptor Kinases
GM-CSF	Granulocyte-macrophage Colony-stimulating Factor
GPCR	G protein-coupled receptor
HCMV	Human Cytomegalovirus
HDAC	Histone Deacetylase
HEK293T	Human Embryonic Kidney cells
HFF	Human Foreskin Fibroblast
HAART	Highly Active Antiretroviral Therapy
HGF	hepatocyte growth factor
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HSCT	Hematopioetic Stem Cell Transplant
HSV	Herpes Simplex Virus
IE	Immediate Early
IF	Immunofluorescence
IFN	Interferon
IgG	Immunoglobulin G
IL4/6/10	Interleukin 4/6/10
IL6R	IL6 receptor
IRF3	Interferon Regulatory Factor 3
ISG	Interferon Stimulated Genes
JAK/STAT	Janus kinase/Signal Transducer and Activator of Transcription
KDM1	Lysine-specific Histone Demethylase
KSHV	Kaposi's Sarcoma-associated Herpesvirus
LacmvIL-10	Latency-associated Viral Interleukin 10
LncRNA	Long non-coding RNAs
LIR1	Leukocyte Immunoglobulin-like Receptor 1
LPS	Lipopolysaccharide
LUNA	Latency Unique Natural Antigen
MACS	Magnetic-activated Cell Sorting
MCL-1	Induced Myeloid Leukemia Cell Differentiation Protein
MCP1/2/3	Monocyte-specific Chemokine-1/2/3
MDBP	Methylated DNA Binding Protein
mDC	mature dendritic cells

MIEP	Major Immediate Early Promoter
miRNA	microRNA
MHC	Major Histocompatibility Complex
MCMV	Murine Cytomegalovirus
MRP-1	Multidrug resistance-associated Protein 1
mRNA	Messenger RNA
ND10	Nuclear Domain 10
NFκB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NK cells	Natural Killer cells
ORF	Open Reading Frame
OPG	Osteoprotegerin
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Pseudomonas Exotoxin-A
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMNL	Polymorphonuclear Leukocytes
pp65	Phosphoprotein 65
PRR	Pattern Recognition Receptor
PCR2	Polycomb Repressor Complex
RCF	Relative Centrifugal Force
REAP	Rapid Efficient And Practical
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RNA Seq	RNA Sequencing
RPE-1	Retinal Pigment Epithelium
RPMI	Roswell Park Memorial Institute
SMC	Smooth Muscle Cells
STAT1/2	Signal Transducer and Activator of Transcription 1
TEAB	Tetraethylammonium bromide
TFA	Trifluoroacetic acid
TGF beta	Tumour Growth Factor
TVS	Transplant Vascular Sclerosis

UL	Unique Long
US	Unique Short
VZV	Varicella Zoster Virus
vIL10	Viral Interleukin-10
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type
YY1	Ying Yang 1

Table 1 List of Abbreviations.

Chapter 1

Introduction

1.1 Phylogeny of HCMV in the *Herpesviridae*

Human cytomegalovirus (HCMV, formally Human Herpesvirus 5 or HHV5) is a member of the family *Herpesviridae* [163], characterised by a large DNA (deoxyribonucleic acid) genome and by their ability to establish a lifelong, latent infection in an animal host. Latency is defined as the maintenance of the viral genome, in an infected host cell, likely with a restricted viral gene transcription profile, and no production of infectious virus. Under certain stimuli, latent infection can reactivate to lytic infection which is defined by full virus gene expression and production of infectious virions.

The *Herpesviridae* family can be divided into three subfamilies (*herpesvirinae*): alpha, beta and gamma, based on rates of reproduction, range of hosts, cytopathology, cell tropism and more recently DNA sequence (see Table 1.2) [354, 355]. HCMV is a member of the *betaherpesvirinae* subfamily, which infect organisms in a species-specific manner. These three major subfamilies are thought to have first infected mammals approximately 180-200 million years ago [253], and to have co-evolved with their hosts, leading to diverse speciation and a wide range of hosts, indeed, eight (or nine, if HHV6 are indeed two separate species) herpesviruses are known to infect humans (see Table 1.1). The large genomes of the *herpesviridae* are thought to allow coding of multiple gene products to modulate host cell behaviour to support the viral life cycle [177].

1.1.1 Global seroprevalence of HCMV

HCMV infection is globally ubiquitous [486], with seroprevalence ranging from nearly 100% in underdeveloped countries in Africa, Asia and South America to less than 30% in adults in

Subfamily	Full name	Abbreviation	Site of latency
Alphaherpesvirinae	Herpes Simplex Virus 1	HSV-1/HHV-1	Neurons (trigeminal ganglia)
	Herpes Simplex Virus 2	HSV-2 / HHV-2	Neurons (sacral ganglia)
	Varicella Zoster Virus	VZV/HHV-3	Neurons (all ganglia)
Betaherpesvirinae	Human Cytomegalovirus	HCMV/HHV-5	Early myeloid cells
	Human Herpesvirus 6	HHV-6	T cells, monocytes and macrophages (including genomic integration)
	Human Herpesvirus 7	HHV-7	T cell
Gammaherpesvirinae	Epstein Barr Virus	EBV/HHV-4	Memory B Cell
	Kaposi's Sarcoma Virus	KSHV/HHV-8	B cell

Table 1.1 Classification of the human herpesviruses, adapted from Crough, 2009: "Immunobiology of human cytomegalovirus: from bench to bedside'.[93, 391, 370, 114, 4, 202, 269]

some areas of North America and northern Europe [55]. Seropositivity increases with age, with around a 2% increase in seropositivity per year after adolescence in North America and Northern Europe, and near universal seropositivity by adolescence in the developing world [413, 414].

Infection is thought to spread naturally via infectious bodily fluids including saliva, breast milk and genital secretions [46, 213, 415], though also through iatrogenic transmission in solid organ, blood and stem cell transplants [44, 80, 153]. Infection is most common during childhood, especially between children in group-care centres [2, 21], though vertical tranmission can occur *in utero*, during birth or through breastfeeding [292, 350, 415]. After adolescence, HCMV is presumed to spread via sexual contact between adults as well as contact with newly infected children [46].

Characteristic	*Alphaherpesviridae*	*Betaherpesviridae*	*Gammaherpesviridae*
Host range	Variable, often broad	Restricted	Limited to family of natural host
Reproductive cycle	Short (24 h)	Long (72 h)	Long (72 h)
Infection in cell culture	Short (24 h)	Long (72 h)	Long (72 h)
Cytotoxicity	Cell destruction	Cells become enlarged (cytomegalia)	Able to induce lymphoproliferation and cancers
Target cells	Primary infection in epidermal cells and latency in neurons	Many cells including secretory glands, lymphoreticular cells, kidneys and others	Specific for either B- or T- lymphocytes, Latent in Lymphoid tissue
Human Examples	HSV-1, HSV-2, VZV	HCMV, HHV-6A, HHV-6B and HHV-7	EBV and KSHV

Table 1.2 Classification of the three herpesvirus subfamilies by biological characteristics, adapted from [425]

1.2 HCMV basic virology

1.2.1 HCMV virion structure and life cycle

HCMV virions are spheres of approximately 230 nm in diameter, characterised by a large, linear, double-stranded DNA genome in the core. The HCMV viral genome is synthesised in the nucleus, where it is also encapsulated by the nucleocapsid proteins. This core is surrounded by the structured nucleocapsid, then a less structured tegument layer and finally a trilaminate membrane envelope [132] (Figure 1.1). The capsid and tegument layers are made of virally-encoded proteins, including viral and cellular RNAs (ribonucleic acids) in the tegument, acquired during the generation of virions in the viral assembly complex [45, 443] while the lipid viral envelope is formed as the nucleocapsid buds from the endoplasmic reticulum (ER) and Golgi apparatus, and is secreted via vesicles [109]. This means that the nucleocapsid acquires the tegument layer and lipid envelope from the nuclear and cellular membranes and the viral lipid envelope therefore contains both cellular proteins as well as virally encoded glycoproteins [132, 464].

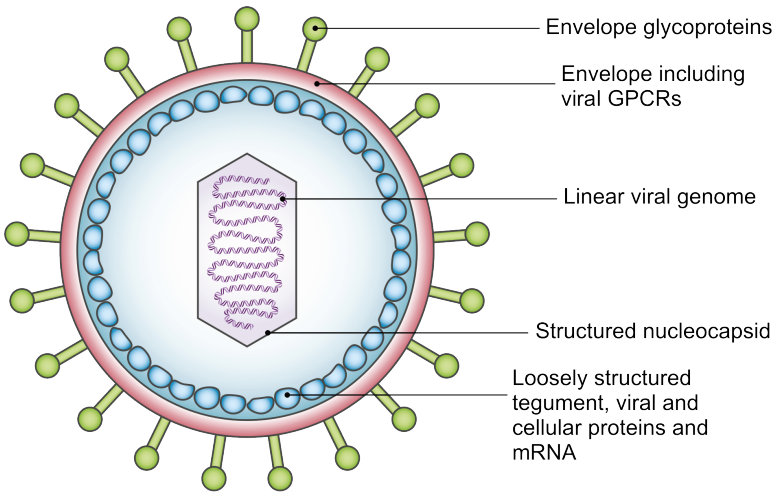

Figure 1.1 The HCMV virion, showing the viral genome, nucleocapsid, tegument layer and envelope with glycoproteins, this image has been adapted from a blog post in "This Week in Virology" by Vincent Racaniello

HCMV infects most cell types via cell receptor-mediated endocytosis triggered by virally encoded glycoproteins binding to target cellular receptors. The current model of HCMV infection is triphasic: firstly, the virion glyocoprotein B (gB), in conjunction with the glycoprotein M/glycoprotein N complex (gM/gN), tethers virus to the cell surface via heparan sulphate glycosaminoglycans [88, 91, 183, 184]. The second phase involves virion binding to HCMV receptors: candidate receptors include integrins and the epidermal growth factor receptors (EGFRs), both of which bind gB [118, 480, 481]. Thirdly, fusion of the viral and cellular membranes is mediated by gB, as well the trimeric complex of glycoprotein H, glycoprotein L and glycoprotein O (gH/gL/gO) in fibroblasts, most likely through interactions with platelet-derived growth factor-α; while a pentameric complex of glycoproteins gH/gL, with Unique Long (UL)128-131 mediate endocytosis in epithelial and endothelial cells [164, 165, 371, 372, 462, 463, 492, 498].

The HCMV lifecycle is summarised in Figure 1.2. After membrane fusion, the viral capsid loses its tegument layer. The released tegument proteins facilitate the migration of the viral capsid towards the nucleus, via microtubules, the release of the viral genome from the capsid, and activation of viral gene expression [25, 119, 179, 478]. Viral genome replication can now occur in the new host cell. HCMV lytic infection is characterised by a temporal cascade of gene expression which be separated into Immediate Early (IE), Early and Late phases of gene expression. Major IE gene expression is driven by the Major Immediate Early

promoter (MIEP), and IE gene products control cell cycle progression (forcing the cell into G1/S phase), prevent cellular antiviral defences and promote early and late gene expression [422]. After this, expression of early genes perform a range of functions, including provision of viral DNA synthesis machinery; generally, both IE and early genes contribute to a cellular environment which is optimal for viral gene expression and viral genome synthesis [490]. Finally, late genes encode viral structural proteins, are expressed from around 48 hours post infection, and their expression begins the production of infectious virions [271].

1.2.2 HCMV genome organisation

HCMV has a relatively large genome, approximately 235 kb, which encodes between 175 - 750 open reading frames (ORFs), including at least 24 microRNAs, and this value depends on whether one classifies genes using the classical analysis of ORFs or uses more recent ribosome profiling analysis [129, 417, 421]. The genome is separated into two unique segments, known as long and short (UL and US), separated by internal repeat segments and terminal repeats at either end of the genome (see Figure 1.3). HCMV genes are numbered sequentially starting from either the UL or US region. Clinical strains generally have the entire HCMV genome intact, but compared to the reference clinical strain, Merlin, the laboratory-passaged isolates of virus have lost a number of genes including portions of the 13kb region known as ULb', which encodes genes that affect cell tropism as well as immune evasion [65, 476, 477].

1.2.3 HCMV cell tropism

HCMV can productively infect a relatively wide range of cell types during primary infection *in vivo*, including fibroblasts, epithelial cells, endothelial cells, hepatocytes, myeloid cells, and neuronal cells [284]. This wide range of target cells is important for HCMV spread. For example, infection of epithelial cells allows for inter-host transmission; infection of endothelial cells and haematopoietic cells facilitates systemic spread within a host, while infection of smooth muscle cells and fibroblasts provides ubiquitous cell types for virus production [393]. Although infection of early myeloid cells is not productive, it creates a site for latent infection (see in detail, below), which is not cleared by the immune system and is therefore important for lifelong infection by HCMV [493].

The tropism for epithelial and myeloid lineages varies greatly among different HCMV strains, and is dependent on difference in the UL128-131 genes of the ULb' locus. UL128-131 encode small proteins which form a complex with the HCMV gH/gL to form the pentamer:

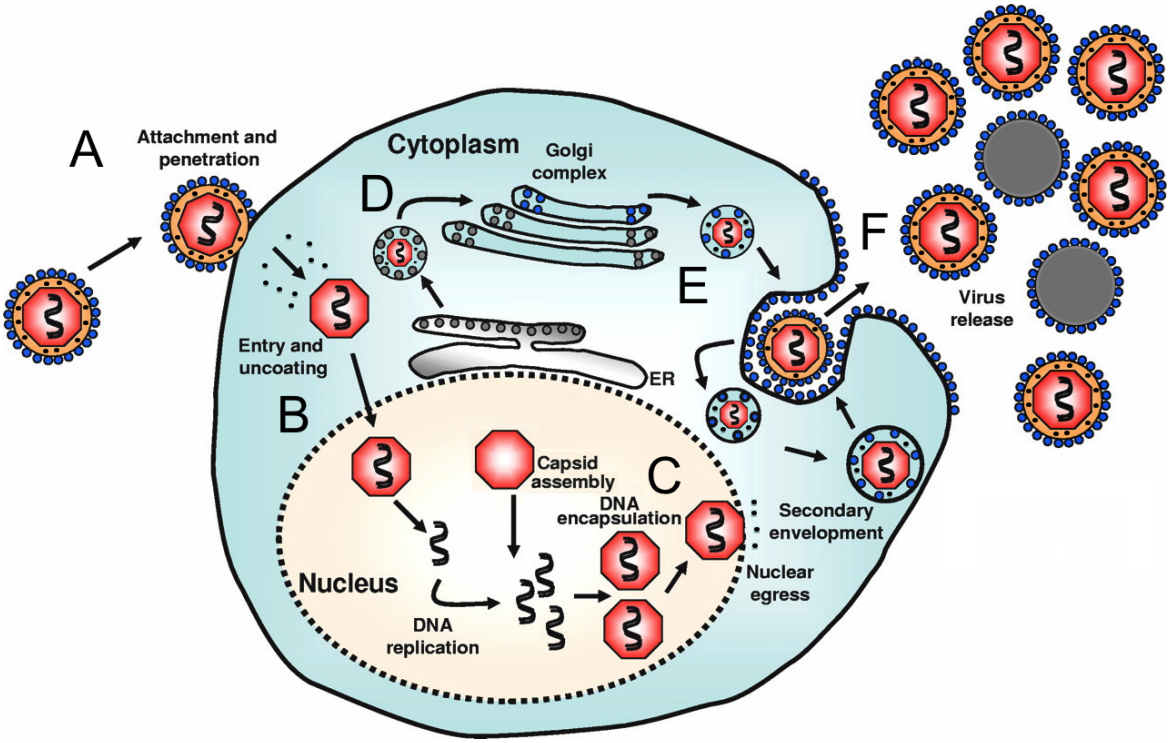

Figure 1.2 The HCMV lifecycle, adapted from [93] based on [172]. (A) Infectious HCMV virions attach to the cell surface via interactions between cellular receptors and viral glycoproteins. Upon membrane fusion, tegument proteins are released into the cytosol, which attenuate cellular innate immune responses. (B) The viral capsid is trafficked to the nucleus, where the genome is delivered via a nuclear pore. The genome is circularised in the nucleus and IE gene are immediately expressed, initiating the viral gene expression cascade. (C) Late gene expression begins capsid assembly within the nucleus. Capsids egress the nucleus, picking up tegument proteins before being trafficking through the endoplasmic reticulum (ER) and Golgi complex. (D) The ER and golgi complex (which form the viral assembly complex during HCMV infection), contain further tegument components as well as viral envelope proteins. (E) Virus budding from the intracellular vesicles of the assembly complex provides the viral envelope, and then (F) viral particles (both infectious and non-infectious dense bodies) are released from the cell.

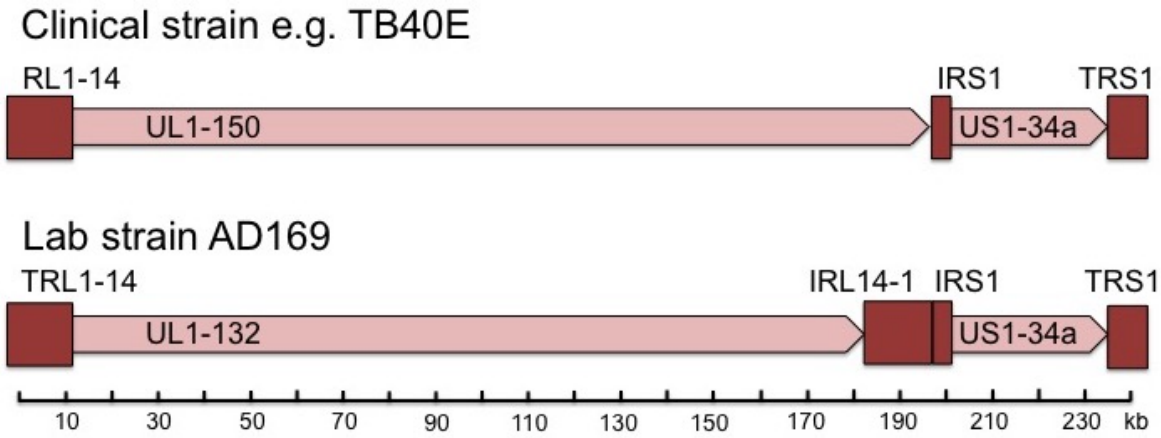

Figure 1.3 The HCMV genome structure, adapted from [279]. Top: a map of the conventional genome structure of a clinal isolate. Containing the long repeat (RL) with 14 genes, the unique long (UL) region with 151 genes, the short internal repeat (IRS), the unique short region (US) with 34 genes and the short terminal repeat (TRS). Bottom: a map of the conventional genome structure of a laboratory-passaged strain such as AD169. The structure is very similar to the above, however the UL132-151 region (also known as ULb') has been deleted and replaced with a duplicated long repeat region (IRL).

gH/gL/UL128-131 [371, 476]. Deletion of the UL128-131 gene locus prevents formation of the gH/gL/UL128-131 pentamer, reducing entry into epithelial and endothelial cells; deletion of any single gene in the gH/gL/UL128-131 complex reduces the efficiency of complex formation and endothelial cell entry [371, 372]. HCMV, without the UL128-131 genes, can infect fibroblasts and is thought to enter fibroblasts via direct fusion of cell and viral membranes, likely because HCMV can infect fibroblast cells in conditions that inhibit endocytosis or endosome acidification [90]. Consequently, viral propagation in fibroblasts fails to select for viruses with high endothelial tropism, while propagation in endothelial cells maintains a broader range of cell tropism [395, 474].

1.2.4 The immune response to lytic infection and immune evasion by HCMV

HCMV elicits a significant immune response in healthy human hosts, which controls the virus, leading to an asymptomatic infection. Despite primary infection being controlled by the immune system, HCMV persists in the host for life due to its ability to establish latent infection (which is not cleared by the immune system), while immune evasion by HCMV

facilitates viral establishment of latency. HCMV has mechanisms to evade both innate and adaptive immunity [327].

Upon infection, a number of host-mediated innate immune responses are triggered which are combatted by expression of viral IE gene products. For instance, glycoprotein binding to cellular receptors triggers NFκB and interferon regulatory factor 3 (IRF-3) [285, 505]. Similarly, HCMV glycoproteins gB and gH are recognised by Toll-like receptor 2, which activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and trigger significant chemokine secretion [37, 38, 48]. However, HCMV IE86 protein interferes with NFκB signalling to attenuate this response [45, 56, 327]. Similarly, HCMV DNA activates IRF-3, which leads to the transcription of interferon-β and the stimulation of Interferon stimulated genes (ISGs) [98, 99] and in turn, HCMV IE72 protein is thought to act antagonistically against this process through interaction with Signal transducer and activator of transcription 2 (STAT2), blocking interactions between phosphorylated STAT2 and STAT1 [304].

In addition to innate immunity, there is also a significant adaptive immune response to HCMV, including both cellular and antibody-mediated immunity. Antibodies are routinely generated against an array of epitopes, including the tegument proteins phosphoprotein 65 (pp65) and pp150 [170], envelope glycoproteins (gB, gH) [47, 458], the pentameric entry complex [125, 489] and non-structural proteins (IE72) [353]. This antibody immunity likely acts to both neutralise the virus by blocking receptor binding and entry, as well as activating complement and antibody-dependent cellular cytotoxicity [82, 234, 499] which may play a protective role against HCMV. This is supported by the observation that prophylactic antibody treatment reduces the risk of HCMV congenital infection (see 1.3.2) [122], which couldn't be reproduced in a randomised control trail [349], however the cellular immune response is now though to be more significant for control of HCMV [276].

The human CD4+ T cell response to HCMV is against a broad array of viral epitopes, with a median of 12 ORFs being recognised by healthy carriers of HCMV, of which 5 immunodominant ORFs (UL55; gB, UL83; pp65, UL86, UL99 and UL123/122; IE-1/2) were recognised by greater than half of those tested, individual donors [432]. Additionally, of 213 HCMV ORFs studied, greater than 70% elicited CD4+ or CD8+ T cell responses. The CD8+ T cell response is also significant; approximately 10% of memory CD8+ T cells in seropositive donors recognised HCMV peptides and this response was focussed, by a small set of T cell clones on specific antigens; including IE1, UL48 and pp65, which is a phenomenon likely due to repeated antigen exposure [432, 475]. Three ORFs: IE1 (UL123), pp65 (UL83) and UL48 elicited a CD8+ T cell response in greater than 50% of seropositive

donors [432]. These CD8+ T cells respond to HCMV infection with expression of cytotoxic effector proteins, perforin and granzyme B, and show direct cytotoxicity *ex vivo* [475].

HCMV has a number of mechanisms to evade this adaptive arm of the immune system. US2, US3, US6 and US11 act in concert to downregulate cell surface presentation of major histocompatibility complex (MHC) class I molecules from the cell surface, preventing antigen presentation and subsequent CD8+ T cell recognition [145, 155]. However, this downregulation of MHC class I risks detection of HCMV-infected cells by the missing self mechanisms on natural killer (NK) cells. To evade this NK cell mediated killing, UL18 and UL40 behave as an HLA-E mimics and are presented on the cell surface to bind Leukocyte Immunoglobulin-like receptor 1 (LIR1) which inhibits NK cell killing [330]. Additionally, the US12 gene family downregulate a number of activators of NK cells, including MICA, MICB and the B7 family [120]; while UL141 downregulates CD112 via US2 mediated recruitment of ubiquitin E3 ligases [155].

1.3 Pathogenesis and treatment of HCMV infection

1.3.1 HCMV diseases

HCMV infection is generally asymptomatic in healthy individuals, due to viral control by a robust immune response, however, primary HCMV infection can sometimes present as symptoms similar to infectious mononucleosis [351, 396]. Disease associated with HCMV occurs when the immune system is compromised, and loses control of viral infection, which can be either a primary infection or reactivation from a latent infection [93]. The severity of CMV disease - correlated to the level of lytic HCMV infection in an immunocompromised host - is a consequence of the wide cell tropism of HCMV, leading to infection of multiple organs, a wide array of potential symptoms, and eventual organ failure [393]. In addition to CMV disease, asymptomic HCMV infection in healthy, seropositive individuals has also been linked with an increased incidence of atherosclerosis, arterial hypertension, glioblastoma (with great controversy) and Guillain-Barré syndrome, and such conditions reduce overall life expectancy in these seropositive individuals [297, 335, 377, 386].

HCMV infection causes significant morbidity and mortality in the immunocompromised, the immunosuppressed, and the immunonaïve and this threat of HCMV infection *in utero* contributes greatly to the reduction in disability adjusted life years associated with HCMV [241, 424]. Congenital HCMV infection leads to cytomegalic inclusion disease (which includes intrauterine growth retardation, jaundice and microcephaly), with a mortality rate

of 30%. Survivors can suffer from multiple disabilities, due to cerebral calcification, with neurological, hearing and visual impairments [54, 76]. The greatest risk of symptomatic congenital HCMV infection is observed upon primary infection of seronegative mothers during the first trimester of pregnancy [414]. However, seropositive mothers are still at risk due to both reactivation of latent virus or reinfection with a different HCMV strain [43].

Before highly active antiretroviral therapy (HAART), roughly 40% of immunocompromised, human immunodeficiency virus (HIV)-infected patients suffered from CMV disease in developed countries; most commonly CMV retinitis [103]. More recently, the increasing numbers of organ transplants that are performed every year has led to an increased number of patients who are immunosuppressed to prevent transplant rejection and are therefore at risk of CMV disease [333]. Solid organ transplant recipients, who are seronegative (R-) are at the greatest risk of CMV disease when receiving an organ from a cadaveric, seropositive donor (D+), due to both lytic and latent virus being carried in the donated tissue [204]. As well as CMV disease, HCMV may be linked to a greater risk of graft rejection, as well as general morbidity and mortality [93, 152, 307]. Prophylactic treatment with the HCMV antiviral ganciclovir, blood screening and donor/recipient matching for HCMV serostatus have decreased the incidence for HCMV disease however, despite this, HCMV remains a significant threat [46].

Allogeneic haematopoietic stem cell transplants (allo-HSCTs) are routinely used as a treatment for several high-risk leukaemias and other non-malignant diseases. Peripheral blood stem cells, for example from Granulocyte-colony stimulating factor (G-CSF) mobilised donors, are now one of the most common sources of stem cells for allo-HSCT and comprise multipotent CD34+ cells, which expand and differentiate to reconstitute the immune system. However, this differentiation can also result in HCMV reactivation in up to 80% of allo-HSCT patients, if not treated with anti-virals [260, 434]. This threat is greatest for R- patients of a graft from a D+ donor. However, for a R+ recipient, subsequent immunosuppression also risks reactivation of the recipient's own latent virus [230]. Whilst prophylactic treatment with anti-virals such as ganciclovir and foscarnet keeps CMV disease incidence below 10% in these patients (this 10% is often due to late-phase HCMV disease), ganciclovir-mediated neutropenia can lead to increased mortality from bacterial and fungal infections [287]. Consequently, the reduction in latent HCMV load in haematopoietic stem cell transplants could have far-reaching clinical benefits [34–36, 231, 232].

1.3.2 Drug treatments for HCMV disease

The current favoured drug treatment for active CMV disease in the immunocompromised is intravenous ganciclovir treatment [243]. Ganciclovir is a nucleoside analogue that preferentially inhibits viral DNA polymerases when phosphorylated to form ganciclovir triphosphate [126]. Initial phosphorylation is catalysed by the HCMV-encoded UL97 kinase, followed by subsequent phosphorylation, catalysed by cellular kinases [227]. As the first phosphorylation step is due to an HCMV-encoded kinase, this leads to drug selectivity for infected over non-infected cells. Ganciclovir has associated cytotoxicity which can cause neutropenia, thrombocytopenia and putative long term infertility [117, 420] and viral resistance to ganciclovir is common after several months of therapy [225]. Other treatments for HCMV include valganciclovir, a ganciclovir prodrug with oral bioavailability, which is provided for solid organ transplant recipients and for patients with HIV as a prophylaxis [154]. Second-line drugs, such as foscarnet and cidofovir, also inhibit the HCMV DNA polymerase, while maribavir inhibits the UL97 kinase, and off-label leflunomide (an immunosuppressive) inhibits nucleocapsid tegumentation, although this is still under investigation [66]. The viral terminase inhibitor, letermovir, has shown great promise in the treatment of CMV acquired after haemotopoetic stem cell transplants [465, 246]. CMV immunoglobulin therapy is approved by the US FDA for prophylactic anti-CMV treatment in conjunction with ganciclovir in high-risk lung transplant recipients. CMV immunoglobulin therapy has also shown success in cardiothoractic transplant recipients [459, 460] though this has had mixed results in preventing congenital CMV disease [288, 289, 349].

1.3.3 Vaccines against HCMV are still in development

An HCMV vaccine was assigned in the highest priority category by the Institute of Medicine, and the second highest priority target after HIV by the Centers for Disease Control [12]. This is due to the recognised combination of widespread infection, large disease burden and limited applications for antiviral drugs and newer therapeutics [12]. Analysis of the immune response to HCMV clearly indicates that a robust antibody and cellular response will be needed to confer protection [222].

The first vaccine candidates against HCMV were live attenuated virus vaccines dating back to the 1970s, which were based on the laboratory-passaged Towne strain of HCMV [112, 317]. This vaccine had low immunogenicity, and attempts have been made to generate chimera with greater immunogenicity [149, 169, 379]. More recently, recombinant vaccines have been based on the two immunodominant epitopes of HCMV: pp65 and gB [432].

Immunisation against these two proteins has been delivered as vaccines using different methods including: delivery of the solubilised proteins [268, 302, 357, 358], DNA vaccines consisting of plasmids containing the genes encoding pp65 and gB [168, 192] as well as viral replicon particles, which are replication-deficient virions that express high levels of encoded proteins and induce a strong immune response [30, 338]. HCMV dense bodies, which are viral tegument and glycoprotein-rich envelopes that lack viral capsids or DNA and are produced by HCMV infection *in vitro*, induce antibody and T cell responses even without adjuvants and, therefore, could be used as a vaccine candidate against HCMV [64]. A recent landmark vaccine consisted of recombinant HCMV gB with MF59 adjuvant; this showed 50% efficacy in protecting young mothers from HCMV infection in Phase II trials as well as protection against viraemia for R-/D+ transplant recipients [139, 303].

The observation that HCMV can superinfect an already persistently infected host [144] suggests that the development of an effective HCMV vaccine will be difficult. Although there is currently no vaccine against HCMV, the relative success of the VZV vaccine [74] as well as advances in vaccines against HSV-2 [308] provide hope that a vaccine against HCMV is possible. Due to the ubiquity of HCMV infection, any vaccine against HCMV which would be given to the general population, would take a very long time to eradicate the disease. Thus, novel antivirals, particularly against latent infection, are still needed.

1.4 Human cytomegalovirus latency

1.4.1 Discovery and brief history of HCMV and latency

HCMV was isolated independently by Smith, Rowe and Weller between 1955 and 1957 [367, 401, 487], from the tissue of an infant with cytomegalic inclusion disease [500]. Although HCMV disease became associated with solid organ transplants quite early on, it was the aquired immunodeficiency syndrome (AIDS) epidemic as well as a large increase in solid organ transplantation in the 1980s and early 1990s leading to a great increase in immunocompromised patients that put the spotlight on human cytomegalovirus [46, 102, 368]. HCMV transmission has been linked to unscreened blood donations, and leukocytes were identified as a viral reservoir because HCMV transmission is reduced when transfused blood has been leukocyte-depleted [3, 96, 448, 502]. The carriage of virus in leukocytes was thought to be latent, as replicating virus was not detectable by the immunohistochemical methods of the time and HCMV is not able to be isolated directly from the blood of healthy donors [176]. The fact that latent infection is as low as one in every 100-5000 monocytes [398] also

made it extremely difficult to identify the site of latency of HCMV in the peripheral blood compartment [176]. However, the invention of polymerase chain reaction (PCR) allowed for amplification of specific DNA sequences, which overcame the problem of low copy numbers of HCMV DNA, and provided a method to interrogate sites of HCMV latency [391].

1.4.2 The haematopoietic cell line

Although HCMV can establish lytic infection in a wide range of cell types, the virus appears to establish latent infection in a more restricted set of cells. One site of latent carriage is in cells of the early myeloid lineage, which includes CD34+ progenitor cells and CD14+ monocytes [215, 258, 391, 439]. CD34+ cells are the self-renewing progenitor cells for all blood-cell lineages (Figure 1.4) [259]. CD34+ cells can develop along the myeloid linage in the bone marrow, via monoblasts and promonocytes. When entering the bloodstream, promonocytes lose their CD34 cell-surface antigens and differentiate into monocytes [185]. Subsequently, monocytes mature into macrophages and dendritic cells [14, 73]. This lineage development is triggered by exposure to growth factors, such as granulocyte-macrophage colony stimulating factor (GM-CSF) and Interleukin-3 (IL-3) for CD34+ cells. In contrast, GM-CSF and Interleukin-4 (IL-4) trigger monocyte maturation to dendritic cells (Figure 1.4) [1]. It is now also clear that, in healthy HCMV-positive donors' cells, differentiation of CD34+ progenitors or CD14+ monocytes to dendritic cells and macrophages, respectively, triggers virus reactivation from latency [322, 346, 408, 440]. Although CD34+ progenitor cells can differentiate into a range of cell types, including lymphoid cells and polymorphonuclear leukocytes (PMNLs) (see Figure 1.4), the HCMV genome has only been detected in the myeloid lineage of cells and never in the lymphoid lineage [438, 439]. CD34+ cells can also differentiate into endothelial cells, however HCMV has not been detected in endothelial cells of the microvasculature, and, it should be stated, that due to ethical reasons it is difficult to analyse the macrovasculature of healthy individuals [171, 332, 343, 388, 394]. Analysing the sites of HCMV latency is complicated further by the fact that death causes reactivation of latent virus, making cadaverous tissue unsuitable for studying latency [449].

Within the monocyte population, the CD14+/CD16+ subset of monocytes have been the subject of some study, suggesting that HCMV may preferably establish latent infection in these cells, over CD14+/CD16- subsets. CD14+/CD16+ monocytes have a patrolling phenotype in mouse models, while CD14+/CD16- cells are inflammatory monocytes, and it is possible that this system could also be true in humans [130, 131]. Work on murine CMV has shown this subset of monocytes to be important for MCMV dissemination [94]

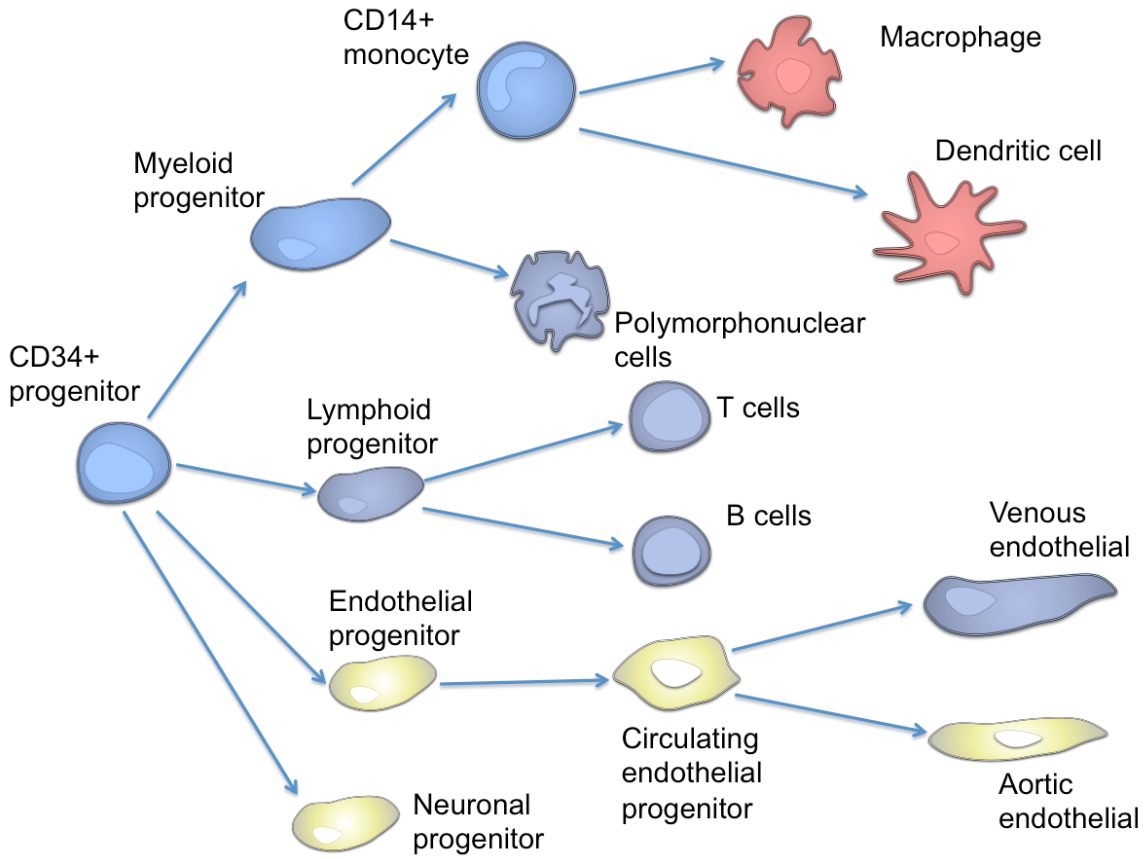

Figure 1.4 The carriage of HCMV through the myeloid lineage, adapted from [108]. HCMV establishes latent infection (blue) in CD34+ haematopoietic progenitor cells, which are resident in the bone marrow. From here, latent HCMV is found in CD14+ monocytes, and reactivates (red) upon monocyte differentiation to macrophages or dendritic cells. HCMV is known not to infect lymphoid cells, polymorphonuclear cells or venous endothelial cells (grey) and viral genome is not carried in these cells. Finally, endothelial and neuronal progenitor cells can be experimentally infected, however it is not clear whether HCMV establishes latent infection in these cells (yellow) *in vivo*.

and, promisingly, this cell type has an immune-privileged phenotype upon differentiation. Although this mechanism would explain important phenomena, such as the low frequency of latently infected monocytes in naturally latently infected individuals [398] and the low percentage of experimentally infected monocytes, even at high MOIs of 5 [208, 217, 325], analysis of latent loads (the percentage of cells latently infected in seropositive individals) from older patients does not suggest any difference in HCMV prevalence for CD14+/CD16+ monocytes *in vivo* [301].

1.4.3 Natural and experimental latency and animal models

A major limitation of working with latent HCMV infection has been the very low frequency of latently infected cells, in HCMV seropositive individuals (termed natural latency) [398]. This makes the detection of viral genes that are expressed during HCMV latency difficult by RT-qPCR, and the detection of expressed viral proteins impossible, due to the high background of non-infected cells. Additionally, there is currently no method for the isolation or enrichment of latently infected cells from a healthy blood donation. This means that much of the work detailing HCMV latency has been performed using *in vitro* experimental models, termed experimental latency [391]. Such models often involve the *in vitro* infection of primary myeloid cell cultures with HCMV at high multiplicities of infection [137, 143, 200, 239, 266, 346].

Different laboratory cell lines have also been experimentally infected as models of HCMV latency. These include THP-1 and Kasumi-3 cells, which are immortalised model cell lines as substitutes for CD14+ monocytic and CD34+ stem cells, respectively [27, 190, 293]. However, these do not always mimic latency exactly [7]. Alternatively, primary early myeloid lineage cells from blood and stem cell donations, which are known sites of HCMV latency *in vivo* are commonly experimentally infected with HCMV, as the best approximation to natural latency [136, 143, 146, 342, 344]. The culture and isolation methods for these cells differ between different laboratories. In our laboratory, CD34+ stem cells and CD14+ monocytes are cultured in a cytokine-free media to prevent any likelihood of differentiation and potential reactivation, thereby maintaining HCMV latency in defined media conditions (see Methods 2.1). This system, importantly, produces insights that can be reproduced in natural latency [136, 157, 326, 346, 347].

The species-specificity of HCMV has precluded tractable animal models of HCMV latency. The detailed sites of mouse CMV (MCMV) latency are not known, but mice with a humanised immune system have been developed, where G-CSF treatment can be used to

reactivate latent HCMV virus [404, 454] and this system is likely to become important in the future.

1.4.4 Reactivation

Reactivation of virus from latency is crucial to viral persistence, as it is very unlikely that a single primary infection would provide sufficient latently infected cells for a life-time of carriage in the host. Although it cannot be ruled out that constant re-infection could be one way of maintaining a latent reservoir, it is likely that subclinical reactivation events likely replenish the pool of naturally latently infected cells [39, 325]. Consistent with this, a consensus has now been established that it is likely that monocyte differentiation to macrophages and dendritic cells causes HCMV reactivation from latency *in vivo*. This was based on early observations that terminally differentiated monocyte-derived macrophages become permissive for lytic infection [162, 216]. This suggested that differentiation of myeloid lineage cell might induce virus reactivation. Subsequently, monocyte differentiation to macrophages, by *in vitro* treatment with granulocyte colony stimulating factor (G-CSF) and hydrocortisone, resulted in lytic gene expression and virus production was detected by reactivating naturally latently infected monocytes with T-cell co-stimulation [405, 408]. Similarly, differentiating CD34+ progenitor cells into dendritic cells [341], confirmed that myeloid differentiation did, indeed, trigger reactivation of HCMV from naturally latent infection. Finally, again consistent with the view that myeloid differentiation can reactivate naturally latent HCMV *in vivo*, isolated alveolar macrophages and blood-derived dendritic cells from HCMV seropositive donors, show reactivation of IE gene expression and virus reactivation, respectively [322, 348].

An important but open question in the field is how latent HCMV can be present in CD34+ progenitor cells, but only persist down the myeloid lineage, and why viral genome is not found in the lymphoid lineage or endothelial cells (see Figure 1.4). Possible answers to this problem include the possibility that latent HCMV directs CD34+ progenitors down the myeloid lineage such that non-myeloid cells do not carry HCMV genome. This could be for a number of reasons; perhaps incompatibility between non-myeloid cell factors and viral functions, or that latent infection kills lymphoid-committed cells before differentiation can occur. Evidence for the former model comes from the observation that downregulation of cellular mir-92a expression, during HCMV latency, leads to increased expression of its target protein GATA2. GATA2 is a transcription factor which directs myeloid cell differentiation,

and so may be directing the lineage commitment of latently infected cells down the myeloid lineage [319, 451].

Although it is far from clear what exact signals associated with myeloid differentiation induce viral reactivation, it is becoming apparent that orchestrated effects of both cellular and viral factors are involved in derepression of the MIEP and induction of the lytic transcription program [49, 221, 161, 189, 228, 336, 496] and they are likely to include ERK-MAP (extracellular signal-regulated kinases-mitogen activated protein) kinase signalling to the MIEP [189].

1.4.5 Gene expression during latency

It is now accepted that there is a specific latency-associated transcriptional profile which the virus has evolved to modulate cell behaviour to support latent carriage of the viral genome [392]. To facilitate this task, the virus has evolved a network of functions, which maintain expression of a latency-associated viral transcriptome. In addition, the virus suppresses expression of IE genes by suppressing the MIEP (thus preventing the viral gene cascade leading to lytic infection) as well as evading intrinsic cell death responses and extrinsic immune responses [340].

Of the 170-750 ORFs that are encoded and expressed by HCMV during lytic infection [107, 129, 138, 278, 310, 421, 484], only a very small subset are expressed in latent infection [392]. Due to the low frequency of latently infected cells and relatively low levels of protein expression, no viral protein has been detected during latent infection to date. However, messenger RNA (mRNA) for a number of putative latency-associated transcripts have been confirmed in natural latency (summarised in Table 1.3).

Work to identify genes expressed during HCMV latency began in the 1990s when a small number of transcripts were discovered around the MIE region [200, 201]. Although antibodies against these encoded proteins have been detected in seropositive individuals, which clearly indicates that they are expressed *in vivo*, their role, if any, during latency has not been well established, as disruption of these ORFs has little effect on latency or reactivation [212, 491]. With the advent of microarray analyses, latency-associated transcription was analysed [78, 137], in 2002 Goodrum et. al, identified a large number of transcripts that are expressed during latency, while Cheung et. al, confirmed only a very small number of these in 2006. This discrepancy is likely because the model of experimental latency used by Goodrum et. al, may have had problems with lytic and abortive infection contaminating the latent population, resulting in a large number of false positive transcripts [135].

Gene product	Latent function	Lytic function	References
CLTs	Unknown	Regulation of anti-viral 2'5' OAS expression (ORF94)	[167, 200] [201, 491]
LacmvIL-10	Downregulation of MHC class II and upregulaton of GATA-2	Unknown, different splice variant expressed	[173, 174]
UL81-82ast (LUNA)	Promotes UL138 gene expression, virus reactivation	Unknown	[26, 190] [347]
UL84	Genome maintenance	DNA replication, UTPase activity, transcriptional regulation	[86, 299] [361, 376] [410]
UL136	Unknown	Unknown	[63, 454]
UL138	Regulation of TNFRI (up) and MRP1 (down), maintained repression of the MIEP	Regulation of TNFRI (up) and MRP1 (down) and virus maturation	[136, 220] [221, 274] [309, 483]
UL144	Unknown	TNF superfamily member, hijacks NF-κB signalling, immune evasion?	[29, 318] [323, 326]
US28	Monocyte adhesion? attenuating signal transduction to the MIEP (one focus of this thesis)	Induction of cell signalling, cell migration and driving the MIEP	[27, 32] [42, 59] [127, 263] [426]
Lnc4.9	Binds Polycomb repressor complex 2, Silencing of the MIEP	Unknown	[361]
Beta 2.7	Unknown	Regulating mitochondria-induced apoptosis	[345]

Table 1.3 Gene products and their function during latency, adapted from [392].

The observation that MHC class-II is downregulated in HCMV-infected granulo-monocyte progenitor cells, led to the discovery of the latency-associated expression of a splice variant of UL111a, or viral IL-10 [173]. Sequencing of a complementary DNA (cDNA) library of peripheral blood mononuclear cells (PBMCs) from a seropositive donor also led to the discovery of the Latency Unique Natural Antigen (LUNA), which is encoded in the anti-sense direction of UL81-82 gene region, otherwise known as the tegument protein pp71. Expression of LUNA in natural latency has been confirmed [347], and knockout of LUNA

abrogrates the ability of latent HCMV to reactivate [190]. Differences in the establishment of latency by different isolates of HCMV have focussed attention on the ULb' region of the HCMV genome. Sequential knock out of the genes in the ULb' region led to the discovery of UL138 as well as UL144 as being involved in latent infection. The expression of UL138 is necessary for the establishment of latency, and was confirmed in naturally latently infected monocytes [136, 221]. The reliable expression of UL138 during latency means that UL138 expression, in the absence of IE gene expression, detected by reverse transcription-quantitative PCR (RT-qPCR), is the current standard for defining an HCMV infection as latent [136, 207, 217, 309, 347]. More recent work on the ULb' region has also shown that specific isoforms of UL136 are expressed during latency, and that this expression is necessary for efficient establishment of latent infection in CD34+ progenitor cells [63]. Additionally, UL135 expression is necessary for reactivation from latency, in order to overcome UL138-mediated suppression of the MIEP [455]. Similarly, UL144 expression has also been confirmed during natural latency [326]. More recently, RNA sequencing (RNA-Seq) analysis of latently infected CD34+ progenitor cells and CD14+ monocytes has confirmed many of the previous observations, as well as identifying viral UL84 expression during natural latency [361]. Although the functions of many of these latency-associated gene products are becoming better understood, their roles in latency, specifically, are still unclear.

At the start of my project, there had only been one report describing the direct therapeutic targeting of latently infected cells. Mass spectrometry analysis of changes in cell surface protein expression, comparing THP-1 cells expressing viral UL138 in isolation with control cells, identified the downregulation of the drug transporter multidrug resistance-associated protein 1 (MRP-1) in these UL138-expressing THP-1 cells and this down-regulation of MRP-1 was also confirmed in experimentally latent CD34+ and CD14+ cells. MRP-1 is necessary for transporting toxic vinka alkaloids out of cells, and its downregulation by UL138 results in latently infected cells being susceptible to killing by vincristine treatment [483].

Regarding US28, the focus of this thesis, US28 mRNA expression was detected in latent infection of the monocytic cell line, THP-1 [27]. US28 mRNA expression was confirmed in subsequent microarray analyses [78, 137], although not by RNA-Seq analysis [361]. During this project, two papers have been published showing that US28 expression in THP-1 cells improves monocyte adhesion to endothelial cells (see section 1.5.10) [497] and, importantly, that US28 expression is necessary for the establishment of latency in CD34+ progenitor cells (and that the deletion of the US28 gene leads to full lytic infection in these cells) [161].

1.4.6 HCMV RNA expression during latent infection

In some experimental models of latency, a burst of what appears to be lytic gene expression has been detected at very early stages after initial infection of early myeloid lineage cells [137, 146, 361], although the functional relevance of this is unclear. Similarly, in herpes simplex virus-1 (HSV-1) transcription from the ICP0 (infected cell polypeptide 0) promoter was detected using the Cre reporter system in latently infected cells in mice [331]. Additionally, in Epstein Barr virus (EBV), this early burst of lytic gene expression has been linked to latency [180]; however, no such evidence currently exists to suggest that this burst of gene expression is necessary for establishment of latency in HCMV.

In addition to protein-coding mRNAs, a number of long non-coding RNAs (LncRNAs) [361] and microRNAs (miRNAs) are also expressed during latency. HCMV encodes 24 mature miRNAs, [140] and the majority of these appear to be expressed, and can be detected, during latent infection [217]. Among them is mirUL-112-1, which has been shown to target and downregulate IE72 transcription during lytic infection [280]. More recently, it was confirmed that this latency-associated expression of miRUL112-2 "mops up" any leaky IE72 transcription from the MIEP, preventing detection of latently infected cells by IE72-specific T cells [218]. Another viral miRNA confirmed to be expressed during latency, miRUL148D, directly targets the mRNA transcript of cellular Activin A receptor 1B (ACVR1B), a protein involved in the activin signalling pathway, for degradation. This downregulation of ACVR1B by latent HCMV infection reduces IL-6 secretion, reducing the potential for this inflammatory cytokine to induce sporadic reactivation [217].

HCMV also expresses two long non-coding RNAs during latency: the long non-coding RNA 4.9 kb (lnc4.9) and beta 2.7 [361]. The role beta 2.7 is unclear, however it may be playing a similar role to the one played during lytic infection; beta 2.7 may interact with complex I to regulate mitochondria-induced apoptosis [345]. Whereas, the expression of viral Lnc4.9 appears to be associated with the polycomb repressor complex (PRC2), which recruits histone methytransferases, which would likely mediate chromatin-repressive phenotypes around the HCMV MIEP, as seen in KSHV and HSV latency [84, 362].

1.4.7 Repression of the MIEP by transcription factors during latency

During lytic infection, viral genes are expressed in a temporal cascade [67, 484], starting with the immediate early genes, that are controlled by the MIEP (see section 1.2.1). During latency, this promoter is heavily repressed in order to prevent lytic infection and promote

latency; and as the MIEP is one of the strongest known promoters in biology [391], it is unsurprising that this repression is mediated by multiple mechanisms.

The MIEP is extremely complex and has multiple binding sites for established activatory and repressive transcription factors, identified during the early 1990's by electrophoretic mobility shift assay (EMSA) and DNAse footprinting analysis. The MIEP is positively regulated by NFκB, the cyclic adenosine monophosphate response element-binding protein (CREB) and Sp1 [255] but can be repressed by transcription factors such as Ying Yang 1 (YY1), methylated DNA binding protein (MDBP), and ETS domain-containing transcription factor (ERF) [18, 228, 507]. One view has been that is is a balance of positive and negative transcription factors which determines MIEP activity and that transcription factors expressed in early myeloid lineage cells, therefore, repressed the MIEP but the balance reverses during myeloid differentiation. However, the mechanisms of action for YY1 and ERF, which recruit histone methylases, also indicated that the MIEP is additionally repressed by cell-type dependent changes in chromatin structure [496].

One factor that may play a role during latency is CCCTC-binding factor (CTCF), which has both transcriptional activatory and repressive functions and has been shown to repress the MIEP during primary, lytic HCMV infection [244]. CTCF has also been implicated in the maintenance of latency by repression of lytic gene expression from EBV [441] and KSHV [182, 418] but also plays a role in driving the lytic infection of HSV-1 (33, 34) and so CTCF may be an evolutionarily conserved mechanism for regulating herpesvirus infection [244].

CTCF is a highly conserved 11-zinc-finger protein that regulates gene expression through the recruitment of transcription factors, and rearrangement of higher-order chromatin structure [312]. CTCF prevents the spread of repressive heterochromatin across the genome, by looping DNA and creating "boundaries" of gene repression [430], and is also known to block communication between enhancer and promoters regions of the DNA, as well as coordinating RNA polymerase II elongation and mRNA processing [384]. CTCF binds to specific DNA sequences, via the 11 zinc fingers, to bind to a 20-base pair core consensus that is ubiquitous in cellular and viral genomes [23, 181, 194]. During this study, I will highlight CTCF as having a possible role in HCMV latency (see Chapter 6).

1.4.8 MIEP chromatin repression and associated proteins

In addition, and in conjunction with transcription factor binding, the MIEP is repressed by post-translational modifications of MIEP-associated histones in early myeloid lineage cells [391]. As latent infection correlates both with cell type and with higher order chromatin

structure, it has been suggested that chromatin structure is dictated by the microenvironment within different cell types. Consistent with this, it has been noted that that the MIEP is intrinsically less active when transfected into undifferentiated myeloid cells [391]. Markers of histone repression found on the MIEP during latent infection include: trimethylation of histone H3, the recruitment of heterochromatin protein-1 (HP-1) and the concomitant absence of histone acetylation on histone H4 in CD34+ progenitor cells [281, 282, 346, 361] and CD14+ monocytes [346, 340]. After myeloid cell differentiation to dendritic cells, and subsequent viral lytic infection, the markers of histone repression of the MIEP are reversed [346].

The reason for this cell-type dependent change in chromatin structure is partly due to transcriptional repressors that are present in undifferentiated myeloid cells, and which recruit histone methyltransferases to the MIEP [228, 390, 496]. In addition, repression of the MIEP may be an intrinsic antiviral response, which is hijacked by HCMV to effect latent infection. Early histone methylation has been detected in lytic infection in fibroblasts before detectable immediate early gene expression [141]. This is thought to be an nuclear domain (ND)-10-mediated antiviral response, which in lytic expression is overcome by tegument protein pp71 and later IE72 expression [179, 250, 416] but exclusion of pp71 from the nucleus of CD34+ progenitor cells may leave this mechanism intact and subsequently help enforce latency [373]. UL138, when expressed during latency, also appears to prevent two cellular Lysine-specific histone demethylases (KDM): KDM1A and KDM6B from associating with the MIEP and triggering activation. UL138 therefore also appears to act to maintain this antiviral activity in order to maintain latency [221].

1.4.9 The latency-associated secretome

Analysis of the secreted proteins (secretome) of latently infected cells has highlighted the secretion of two key factors, tumour growth factor (TGF)-β and cellular interleukin (cIL)-10, which create an immunosuppressive microenvironment around latently infected cells [248]. TGF-β induces the downregulation of hsa-miR-92a [207, 320] and increases signalling via the GATA-2 pathway. GATA-2 is already known to be associated with haematopoiesis and myeloid cell production [224, 450], but additionally, the latency-associated genes UL144 and LUNA have GATA-2-binding consensus sequences in their promoters [326, 347]. This has led to the suggestions that GATA-2 signalling may selectively target key latency-associated genes for expression during latent carriage in myeloid cells, while the rest of the genome, such as IE, is repressed.

TGF-β secretion has a paracrine and autocrine effect, inducing histone deacetylase (HDAC)4 expression, which is necessary for the repression of IE gene expression by histone deacetylation around the MIEP [207]. Inhibition of HDAC4, with the Class II HDAC inhibitor MC1568 or the more general HDAC inhibitors topirimate or valproic acid, therefore, triggers IE gene expression in latently infected cells and has allowed detection and killing of these cells by IE-specific CTLs from a healthy immune system. This approach constitutes only one of two proposed treatments for HCMV latency in the literature [207].

1.4.10 Genome maintenance during latency

It is not clear whether the viral genome is actively replicated during latency, or simply maintained in latently infected myeloid cells, with poor replicative capacity until reactivation. However, recent evidence suggests that the viral genome may be replicated [361, 436]. In these publications, two HCMV ORFs were found to be transcribed in latently infected cells: UL84 and UL44, which are associated with viral genome replication during lytic infection. Additionally, a small transcript from exon 4 of IE1 was found to be essential for viral genome persistence in CD34+ progenitor stem cells. However, unpublished observations from our laboratory (Poole et al, unpublished) have shown that an IE1 deletion mutant is still able to maintain latent genome in latently infected CD14+ monocytes.

1.4.11 Viral evasion of cell death during latency

Viral entry into cells, via receptor binding, triggers the apoptosis signalling cascade. This innate response is due to glycoproteins B and H, which, because they are pathogen associated molecular patterns (PAMPs), bind to Toll-like receptor 2, a pattern recognition receptor (PRR) [38, 89, 115]. However, in the cell types associated with a latent infection, the binding of the virus instead activates cell survival signals. Induced myeloid leukemia cell differentiation protein (MCL-1), an anti-apoptotic protein, is upregulated by the ERK-MAPK pathway triggered by viral gB in CD34+ cells, and by a Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-dependent signal in CD14+ monocytes [69, 342, 188].

After establishment of latency, virus-mediated changes to gene transcription help to protect latent cells from pro-apoptotic signals. Protein and mRNA of the anti-apoptotic factor PEA-15 has been detected in experimentally infected early myeloid lineage cells [324, 400], which blocks tumour necrosis factor receptor 1 (TNFR1) and Fas ligand-triggered apoptosis [92]. It has been shown that latently infected CD34+ cells are therefore protected from Fas ligand-induced death [320]. As well as having general pro-surivival benefits, the upregulation

of PEA-15 RNA may have evolved to block the increased expression of TNFR1 by the latency-associated viral gene UL138 [483]. In addition to this intracellular signalling, cIL-10 from the latency-associated secretome likely plays an anti-cell death role [248, 320].

1.4.12 Viral evasion of host immunity during latency

Latently infected cells express only a relatively small number of latency-associated viral genes and do not produce infectious virus. This strategy means that latently infected cells express only low levels, if any, of lytic genes which are known to be immunodominant antigens such as IE, pp28 and gB which, in itself, means that latency likely aids escape from host immune surveillance [493]. However, latently infected cells are not cleared by the adaptive immune response, despite the known presence of T cell responses against latency-associated antigens in healthy HCMV carriers [247, 444]. Work in these papers suggests that latency-associated transcripts, UL138, LUNA, US28 and UL111a trigger cIL-10 secretion from CD4+ T cells specific to these antigens; which has been argued to drive an immunosuppressive environment around latently infected cells [247].

Consistent with this, TGF-β and cIL-10 that are secreted by latently infected cells have an immunomodulatory effect that can block CD4+ effector cell functions [248]. Additionally, the CD4+ response to latency-associated antigens is dominated by T regulatory cell responses, which express cIL-10 and would augment the immunosuppressive nature of the latency-associated secretome [248]. Overall this would create a microenvironment around latently infected cells which would allow evasion of T cell-mediated killing, without affecting overall, normal, immune homeostasis [392].

In addition to upregulating cIL-10 expression, HCMV encodes two cIL-10 homologues, viral IL-10 (vIL-10) and latency-associated viral interleukin 10 (LacmvIL-10) both of which are expressed during lytic infection, but only LacmvIL-10 is expressed during latent infection respectively [173, 174, 203, 233]. LacmvIL-10 is a splice variant of vIL-10 and does not function in the same way; it does not bind the cIL-10 receptor but does downregulate the expression of MHC Class II [146, 173, 174, 399]. Deletion of the LAcmvIL-10 gene locus, UL111a, leads to the recognition and killing of latently infected cells by CD4+ T cells [79] as well as impacting on infected myeloid cell differentiation to dendritic cells [16]. It is believed, therefore, that LacmvIL-10 contributes to the persistence of latent infection in the host [392].

1.4.13 The effects of latent infection on myeloid differentiation

As already stated, HCMV infection is found along the myeloid lineage but never the lymphoid lineage (see section 1.4.2). Changes induced by HCMV infection of early myeloid lineage cells, rather than factors associated with viral latency, appear to induce gradual differentiation of infected cells to macrophages [68, 290, 402, 403]. Virus binding to myeloid cells activated NFκB and phosphoinositide 3-kinase (PI3K) which can cause cells to differentiate into an atypical macrophage that is a combination of M1 and M2 types [71] and activation of basal levels of caspase-3 that promote differentiation over apoptosis [70]. Other clues as to the role of HCMV in myeloid lineage commitment suggest that latency-associated secretion of LacmvIL-10 appears to inhibit dendritic cell maturation [16], while upregulation of GATA-2 may also drive myeloid over lymphoid differentiation [319].

1.5 The HCMV gene US28

1.5.1 Chemokine receptors

Chemokine receptors (CCRs) are 7-transmembrane G protein-coupled receptors (GPCRs) which, themselves, are a large and diverse group of eukaryotic signalling proteins with a 7-transmembrane structure that respond to a diverse array of different stimuli, acting via a common signalling mechanism [6]. GPCRs interact with heterotrimeric G (guanine nucleotide-binding regulatory) proteins (the three subunits of the trimer are termed Gα, Gβ and Gγ) which are embedded on the intracellular face of the plasma membrane. External signals cause a conformational change in GPCR structure which allows the binding and activating of the Gα subunit, by exchanging the bound guanosine diphosphate (GDP) molecule with guanosine triphosphate (GTP). This change allows that G protein heterotrimers to dissociate from the GPCR and diffuse laterally across the plasma membrane, allowing them to interact with other proteins and activate downstream cellular signalling. GPCRs are a diverse group of signalling proteins, and are located on the cell surface, making them good drug targets, as such, GPCRs constitute a large number of drug targets in modern medicine [104, 437].

CCRs are a subfamily of Class A GPCRs and bind extracellular chemokine proteins which activate cellular signalling, most often, causing cellular migration [17]. Chemokine receptors have a number of common characteristics in their structures. They require their N terminus for "hooking" onto chemokines, before pulling the chemokine into the barrel structure by forming more interactions to induce a conformational change in the CCR. This

N terminus is very often necessary for chemokine interactions and usually includes both acidic residues as well as a sulphonated tyrosine residue [5, 219, 275, 283]. Ligand binding stabilises an open conformation on the intracellular face of CCRs, exposing a conserved DRY motif which interacts with the Gα protein and disruption of this DRY motif has different effects for different CCRs; however, often, removal of the arginine residue in DRY motif ablates signal transduction [15, 148, 381]. Certain CCRs can bind multiple ligands which stabilise different open conformations, leading to signalling via distinct G proteins [374, 494, 506]. CCRs are also regulated at the C terminus by phosphorylation of serine and threonine residues which allows binding by arrestin proteins [187]. CCRs can be phosphorylated either by GPCR kinases, or by second messenger kinases such as protein kinases A and C [447]. Arrestin binding desensitises CCRs by preventing further signal transduction and allows internalisation of the desensitised receptor. The receptor can then be recycled to the cell surface or degraded; phosphorylation and arrestin binding varies in a ligand and tissue-specific manner [187, 209, 313, 447].

Ligand binding to GPCRs causes a conformational change which opens the intracellular face of the GPCR, allowing it to interact with Gα proteins. GPCRs can interact with Gα proteins which are subdivided into subfamilies based on sequence similarity: Gαi, Gαq, Gα12/13 and Gαs. Members of these different subfamilies show different tissue distribution and signalling properties, for example Gαi and Gαq have wide tissue distribution but Gα15/16 is found in the myeloid and lymphoid lineages of cells [328, 446].

1.5.2 Chemokines

Chemokines constitute a large family of cytokines (secreted inter-cellular signalling proteins) which can be divided into four subfamilies: CXC, CC, CX3C and C, based on the positioning of cysteine residues in the N-terminal region. These cysteines form structural disulphide bonds which affect the tertiary folding of the protein. These marginal differences in chemokine structure between the four subfamilies mean that most CCRs only bind one subfamily of chemokine, but most CCRs will respond to multiple chemokines within one subfamily. The chemokines and their receptors are named based on the subfamily of the chemokine, and the order of discovery [17, 87].

Pertinent to this thesis is the CX3C subfamily, which has only one human chemokine: CX3CL1, also known as fractalkine, that binds its receptor CX3CR1. Fractalkine acts as both an inflammatory factor and a cell adhesion factor and is also anchored to the extracellular face of the plasma membrane with a mucin-like stalk, a transmembrane domains and a

cytoplasmic domain [24, 363, 456]. Fractalkine can also be shed from its membrane anchor, released as soluble fractalkine, by proteolytic cleavage by ADAM17 (a disintegrin and metalloproteinase domain 17) [128, 453]. This suggests a broad role for fractalkine in human biology and dysregulation of fractalkine expression leads to a number of inflammatory conditions including vascular disease, acute allograft rejection and renal disease [456].

1.5.3 HCMV-encoded chemokine receptor homologues and their interactions

The large DNA viruses, such as poxviruses and herpesviruses, encode chemokine receptor homologues [8]. HCMV encodes four chemokine receptor homologues: UL33, US27 and US28 were discovered by sequence homology analysis of the HCMV genome to rhodopsin [75], and UL78 was discovered slightly later after more sensitive analysis compared the HCMV genome to that of HHV-6 [133].

All four CCRs are expressed during lytic HCMV infection, [242, 485], but only US28 mRNA has been detected in latent infection and models of latency [27, 78, 137, 326]. Supporting this assertion, deletion of the US27, UL33 and UL78 genes does not affect HCMV latency, however deletion of US28 in CD34+ progenitor cells [161] leads to lytic infection (see section 1.5.10). US27, UL33 and UL78 are orphan receptors with no known ligand [11, 433] and are also not necessary for lytic infection of fibroblasts [106, 175, 261]. US27 does not appear to have constitutive signalling activity [473] but has recently been shown to potentiate CXCR4-mediated calcium release [11], and for extracellular viral transmission [294]. UL33 causes constitutive activation of Phospholipase C (PLC) in COS-7 (CV-1 in Origin, and carrying the SV40 genetic material) cells [60, 473]. Despite being highly conserved, UL78 activity is not known and it is non-essential for viral replication [261], but expression of UL78 is required for efficient entry and virion delivery into epithelial cells, but not fibroblasts [295].

HCMV-encoded CCR homologues appear to interact with other CCRs: both UL33 and UL78 have been shown to heteromise with CCR5 and CXCR4 and negatively impact on their respective functions when overexpressed in THP-1 cells [433]. Overexpression studies in 293T human embryonic kidney (HEK 293T) cells have revealed that US28 dimerises with the other HCMV chemokine receptors. Although no functional changes were observed between the US28:US27 dimer, the US28:UL33 and US28:UL78 show reduced activation of NFκB signalling pathway relative to US28 alone [452].

Analysis of the mouse and rat CMV genomes showed that MCMV and RCMV have two CCRs: M33 and M78 in MCMV (or R33 and R78 in RCMV) which are homologues most closely related to UL33 and UL78 [337]. Knock out of M33 leads to poor replication of MCMV in the salivary glands of mice, suggesting that the other CCR homologues may play a role in viral transmission and whole organism pathogenesis [28, 62, 95]. R33, M33 and UL33 all cause constitutive activation of PLC in COS-7 cells [60, 142, 473]. Rhesus CMV has five US28 homologues, all of which appear to have chemokine binding capabilities similar to US28, but signalling properties have not, so far, been assaysed [306]. Due to its homology with CX3CR1, US28 may also be a cofactor in HIV entry [316, 369].

1.5.4 US28 localisation during lytic infection

US28 mRNA and protein (with a green fluorescent protein (GFP)-tag) have been detected early during lytic infection [466, 508] and transient expression of US28 in HeLa and COS-7 cells, followed by immunogold staining, showed that US28 is localised mainly to endocytic vesicles, with only around 20% of the protein localised to the plasma membrane [123]. US28 protein has also been detected in the envelopes of HCMV virions [123, 161, 306, 315, 464] along with other GPCRs [52]. Ligand binding at the plasma membrane causes internalisation of US28 by phosphorylation of the US28 C-terminus and arrestin binding, which triggers endocytosis. This is followed by recycling of US28 back to the plasma membrane [33, 123]. This recycling removes chemokines from the cellular milieu, and, consistent with this, HCMV infected fibroblasts are deficient in certain chemokines known to bind US28: MCP-1 and RANTES [33, 334]. The biological relevance of this "chemokine sink" model has been challenged as US28 overexpression in endothelial cells does not prevent chemokine-induced monocyte adhesion, suggesting that physiological concentrations of chemokine are too high to be removed in a significant manner by US28 [41].

1.5.5 US28 structure and function

US28 has strong homology to CCR1 and CCR5 [127] but also has low homology to CX3CR1 [196] and the crystal structure of US28 has recently been solved [50]. The N-terminal domain of US28 contains the chemokine binding site: TTEFDY, and the removal of this site greatly reduces the affinity of US28 for all chemokines, but disruption of different residues differently affects binding for RANTES or fractalkine. Mutation of the putatively sulphonated tyrosine residue to an alanine (US28-Y16A) abrogates trafficking to the cell surface while mutation to the aromatic residue phenyl-alanine (US28-Y16F) abrogates binding, while

only reducing plasma membrane localisation [61, 121, 223]. Threonine-12 residue is also putatively O-glycosylated [22, 61]. Despite some suggestions, no N-glycosylation has been detected in the AA30-32 region of US28, (see Figure 1.5) [291].

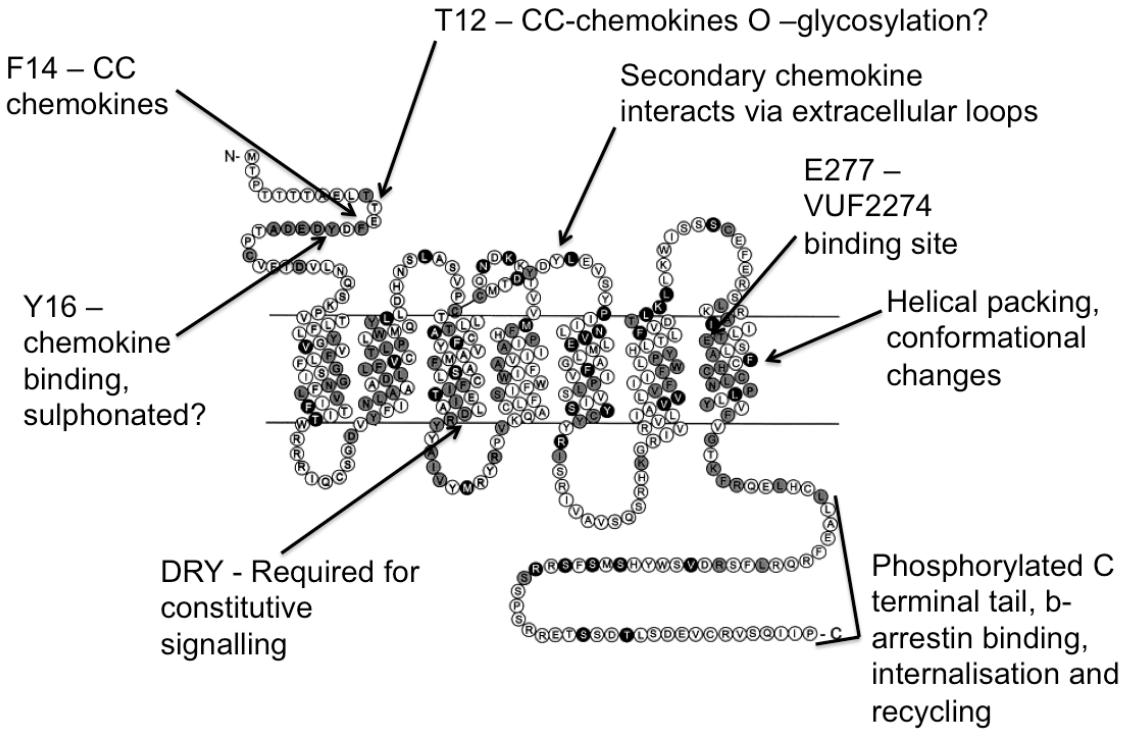

Figure 1.5 Structural elements of the US28 protein, in a serpentine diagram. Showing the seven transmembrane helices, key residues in the N terminal region for CC chemokine binding (T12 and F14) as well as Y16, which is necessary for trafficking and chemokine binding, the DRY motif on the second intracellular loop and the the C terminal region, adapted from [196].

US28 contains the highly conserved DRY motif, found in most GPCRs and all CCRs [366]. A point mutation in the arginine residue of DRY (US28-R129A) which disrupts this binding and uncouples G-protein binding, greatly reduces both constitutive US28 signalling capability and ligand-induced signalling [251, 252, 264, 473] but not ligand-dependent smooth muscle cell migration via Gα_{12}. The US28 C-terminus is not required for constitutive signalling [472] but is heavily phosphorylated by multiple cellular kinases including G-coupled receptor kinases (GKR)2 and GKR5, protein kinase C and casein kinase, which modulates US28 signals by binding of β-arrestin, causing internalisation and recycling to the plasma membrane (see Figure 1.5) [124, 263, 273, 383].

1.5.6 US28 constitutive signalling during lytic infection

US28 is the best characterised HCMV CCR homologue and we also have a broad understanding of US28 activity during lytic infection. The structure of US28, and models for GPCR signalling, allow for the possibility that US28 can spontaneously adopt an open conformation, allowing ligand-indepedent Gα protein binding which would explain the constitutive activity of US28 [50, 381, 471].

US28 signals constitutively via multiple different G-alpha proteins [59, 272, 468], activating different signalling pathways (summarised in Table 1.4). Some signalling activities have been confirmed by *in vitro* infection of cells with US28-expressing HCMV isolates, but other activities have not been confirmed. The role for US28 protein during HCMV lytic infection appears to be the promotion of proliferative signals, such as PLC, MAPK, Wnt and NFκB [59, 214, 264, 397] to promote chemotactic and mitotic properties (discussed below) and to transactivate the MIEP, which has been proposed to help drive lytic HCMV infection [42, 100, 488].

1.5.7 US28 interaction with chemokines during lytic infection

US28 binds both CC and CX3C chemokines, but not CXC chemokines, which has been shown in models of both ectopic expression of US28 as well as in infected cells [32, 33, 196, 286, 470]. US28-mediated release of intracellular calcium, activation of the small G-protein Rho and activation of the Focal Adhesion Kinase (FAK); all require ligand binding, which demonstrates that these ligands must induce a conformational change in the receptor that activates a subset of the US28-driven signalling pathways [32, 127, 257, 427].

US28 signalling is modulated by this chemokine binding and ligand bound US28 shows "functional selectivity": modulating different pathways depending on the binding of CC or CX3C chemokines [457, 471]. The CC chemokines have no effect on constitutive US28 signalling, but the binding of CC chemokines mobilises calcium signalling in K562 cells, similar to the MIP1α / RANTES receptor, CCR5 [127], as well as in response to monocyte-specific chemokine-3 (MCP-3) in HEK 293Ts [32]. Fractalkine acts as an inverse agonist of US28 activation of PLC (significantly reducing PLC activation upon binding), but as an agonist for calcium mobilisation [59, 60, 252]. This effect is most clear when analysing the effect of US28 on ligand-dependent cellular chemotaxis: CC-chemokines bind US28 to promote migration of HCMV-infected smooth muscle cells and activation of FAK, which can be blocked by fractalkine. Fractalkine, however, binds US28 to promote HCMV-infected macrophage migration, which is blocked by RANTES [426, 470, 471]. CC-chemokines and

fractalkine interact differently with the N terminus of US28 [61]. Hypothetically, the ability of different ligands to bind US28 likely stabilises different open confirmations, leading to differential binding of Gα proteins. However, the data highlight the fact that mechanisms involved in ligand-regulation of US28 signalling remain poorly understood.

Cell system expressing US28	Ligands	Phenotypic change
HEK 293T	MIP1-α/MIP1-β RANTES/MCP-1	None [286]
K562 cells	MIP1-α/RANTES	Calcium release [127]
COS-7 cells	MCP-1/RANTES	None [210]
COS-7 cells	Fractalkine	None [196]
Infected HUVEC cells and transfected HEKS	MCP-3 and RANTES	Calcium release and MAP kinase activation via Gα_{16} [32]
Infected fibroblasts	MCP-1/RANTES	Calcium [466]
Infected arterial smooth muscle cells	RANTES inhibited by fractalkine	Chemotaxis via G$\alpha_{12/13}$, Src and FAK [426, 427] hemotaxis via RhoA [257, 470]
COS-7 cells	Constitutive, downregulated by fractalkine, no effect from MCP-1 or RANTES	PLC, NFκB [59, 123] via Gα_{q}
Infected fibroblasts	Constitutive	PLC via Gα_{q} [265]
COS-7 cells	Constitutive, antagonised by RANTES	PLC and NFκ [20] via Gα_{o} and Gα_{q11}
HEK 293T	Constitutive	CREB/NFAT via Gα_{q}[286]
NIH-3T3 cells	Constitutive	VEGF secretion, via Gα_{q}, and MAP kinase and proliferation/ angiogenesis [252]
COS-7 cells	Constitutive	Serum response factor (SRF) via Gα_{o}/Gα_{q11} (inhibited by Gα_{16} [272]

Cell system expressing US28	Ligands	Phenotypic change
Infected macrophages	Fractalkine (inhibited by RANTES)	Chemotaxis via Gα_q [470]
NIH-3T3 cells and infected fibroblasts	Constitutive	COX2 and VEGF via Gα_q via NFκB [251]
NIH-3T3 cells, HEK 293T cells and infected U373-MG cells	Constitutive	NFκB induction of IL-6 with "G protein coupling" VEGF secretion which induces JAK/STAT3 [397]
US28 expression in non-proliferating hippocampal cells and HUVECS	RANTES	Invasive phenotypes via STAT3, AKT, ERK1/2, FAK, Src and eNOS[409]
NIH-3T3 and U373-MG cells and HEK 293T and Infected Hffs	Constitutive	Beta-catenein via both Gα_{12}, Gα_q together and Rho-ROCK[214]
HCMV infected HASMC, U373MG, HFFs and HUVECs, transient HEKS	Constitutive	PLC-β via Gα_q and Gα_{11} in all cell types [264]
HCMV infected HASMC and, HFFs	RANTES	Calcium release via G$\alpha_{12/13}$ [264]
Infected ARPE-19 cells		Poor cell-cell growth kinetics in epithelial cells [291]
U251 and NIH-3T3 cells constitutively expressing US28	Constitutive	VEGF secretion and HIF1-α activation with Akt and PKM2 [97]

Cell system expressing US28	Ligands	Phenotypic change

Table 1.4 US28 signalling properties, by cell type and ligand, demonstrating the diversity in different responses to US28 expression between cell types, either expressing US28 in isolation or in the context of HCMV infection.

1.5.8 US28 and cancers

HCMV infection was originally linked to cancers because HCMV proteins and DNA have been detected in a number of cancers including prostate cancer, colon cancer, EBV-negative Hogkins lymphoma and glioblastoma multiformae (GBM) [85, 147, 156, 267, 375, 378]. This observation could, of course, be artifactual. However, it could be that tumour formation promotes HCMV infection or interferes with HCMV detection techniques and, indeed, some groups struggle to detect HCMV in similar tissue samples, which has led to significant controversy in the field [101, 406, 435, 501]. A number of mechanisms, however, could link HCMV to cancer as an oncomodulatory virus. This model could result in HCMV infection of a cell resulting in the improval of cell survival, better evasion of the immune system, increased secretion of pro-inflammatory and angiogenic cytokines, as well as aggravation of mitotic signals in such a manner as to have an oncomodulatory effect on an already neoplastic, HCMV-infected cell [40, 83, 105]. Much work has linked HCMV to glioblastoma, a highly aggressive cancer of astrocytic origin [235], where the mean two-year survival with GBM is 15-25%. HCMV DNA and protein has been found in glioblastoma biopsies, [85, 267, 378] and treatment of patients, who have GBM, with valganciclovir improves overall life expectancy [407]. Additionally, HCMV infection of p53-/+ mice is known to accelerate the progression of GBM [329].

US28 is also a primary candidate gene for oncomodulatory effects of HCMV on tumour cells. US28 activates a number of proliferative signals in models of neuronal infection, including PLC, endothelial nitric oxide synthase (eNOS), MAP kinase, Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT), cyclooxygenase (COX2) and NFκB as well as promoting the secretion of IL6 and Vascular endothelial growth factor (VEGF); all of these pathways contributing to cell survival and proliferation as well as an inflammatory cellular environment [397, 409, 469]. Expression of US28 in models of GBM also promotes tumouriogenesis [251, 252, 397].

1.5.9 US28 in vascular disease

Atherosclerosis is the build up of smooth muscle cells (SMCs) and extracellular matrix components on the lumen of large arteries. Atherosclerosis is driven primarily by chronic inflammation - which promotes cell migration to atherosclerotic plaques and in turn induces production of inflammatory cytokines, generating a positive feedback loop. This loop is aggravated by the intake of low density lipoprotein as well as the presence of monocytes (which rapidly differentiate into macrophages) thereby driving inflammation and occlusion of blood flow and/or rupturing of these plaques. Atherosclerosis is a leading cause of death in developed countries [236]. HCMV seropositivity is linked to a higher rate of mortality from atherosclerosis [277] and HCMV DNA has been found both in diseased vessels and specifically in SMCs in early stage atheroschlerotic lesions (albeit also in non-diseased SMCs) [150, 151, 256, 298]. Similar to atheroschlerosis, HCMV is also linked to transplant vascular sclerosis (TVS), an accelerated form of atherosclerosis which is a major cause of transplant rejection [31].

US28 likely plays a key role in this HCMV-mediated aggravation of atherosclerosis. For instance, HCMV infection of SMCs induces US28-dependent migration in the presence of CC-chemokines, and macrophage migration in the presence of fractalkine [426, 470]. US28 could, therefore, contribute to atherosclerotic disease by promoting the build up of SMCs and the infiltration of inflammatory macrophages into this, inflamed, milieu. US28 also induces the release of pro-inflammatory and pro-angiogenic cytokines (see section 1.5.8) which contributes to chemotaxis, cell survival and the overall spread of HCMV infection.

1.5.10 The roles of US28 in latent infection

Latency-associated US28 transcription was first detected in experimentally infected THP-1 cells, a now established model of latency [27]. Since then, a number of microarray screens have been performed on latently infected early myeloid lineage cells which have also detected US28 mRNA [78, 137]. Although US28 mRNA was not detected by a recent RNA sequencing (RNA-seq) screen of latently infected cells [361], US28 mRNA has been detected in naturally latently infected monocytes from seropositive donors [326]. The presence of US28 during latency seems to be paradoxical with its known functions during lytic infection.

During this project two papers were published which pertained to the role of US28 during latent infection. Most importantly, Humby and O'Connor showed that US28 is necessary for the establishment of latent infection in CD34+ cells and the similar Kasumi-3 cell line [161]. This paper also demonstrated that US28 protein, carried by the incoming virion, is not

capable of maintaining latent infection and US28, therefore, needs to be expressed *de novo* in the latently infected cell [161]. Wu and Miller were the first group to detect US28 protein expression in latently infected THP-1 monocytic cells and showed that US28 activates PLC-β when expressed alone in THP-1 cells, which promotes THP-1 cell adhesion to endothelial cells [497].

1.5.11 Inhibitors of US28

Inhibition of US28 is complicated by its multifaceted signalling properties - inhibitors designed against US28 have been shown to block chemokine binding to US28 (antagonists) or reduce constitutive signalling (inverse agonist) while also mobilising intracellular calcium (agonists) [469]. Indeed, chemokines can be both agonists and inverse agonists of US28 in some scenarios [59, 61, 470]. The CCR1 antagonist, VUF2274 has been found to be a full inverse agonist of US28, while also sterically blocking chemokine binding [60]. More recently, two more inverse agonists of US28 were identified, based on antagonists of CX3CR1 [206], or screening of compound libraries [159, 160, 205, 467]. However, these inhibitors have not been used in any analysis of the role of US28 in HCMV latency.

1.6 Fusion Toxin Proteins and F49A-FTP

Fusion toxin proteins (FTPs), also known as immunotoxins, are a therapeutic strategy designed to kill pathogenic cells with significant specificity. They consist of a protein which binds the pathogenic cell at high specificity, usually a monocloncal antibody, fused to a toxin protein domain, normally from ricin, diphtheria or *Pseudomonas*. The high-affinity receptor-ligand interactions direct these highly cytotoxic molecules to target cells and these have been developed since the 1970s [10]. A number of these have been approved for clinical use. These include: DT-Il2 a diphtheria toxin-IL2 fusion protein which treats recurrent cutaneous T cell lymphoma [110]; the anti-HER2 monoclonal antibody trastuzumab, covalently linked to DM1 as a treatment for breast cancer [311]. Others also exist, and are used largely in conjunction with standard chemotherapy to overcome the problems of chemotherapeutic resistance [81, 482].

This technology also has a great potential against viral infections, since virally-encoded targets provide superior sites for highly specific binding [411]. Consequently, there have also been attempts to target infectious diseases with such FTPs, largely HIV [13]. However, the major drawback of FTPs is the need to carry the toxin protein into the cell, requiring

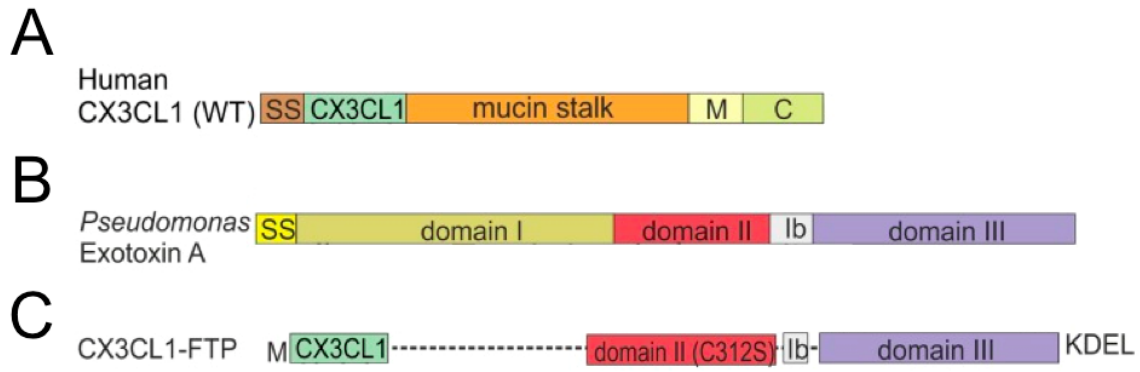

Figure 1.6 F49A-FTP (C) was generated by recombining the soluble N-terminal domain of fractalkine (including an F49A mutation to abrogate binding to CX3CR1) (A), with the toxic C-terminus of *Pseudonomas* exotin A (B), and a KDEL motif to localise F49A-FTP to the ER. This figure was borrowed from the original publication describing F49A-FTP [412]

its efficient internalisation [10, 58, 229, 380]. Many of these anti-viral FTP strategies have exploited the *Pseudomonas* exotoxin-A (PE), which in nature enters cells via CD91 and catalyses adenosine diphosphate (ADP) ribosylation of elongation factor 2 of the cellular translation machinery, leading to inhibition of translation and cell death [305, 385]. PE is a multidomain protein, and removal of domain I abrogates CD91 binding and cellular internalisation (see Figure 5.13 [385].

Recently, a novel fusion toxin protein (F49A-FTP) has been described, which targets and kills cells lytically infected with HCMV [412]. F49A-FTP is based on the soluble extracellular domain of fractalkine, fused to PE (see Figure 5.13). This fractalkine domain binds both CX3CR1 and US28 but the point mutation F49A greatly reduces CX3CR1 binding, thereby generating high selectivity towards US28. After the soluble fractalkine domain binds US28, F49A-FTP is internalised and cell killing is mediated via PE domains II and III. These domains are a cytosol translocation domain and the ADP ribosyltransferase (and part of domain Ib which has poorly defined functions). Additionally, a C-terminal KDEL motif mediates localisation to the ER via KDEL receptors, which greatly increases cytotoxicity, once PE has been internalised (see Figure 5.13) [382, 423]. F49A-FTP specifically kills cells expressing US28 in isolation as well as fibroblasts productively infected with HCMV [412]. This immunotoxin is therefore a potent and efficient drug candidate against HCMV which could be used to target latently infected cells due to their expression of US28 during latency.

1.7 The aims for this project

1.7.1 Detecting US28 protein in latently infected cells

Latency-associated protein expression has not been detected before in latently infected cells. When this project began, the role of US28 during latency was not known and, although US28 mRNA had been detected during latency [27, 78, 326], it was not clear if US28 protein was actually expressed. Therefore, I decided to utilise a new, GFP-expressing TB40E isolate of HCMV to optimise a cell sorting protocol in order to isolate latently infected monocytes, which would hopefully allow me to detect US28 protein by immunoblot. A protocol to isolate or enrich latently infected monocytes would have many other applications as well, and this technique has been used for other projects [208, 217].

1.7.2 Investigating the role of US28 expression during HCMV latency

During this project, Humby and O'Connor published the observation that infection of CD34+ cells with a TB40E isolate of HCMV with a US28 gene deletion, leads to lytic infection instead of latency [161]. I had made similar observations in CD14+ monocytes and endeavoured to elucidate the mechanism by which US28 helps to establish or maintain HCMV latency in myeloid lineage cells.

1.7.3 Optimising the use of F49A-FTP, an anti-US28 fusion-toxin protein to target and kill latently infected cells

The observation that F49A-FTP can kill lytically infected cells led to the possibility that this novel fusion toxin protein could also be used to clear latently infected cells, which could have immense potential benefits for haematopoeitic stem cell transplant recipients. I tested whether F49A-FTP showed any efficacy in killing latently infected cells, and optimised a protocol to clear latent cells in both experimental and natural models of HCMV latency and reactivation.

1.7.4 Confirming and further investigating proteins that change during ectopic expression of US28 in THP-1 cells, according to mass spectrometry proteomic screening

While investigating the role of US28 in HCMV latency of monocytes, I helped perform a proteomic screen, by mass spectrometry, looking at changes in cellular protein expression in THP-1 cells expressing wild type US28 in isolation. These screens highlighted a number of interesting hits, including CTCF. With the assistance of a Master's Degree student, Elizabeth Elder, we confirmed that CTCF changes in THP-1 cells expressing US28 in isolation and endeavoured to investigate how US28 may be using CTCF to maintain HCMV latency.

1.7.5 Analysing how well F49A-FTP resistant mutants of US28 maintain latency

Work optimising the use of F49A-FTP for the clearence of latently infected cells led to the discovery and/or generation of two viral isolates with mutations in the US28 gene. I sequenced these mutants to see how US28 may gain resistance to F49A-FTP, and investigated whether these mutants could still establish latent infection in monocytes. Insights from this work could be beneficial to future recipients of F49A-FTP treatment, while also providing insights into how US28 functions to maintain latency.

1.7.6 Determining if HCMV specifically infects CD16+ monocytes

The observation that F49A-FTP kills latently infected cells specifically, raised the possibility that F49A-FTP was actually binding CX3CR1 and that the expression of CX3CR1 could be changing on latently infected cells or that HCMV preferentially infects monocyte subpopulations which express high levels of CX3CR1 (CD14+/CD16+ monocytes). I investigated whether HCMV does preferentially infect CD14+/CD16+ monocytes, as this could provide useful information on how HCMV establishes latency and spreads *in vivo*. If true, it would also provide a method to isolate naturally latently infected cells, be selectively isolating the CD14+/CD16+ monocytes subset.

Chapter 2

Methods

2.1 Cell culture

All cell types during this project were incubated at 37°C in 5% CO_2, unless otherwise specified.

Human foreskin fibroblast cells (HFFs), HEK 293T, and Retinal pigment epithelium (RPE-1) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% inactivated foetal bovine serum (FBS) (PAA) and penicillin/streptomycin (Sigma). Primary CD14+ monocytes were cultured in X-vivo 15 (Lonza); monocytes were differentiated to immature dendritic cells by GM-CSF and IL-4 (Peprotech) stimulation at 1000 U/ml for 5 days and mature dendritic cells were produced by stimulation for 2 days with lipopolysaccharide (LPS, Sigma) at 500 ng/ml, as described previously [146, 389]. Frozen CD34+ progenitor cells were a kind gift from Sebastian Voigt (Department of Pediatrics, Berlin) and were resuscitated by first washing the frozen cells in warm X-vivo15 media with 1U/ml benzonase (Roche), and then cultured in X-vivo 15 media without benzonase thereafter. CD34+ progenitor cells were differentiated to immature dendritic cells by G-CSF, tumour necrosis factor-α (TNF-α), GM-CSF and IL-4 (Peprotech) stimulation at 1000 U/ml for 5 days incubation. Mature dendritic cells were produced by stimulation for 2 days with LPS at 500 ng/ml. THP-1 and kasumi-3 cells were grown in Roswell Park Memorial Institute (RPMI) medium (Sigma), with 10% inactivated FBS (PAA) and penicillin/streptomycin (Sigma). THP-1 and kasumi-3 cells were differentiated into a macrophage-like cell using 50 ng/μL phorbol myristate acetate (PMA) treatment for 2 days [293, 300].

2.1.1 Peripheral blood mononuclear cell preparation from peripheral venous blood and apheresis cone donations

Peripheral blood mononuclear cells (PBMCs) was obtained from either venous blood by venipuncture by a trained phlebotomist or obtained from apheresis cones from NHS Blood and Transplant (UK). Blood was diluted in phosphate-buffered saline solution (PBS) with 100 U/ml heparin sodium (Wockhardt, Wrexham, UK) and erythrocytes were removed by density gradient centrifugation through Lymphoprep^{TM} solution at 800 relative centrifugal force (RCF) for 20 minutes at room temperature, without the centrifuge brake. Mononuclear cells were removed from the interface between Lymphoprep^{TM} and blood plasma. Cells were ennumerated by haemocytometer using trypan blue staining (Sigma).

2.1.2 HCMV seropositivity testing of blood donations

HCMV serology of donors was tested using an HCMV-specific Immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) kit, Capita^{TM}, according to manufacturer's instructions (Trinity biotech).

2.1.3 Selection of CD14+ monocytes, T cells and CD14+CD16+ monocytes from PBMCs

Positive selection of monocytes was performed using magnetic activated cell sorting (MACS) with CD14+ microbeads (Miltenyi Biotec) as detailed in the manufacturer's protocol. A 10 μl aliquot of these monocytes was taken and the number of CD14+ monocytes was quantified by counting with a haemocytometer and using trypan blue staining (Sigma) to exclude dead cells. Cells were plated in at a density of 2.5x10^5 cells per 1 cm^2 area. To adhere monocytes to a tissue culture dish, monocytes were incubated in PBS with calcium and magnesium but without FBS, at 37°C and in 5% CO_2 for up to 3 hours before changing the media to X-vivo15 (Lonza).

CD8+ T cells were isolated from PBMCs which had been depleted of CD14+ monocytes by positive selection. Positive selection of CD8+ T cells out of this population of depleted PBMCs was then performed using MACS with CD8 microbeads (Miltenyi Biotec) and manufacturer's protocol.

CD14+/CD16+ monocytes were isolated using MACS and a human monocyte isolation kit II (Miltenyi Biotec), which initially selects for all cells except monocytes. These

Species	Virus	Tag	Deletion	Origin
HCMV	TB40E	SV40 GFP	-	Eain Murphy
	TB40E	SV40 GFP	Mutations in US28 N terminus	Generated during project
	TB40E	SV40 GFP	Stop codon at US28 amino acid 220	Generated during project
	Titan	UL32-GFP	-	Martine Smit
	Titan	UL32-GFP	US28 gene region	

Table 2.1 Table of viruses used for this work. Eain Murphy is from the Blumberg Institute, Philadelphia, USA. Martine Smit is from the Division of Medicinal Chemistry, University of Amsterdam, Netherlands.

monocytes were then treated with CD16+ microbeads (Miltenyi Biotec), in order to separate CD16+/CD14+ monocytes from CD16-/CD14+ monocytes.

2.1.4 Experimental infection of cells with HCMV

For adherent HFFs, RPE-1, CD14+ monocytes and CD34+ progenitor cells, media was removed and cells were then washed in PBS. Cells were incubated in a covering volume of media (roughly 1ml of media per $1x10^6$ cells) for 3 hours, rocking, at room temperature, with the relevant HCMV virus (see Table 2.1) at a calculated MOI of 0.1 for HFF cells, 0.5 for RPE-1 cells or an M.O.I of 5 for monocytes and CD34+ cells (or as otherwise specified). After three hours rocking at room temperature, media was changed, cells were washed in PBS and returned to incubation in the relevant cell culture media.

For cells in suspension - THP-1, kasumi-3 and CD14+ monocytes - cells were pelleted and washed in PBS. Cells were incubated in 1ml of media per $1x10^6$ cells with the relevant HCMV virus at a calculated MOI of 5. Cells were returned to the incubator for 3 hours, and cells were shaken manually once an hour. After three hours, cells were washed in PBS and returned to fresh media.

2.1.5 UV inactivation of virus

200 μL aliquots of virus were transferred into one well of a 24-well polystyrene plate. The lid was removed and supernatants exposed to UV light of 254nm wavelength emitted at a frequency of 50Hz from a 30W germicidal lamp for 45 minutes. UV inactivated, control virus, was generated from the same batch as active virus and used at the same concentration.

2.1.6 Titrating virus release

Media was harvested from infected monocyte cultures and titrated onto HFFs, as described in Section 2.1.4. Cells were then incubated for 24 hours and then stained for immediate early as described in Section 2.3.5, and the virus titre was calculated as the number of IE-positive cells per Hoechst-stained nucleus, per μL of media added to that culture.

2.1.7 Quantifying reactivation events after F49A-FTP treatment

Monocyte and CD34+ -derived dendritic cells (see Section 2.1) were co-cultured with $3x10^3$ HFFs per cm^2 of growth area in a 50:50 mixture of DMEM-10 and X-vivo15 (Lonza). THP-1 and kasumi-3 derived macrophage-like cells were co-cultured with fibroblasts in a 50:50 mixture of DMEM-10 and RPMI. Cultures were incubated for 14 days and then stained for Immediate Early (IE). For natural latency experiments, monocyte-derived dendritic cells were co-cultured with fibroblasts for 21 days.

2.1.8 Treatment with F49A-FTP

Latently infected monocytes were treated with F49A-FTP 24 hours post infection, or 24 hours after isolating the naturally latently infected monocytes. In Figure 5.11, where stated, this treatment was delayed across the time course of latent infection. Based on preliminary studies using titrations of F49A-FTP (Figure 5.4), cell cultures were treated with $5x10^{-8}$M F49A-FTP for 3 days, unless otherwise specified.

2.1.9 Generation of F49A-FTP-resistant virus

HFFs were seeded at $3x10^5$ cells per T25 flask and incubated for 24 hours. Cultures were then infected at an MOI of 0.01 with SV40-GFP-TB40E. After 24 hours incubation, the media was changed and $1x10^{-9}$ M F49A-FTP was added (including an untreated control). The media was then changed 3 times a week, replenishing the F49A-FTP at $1x10^{-9}$ M for 4 weeks, until the entire HFF culture was infected.

2.1.10 Plaque Purification of viral isolates

10 cm^2 dishes with HFFs at a confluency of 40% were infected with virus stocks, using either 10, 1, 0.1 and 0.01 μL of stock per plate. One day post infection, warm, sterile, 1.25% agarose was melted in a microwave and heated to 55°C in a water bath. Once at 55°C,

agarose was mixed with Nuserum, 2X DMEM, xanthine, mycophenolic acid and HCl which had been warmed to 37 °C. When the mixture reached 40 °C, the mixture was poured over the infected HFF cells, and overlays were left to set and then returned to the incubator. Plaque formation was observed over the next 14 days and marked on the plates. Once plaques were sufficiently large (14 days post infection), using a sterile pasteur pipette, small plugs of the agarose were removed and transferred to fresh HFFs. These HFFs were left, to be infected, for 3 hours rocking at room temperature, the media was then replaced. These HFF cultures were each infected with a single plaque, from a single viral isolate.

2.1.11 Quantifying cell survival by trypan blue staining

Cell death was quantified using trypan blue staining at a 1:20 dilution in PBS. Cells were immediately observed by light microscopy and both white (live) and blue (dead) cells were counted. Cell survival was calculated as the number of white cells as a percentage of total cells.

2.1.12 IE-specific T cell, and PBMC killing assays

CD14+ monocytes were plated at $1x10^5$ cells per well of a 96 well plate and infected with either Titan-WT, Titan-ΔUS28 or SV40-GFP-TB40E. Three days post infection, the monocytes were then co-cultured with HLA-matched (HLA-A2) immediate-early-specific CD8+ T cells [166] at an E:T ratio of 5:1 or donor PBMCs at an E:T ratio of 1:3. VUF2274 was added, at a concentration of $6x10^{-7}$ M where indicated. In order to measure the killing of latently infected monocytes, the number of HCMV-infected, GFP-expressing, or UL32-GFP-expressing monocytes were enumerated, by fluorescence microscopy, over several days as indicated. Media from these cultures was collected to titre any virus that was produced by Titan-ΔUS28 infected monocytes before being differentiated to dendritic cells by cytokine treatment. Virus release was quantified as specified in Section 2.1.6.

2.1.13 Generation of lentivirus vectors

US28 with an N-terminal haemagglutinin tag (HA) (YPYDVPDYA) was kindly provided in a pAdtet7 vector by Daniel Streblow (Oregan Health and Science University). The CTCF cDNA sequence in a pI vector was kindly provided by Elena Klenova. These genes were excised from their vectors using EcoR1 and Xba1 (Promega) restriction enzyme digestion. The pHR-SIN vector (see Figure 2.1) was opened by digestion with EcoR1 and Spe1 (Promega)

Vector	Drug Resistance Marker	Insertion	Origin of Insert
pHR-SIN (1253)	puromycinR	HA-US28 WT	Daniel Streblow
pHR-SIN (1253)	puromycinR	HA-US28 R129A	Daniel Streblow
pHR-SIN (1253)	puromycinR	HA-US28 Y16F	Daniel Streblow
pHR-SIN (1253)	puromycinR	CTCF	Elena Klenova
sc-35124	puromycinR	shRNA CTCF	Santa Cruz Biotech
iLenti^{TM}	puromycinR	siRNAUS28	NBS Biologicals
iLenti^{TM}	G418R	siRNA US28	NBS Biologicals

Table 2.2 Lentivirus vectors used during the project. WT = wild type. Paul Lehner provided the pHR-SIN (1253) vector and is from the Cambridge Institute of Medical Research, UK. Daniel Streblow is from Oregan Health and Science University, USA. Elena Klenova is from the University of Essex, UK.

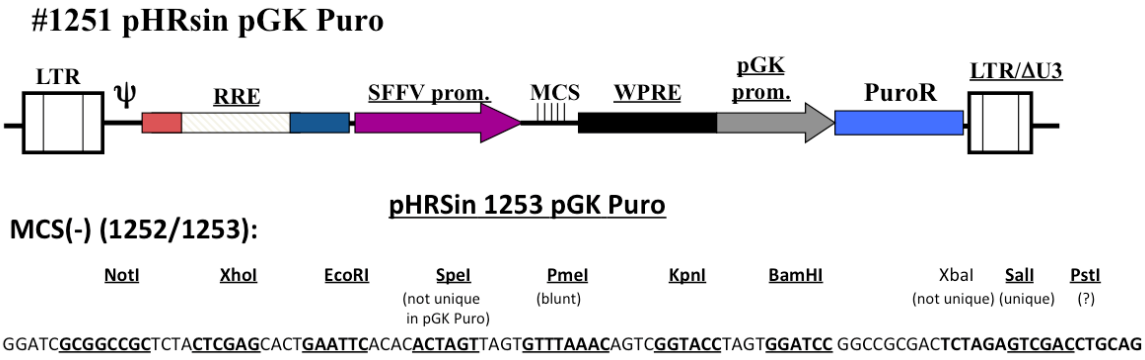

Figure 2.1 pHR-SIN 1253 map, including the multiple cloning site (MCS), a kind gift from Paul Lehner (Cambridge, UK) and described previously [51, 483].

and both insert and vector were purified by 1% agarose DNA gel electrophoresis. Inserts were ligated into pHR-SIN using T4 ligase (Promega), overnight incubation at 4°C. Xba1 and Spe1 digest DNA which then has complimentary overhangs, allowing ligation. Successful ligation was confirmed by digestion of pHR-SIN with EcoR1 and BamH1, because the Xba1/Spe1 site was destroyed after ligation. These inserts were cloned into pHR-SIN which leaves the gene flanked by a self-inactivating (SIN) viral long terminal repeat (LTR) and psi sequence (packaging signal), which allows packaging into pseudotyped lentivirus and subsequent lentivirus transduction of these genes.

2.1.14 Transfection of HEK 293T cells

HEK 293T cells were cultured in DMEM media. When cells were between 40-60% confluent, transfections were performed using *Trans*IT®-293 Transfection reagent (Mirus), according to manufacturer's protocol, using 1μg of total DNA per well of a 24 well plate.

2.1.15 Transduction of THP-1 cells with VSV-G pseudotyped lentivirus

Lentiviral vectors (600 ng per well of a 24 well plate, see Table 2.2) were co-transfected with packaging and VSV-G envelope plasmids (200 ng each per well of a 24 well plate) into HEK 293T cells in RPMI-10 media, using *Trans*IT®-293 reagent (Mirus) and manufacturer's protocol. Lentivirus was harvested 48 and 72 hours after transfection and incubated with 10,000 THP-1 cells in the presence of 8μg/ μL polybrene, and centrifuged at 800 RCF for 60 minutes to enhance lentivirus transduction. 24 hours post-transduction, media was changed to remove the polybrene and cells were incubated for a further 48 hours. Transduced THP-1 cells were then selected by puromycin treatment (1μg/mL) for up to one month, until a puromycin-resistant cell population emerged; during this time, media was changed with fresh puromycin once a week.

2.2 Flow cytometry analysis

2.2.1 Quantifying survival of GFP-positive monocytes by flow cytometry

Latently infected monocytes in suspension were washed twice in PBS before being resuspended in PBS for flow cytometry analysis. Monocytes which were adhered to a tissue culture plate were washed twice in PBS before being incubated with ice cold Cell Dissociation Solution (Non-enzymatic) (Sigma-Aldrich) for 2 hours before being lifted, gently, by pipetting and scraping with a Pasture pipette. These monocytes were then washed twice in PBS before flow cytometric analysis. Samples were processed on a 3-colour FACSort (BD) and analysed using FlowJo software (Tree Star Inc).

2.2.2 Analysing cell surface markers by flow cytometry

For antibody labelling and flow cytometry, samples were washed twice in PBS and then blocked with 20% normal goat serum (Abcam, 156046) for 15 minutes at room temperature.

Cells were then labelled with the relevant antibody (see Table 2.3) by incubation for 1 hour at 4°C, diluted 1/100 in PBS with 10% normal goat serum (see Table 2.3). Cells were then washed twice in PBS at 4°C. For flow cytometry, samples were processed on a FACS Calibur or Fortessa (Becton Dickinson) and analysed using FlowJo software (Tree Star Inc). Dead cells were removed from analysis using LIVE/DEAD Fixable Aqua Dead cell stain (Invitrogen).

2.2.3 Analysing T cell activation by flow cytometry

Experimentally infected monocytes were co-incubated with CD8+ T cells from the same, seropositive donor, overnight in the presence of CD107a Alexa fluor 647, 5μg/ml, Brefeldin A and 2μM Monensin (all from BioLegend) at 37°C. CD8+ T cells were harvested and washed, then stained with a combination of surface antibodies (CD3 brilliant violet 650, CD14 Brilliant Violet 510 and CD19 Brilliant Violet 510 (BioLegend)) and LIVE/DEAD Fixable Aqua Dead cell stain (Invitrogen) at 4°C. Cells were fixed and permeabilised using FIX&PERM (ADG, Kaumberg, Austria) and stained intracellularly with antibodies (CD69 Pacific Blue, 4-1BB PE-Cy5, CD8 Brilliant Violet 570, Granzyme A FITC (BioLegend), Granzyme B FITC (Miltenyi Biotec), Granzyme K FITC (Santa Cruz Biotechnology, Texas, USA), TNFα Brilliant Ultra Violet 395 and IFN-γ Brilliant Violet 786). Responding CD8+ T cell populations were identified by the expression of CD69 and 4-1BB, above background and their expression of CD107a, TNF-α and IFN-γ were then measured. In all cases, cell doublets, monocytes, B cells and dead cells were eliminated from the analysed populations.

2.2.4 Sorting GFP-positive monocytes by FACS to generate an enriched population of latently infected monocytes

Approximately 2x10^8 monocytes were isolated and latently infected at an MOI of 5 with SV40-GFP-TB40E. Four days post infection, monocytes were sorted for GFP-positive cells by fluorescence activated cell sorting (FACS), using an FACSJazz cell sorter (BD) with help from the NIHR Cambridge BRC Cell Phenotyping hub. This yielded roughly 3% of total cells.

Target	Origin	Clone	Fluorophore	Supplier
CD3	Mouse	OKT3	Brilliant Violet 650	Biolegend
CD4	Mouse	OKT4	Brilliant Violet 605	Biolegend
CD8	Mouse	RPA-T8	Brilliant Violet 570	Biolegend
CD14	Mouse	M5E2	Brilliant Violet 510	BD
CD16	Mouse	3G8	PerCPCy5.5	Biolegend
CD19	Mouse	3G8	Brilliant Violet 510	Biolegend
CD83	Mouse	HB15e	APC	BD
CX3CR1	Rat	2A9-1	PerCPCy5.5	Biolegend
CD107a	Mouse	H4A3	AlexaFluor 647	Biolegend
CD69	Mouse	FN50	Pacific Blue	Biolegend
4-1BB	Mouse	4B4-1	PE-Cy5	Biolegend
Granzyme A	Mouse	CB9	FITC	Biolegend
Granzyme B	Human	REA226	FITC	Miltenyi
Granzyme K	Mouse	GM6C3	FITC	Santa Cruz
IFN-γ	Mouse	4S.B3	Brilliant Violet 786	BD
TNF-α	Mouse	MAb11	Brilliant Ultra Violet 395	BD

Table 2.3 Antibodies used for flow cytometry.

2.3 Molecular Biology

2.3.1 Extraction of viral DNA, PCR amplification of the US28 gene region and qPCR quantification of viral genomes

Cells were washed in PBS then resuspended at 200μL per million cells in solution A (100mM KCl, 10mM Tris-HCl pH 8.3 2.5mM MgCl) followed by 200μL solution B (10mM Tris-HCl pH8.3, 2.5mM MgCl 1% Tween20, 1% NP-40, 0.4 mg/ml proteinase K). Cell solutions were heated at 60°C for one hour then 95°C for ten minutes. 5μL of each solution was used to PCR amplify the US28 gene region. Viral genomes were quantified using primers against the MIEP and using the Taqman® one-step RT-qPCR using Quantitect +ROX virus kit (Qiagen) without added reverse transcriptase, with the primers and probes described below in Table 2.4.

2.3.2 Amplifying the whole *US28* gene region by PCR

The whole US28 gene region was amplified in order to demonstrate that Titan-ΔUS28 lacked the *US28* gene, for Sanger sequencing *US28* mutants as well as cloning the US28 1-219 construct into pHRSIN. Due to slight differences in genome sequence, different primers

were used to amplify the *US28* gene region, however both sets of forward primers bind just downstream of the *US27* gene, while the reverse primers bind just upstream of the *US29* gene. For TB40E the following primers were used: fwr: CTATTGTAATTCGATCCTC and rev: CTACATGATCCTTTCCTACGTC while for Titan the following primers were used to amplify the *US28* gene, fwr: CAGGTGAGCAACATGACAAATCACACC and rev: CAACAACCCCAGTATCAGCAGCACACTG.

Sanger sequencing the US28 gene region was performed using the primers above by SourceBioscience.

2.3.3 RT-qPCR quantification of gene expression

Monocytes were infected with Titan wild type or Titan-ΔUS28. After three hours incubation, cultures were citrate washed (1 minute incubation in 40mM sodium citrate, 10mM KCl, 135mM NaCl pH 3.0, followed by twice washing in PBS) to remove cell-associated virus. Control wells for each condition, representing input mRNA from incoming virions, were then harvested in TRIzol (Life technologies).

Cells were washed twice in PBS and RNA was harvested by incubation in 700 μL TRIzol reagent (Life technologies) per 1x10^6 monocytes for 5 minutes, and isolated using miRNeasy mini kit (Qiagen), following manufacturer's instructions. UL138, IE, pp28 and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping transcript were quantified using a Taqman® one-step RT-qPCR using Quantitect +ROX virus kit (Qiagen), with the primers and probes described below in Table 2.4. US28, CX3CR1 and CTCF were quantified using the Quantitect® SYBR® Green RT-qPCR system (Qiagen), with primers described below in Table 2.4. Samples were analyzed and processed with an ABI 7500 Fast Real Time machine using MicroAmp Fast Optical 96-well reaction plates with the following RT parameters: 50°C for 20 minutes, followed by heat inactivation at 95°C for 5 minutes and then the PCR cycle steps: (50 cycles of 95°C for 15 s and 60°C for 45 s).Values were calculated using the ΔΔCT method, which measured relative differences between the input control and the relevant time point. Samples were normalised to GAPDH, as described previously [319].

2.3.4 Immunoblot detection of proteins

5x10^5 cells were washed twice in PBS and lysed in Radioimmunoprecipitation Assay (RIPA) buffer: 25 mM Tris pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100 and 1% NP-40 with protease inhibitor cocktail 1/50 (Promega), for 10 minutes on ice. Cell

Primer or Probe Target	Sequence
Immediate Early Forward	CAAGAACTCAGCCTTCCCTAAGAC
Immediate Early Reverse	TGAGGCAAGTTCTGCAATGC
Immediate Early Probe	(FAM)CCAATGGCTGCAGTCAGGCCATG(TAM)
UL138 Forward	CGCTGTTTCTCTGGTTAG
UL138 Reverse	CAGACGATACCGTTTCTC
UL138 Probe	(Cy5)CCGACGACGAAGACGATGAAC(BHQ2)
UL99 Forward	CGAACTCTGCAAACGAATA
UL99 Reverse	GAGGGATGTTGTCGTAGG
UL99 Probe	(Cy3)CGTAGAGACACCTGGCGACC(BHQ2)
GAPDH Forward	GGAAGCTTGTCATCAATG
GAPDH Reverse	CCCCACTTGATTTTGGAG
GAPDH Probe	(JOE)ATCACCATCTTCCAGGAGCGAG(BHQ1)
MIEP Forward	CCAAGTCTCCACCCCATTGAC
MIEP Reverse	GACATTTTGGAAAGTCCCGTTG
MIEP Probe	(FAM)TGGGAGTTTGTTTTGGCACCAAA(TAM)
US28 Forward	ATCGCTACTACGCTATTG
US28 Reverse	GCATGAGTTCTACGTTGA
CX3CR1 Forward	CTGCCTCTTAGACTTCTG
CX3CR1 Reverse	GGCTATCACTCTGTAGAC
CTCF Forward	ACCAACCAGCCCAAACAGAAC
CTCF Reverse	GTATTCTGGTCTTCAACCTGAATGATAG

Table 2.4 Primers and probes used for RT-qPCR during this project. All primers generated by Sigma aldrich. FAM = 6-carboxyfluorescein, TAM = 6-carboxytetramethylrhodamine, JOE = 6-carboxy-2',7'-dimeoxy4',5'-dichlorofluorescein, BHQ = black hole quencher.

debris were removed by centrifugation at 13,000 $\times$ g for 10 min at 4° C and proteins from the supernatant were denatured in Laemmli buffer (2% SDS, 10% glycerol, 60 mM Tris pH 6.8, 0.1% beta-merceptoethanol and 0.01% bromophenol blue) [211] followed by heating at 95°C for 5 minutes (but not for US28 samples).

For NFκB immunoblots, cells were fractionated using the Rapid Efficient And Practical (REAP) method [431]. 8 x 10^8 THP-1 cells were washed twice in ice cold PBS and resuspended in 900μL of ice cold PBS with 0.1% NP40. This was pipetted up and down exactly five times with a p1000 gilson pipette and 300μL of this was removed and added to 4x Laemmli Buffer as "whole cell lysate". After this, the remaining solution was spun for 10 seconds using the "short-spin" function, and a further 300μL of this was removed and added to 4x Laemmli Buffer as "cytoplasmic fraction". Finally, the pellet was washed twice in ice cold PBS with 0.1% NP40 (using pop-spinning for 10 seconds each time) and then the pellet was resuspended in 180μL of 4x Laemmli buffer. Fractionation was confirmed by immunoblotting for p84 (a nuclear protein) and GAPDH (a cytoplasmic protein).

Proteins were separated on SDS-polyacrylamide gels (10% v/v acrylamide, 0.375M Tris pH 8.8, 0.1% SDS, 0.1% ammonia persulphate, 0.001% N,N,N',N'-tetramethylethylenediamide) with a 5% stacking gel (5% v/v acrylamide, 0.125M Tris pH 6.8, 0.1% SDS, 0.1% ammonia persulphate, 0.001% N,N,N',N'-tetramethylethylenediamide) in SDS running buffer (2.88% glycine, 0.6% Tris OH, 0.1% SDS) using the Hoefer^{TM} Electrophoresis system. After separation, proteins were transferred to nitrocellulose membranes (Axygen, Corning) in transfer buffer (25mM Tris OH, 19mM glycine, 20% methanol) overnight. After blocking for 1 hour in 5% skimmed milk at room temperature, blots were incubated with primary antibodies (see Table 2.6) in 5% skimmed milk overnight at 4°C, washed with either PBS or TBS with 0.5% Tween20 three times for 10 minutes, and then incubated with secondary antibody (see Table 2.6) for 1 h at room temperature. Blots were developed with the use of enhanced chemiluminescence (GE Healthcare) and visualised with autoradiography film. Minor variations to this protocol are shown in Table 2.5.

2.3.5 Immunofluorescent staining and microscopy

Cells were fixed and permeabilized in 4% paraformaldehyde in PBS for 15 minutes, rocking at room temperature, followed by 20 minutes at -20°C in 70% ethanol and then washed in PBS [321]. Fc receptors on monocytes were blocked with 10% goat serum (Abcam) for 15 minutes at room temperature, while HFFs and RPE-1 cells did not need such blocking. Cells were then stained with relevant primary antibodies in PBS, washed twice in PBS and then

Target Protein	Buffer	Gel %
HA-US28	RIPA with 0.1% SDS and 2 mM EDTA	10% and no beta-mercaptoethanol
US28	RIPA with 0.1% SDS and 2 mM EDTA	10% and no beta-mercaptoethanol
ERK1/2	RIPA	12%
Phosphor-ERK1/2	RIPA	12%
MSK-1	RIPA	12%
Phosphor-MSK-1	RIPA	12%
NFκB	REAP	12%
p84	REAP	12%
GAPDH	REAP	12%
Actin	RIPA	Variable
CTCF	RIPA	8%

Table 2.5 Variations on immunoblotting protocols for different target proteins. RIPA = radioimmunoprecipitation assay buffer, REAP = Rapid Efficient And Practical method, beta-mercaptoethanol was not included in US28 blots to prevent US28 aggregation during gel electrophoresis.

Target	Species	Clone	Manufacturer	Concentration
US28	Rabbit	Polyclonal	Origene technology	1/500
HA-tag	Mouse	F-7	Santa Cruz Biotech	1/200
Total ERK1/2	Rabbit	137F5	Cell Signalling	1/1000
Phosphor-ERK1/2 (T202 &Y204)	Rabbit	D13.14.4E	Cell Signalling	1/1000
Total MSK-1	Rabbit	C27B2	Cell Signalling	1/500
Phosphor-MSK-1 (S360)	Rabbit	092015	Cell Signalling	1/500
Total CREB	Rabbit	Polyclonal	Merck	1/500
Phosphor-CREB (S360)	Rabbit	Polyclonal	Merck	1/500
NFκB	Rabbit	Polyclonal	AbCam	1/1000
p84	Mouse	5E10	Thermo	1/1000
GAPDH	Rabbit	Polyclonal	Millipore	1/500
Actin	Rabbit	Polyclonal	Abcam	1/1000
CTCF	Rabbit	Polylconal	Abcam ab70303	1/500
CTCF	Rabbit	EPR7314	Abcam ab128873	1/1000
Rabbit IgG	Chicken	Polyclonal	Santa Cruz Biotech	1/5000
Mouse IgG	Bovine	Polylconal	Santa Cruz Biotech	1/5000

Table 2.6 Antibodies used for immunoblotting.

Target	Origin	Clone	Fluorophore	Concentration	Supplier
US28	Rabbit	Polyclonal		1/100	Origene
IE72 and IE86	Mouse	11-003	-	1/1000	Argene
GFP	Rabbit	Polyclonal		1/1000	AbCam
Mouse IgG	Goat	Polyclonal	AF488	1/1000	Thermo
Mouse IgG	Goat	Polyclonal	AF594	1/200	Thermo
Rabbit IgG	Chicken	Polyclonal	AF488	1/1000	Thermo
UL99	Rabbit	SC3	-	1/100	Abcam

Table 2.7 Antibodies used for IF staining. AF = Alexa Fluor®

stained with relevant secondary antibodies in PBS (see Table 2.7), both times for one hour, rocking, at room temperature. Hoechst 33342 nuclear stain (1/50,000, Thermo) was used to stain cell nuclei. Samples were visualised on a Nikon TE200 microscope and pictures taken using Image-Pro Plus software.

2.3.6 Phosphokinase and cytokine antibody arrays

For the phosphokinase antibody array, $1x10^6$ THP-1 cells transduced with empty pHR-SIN vector, or pHR-SIN vector containing US28, US28-R129A or US28-Y16F were harvested and lysed using the manufacturer's protocol. These lysates were incubated with membranes and analysed following manufacturer's protocol (Proteome Profiler Human Phospho-Kinase Array Kit; R&D systems) and spot intensity was analysed using ImageJ software. P values were calculated from two technical repeats on each

For the cytokine arrays, CD14+ monocytes were infected with either Titan-WT or Titan-ΔUS28, or with UV-inactivated Titan-WT virus, and incubated for 5 days in order to establish latency. The media was then changed and cells were washed in PBS. Two days later, media from these cultures was harvested and assayed by RayBio® Human Cytokine Antibody Array C1000 Combination, of Array VI and VII) following the manufacturer's instructions (Raybiotech). Spot intensity was analysed using ImageJ software.

2.3.7 Luciferase assays

THP-1 cells expressing various HA-US28 constructs were generated as described in Section 2.1.13. These cells were transfected, by nucleofection, with an MIEP-luciferase construct [309], as well as an SV40-Luciferase (Renilla) construct (pRL, Promega) as a transfection control. Two days after transfection, Luciferase assays were then performed, or THP-1 cells

were treated with PMA for a further two days to allow time for differentiation. After this, luciferase assays were performed using Dual-Luciferase® Reporter Assay System (Promega), following manufacturer's protocol, using a GloMax®-96 Microplate Luminometer.

To analyse the role of CTCF expression on the MIEP, THP-1 cells which had not been transduced by lentivirus were transfected, by nucleofection, with an MIEP-luciferase construct [309], as well as an SV40-Luciferase (Renilla) construct (pRL, Promega) as a transfection control, as well as with control siRNA, anti-CTCF siRNA, a CTCF vector or empty vector as a control. After this, luciferase assays were performed using Dual-Luciferase® Reporter Assay System (Promega), following manufacturer's protocol, using a GloMax®-96 Microplate Luminometer.

2.3.8 Chromatin Immunoprecipitation of the MIEP

Chromatin Immunoprecipitation (ChIP) of CTCF was performed on monocytes infected with Titan-WT, Titan-ΔUS28 or mock infected cells. $1x10^6$ cells were fixed in formaldehyde and lysed in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH8.1, 1mM PMSF, 1mM pepstatin A, 1mM aproprotin), and sonicated twice for 2 minutes on ice at 40% continuous pulse (XL2020 Heat Systems, Sonicator). Samples were centrifuged at 3500 RCF for 5 minutes and the supernatent was diluted in buffer 1 (0.1% SDS, 1% Triton-X100, 2mM EDTA, 20mM Tris pH 8.1, 150mM NaCl) and an aliquot of this was taken as an input control. The lysate was then precleared with 160uL of protein A beads with 1% BSA and 200 μg/ml sonicated Herring Sperm DNA for 30 minutes at 4°C. The sample was then centrifuged twice at 3500 RCF for 2 minutes to precipitate the beads and the supernatent split into two aliquots, one of which was treated with 5μg of anti-CTCF antibody (ab70303, AbCam), the other with an isotype control antibody (ab27478, AbCam). These were incubated, rotating, overnight at 4°C and then 60μL of protein A beads added, with 1% BSA and 200μg/ml sonicated Herring Sperm DNA, and incubated for a further 1 hour at 4°C. The samples were then centrifuged at 3500 RCF for 2 minutes to precipitate the beads, and washed in buffer 1, followed by buffer 2 (0.1% SDS, 1% Triton-X100, 2mM EDTA, 20mM Tris pH 8.1, 100mM NaCl) and then buffer 3 (0.25M LiCl, 1% NP40, 1% Sodium deoxycholate, 1mM EDTA, 10mM Tris pH 8.1). The beads were then washed twice in Tris-EDTA buffer, and CTCF eluted by washing twice in 250μL elution buffer (1% SDS, 0.1 M $NaHCO_3$).

DNA can be extracted from these two samples, as well as the input control sample using QIAquick PCR Purification Kit (Qiagen), following manufacturer's procotol. 5μL of this

50μL DNA sample was used for qPCR amplification of the MIEP genome region, as specific in Section 2.3.1.

2.3.9 Homologous binding experiments on transient transfected COS-7 cells.

This protocol was carried out by Dr Katja Spiess in the laboratory of Professor Mette Rosenkilde at the Technical University of Denmark. 3x10^6 COS-7 cells were transfected with 20μg of receptor cDNA (US28 or CX3CR1) using calcium precipitation [419] and transferred to 24 well culture plates coated with poly-D-lysine one day after transfection. The number of cells seeded per well was determined by the apparent expression efficiency of the receptors and was aimed at obtaining 5-10% specific binding of the added radioactive ligand (2.5x10^4 cells/well for US28 and 1x10^5 cells/well for CX3CR1-expressing cells). Two days after transfection, cells were assayed by competition binding for 3 hours at 4°C using 10-15pM ^{125}I-CX3CL1 as well as unlabelled CX3CL1 in 50mM HEPES buffer, pH 7,4 supplemented with 1mM CaCl2, 5mM MgCl2 and 0.5 (w/v) bovine serum albumin (BSA) (binding buffer) [360]. After incubation, cells were washed twice in ice-cold binding buffer, supplemented with 0.5M NaCl. Determinations were made in duplicates.

2.3.10 Homologous binding experiments on uninfected and latent HCMV infected monocytes.

Approximately 2x10^8 monocytes were infected with TB40E with an SV40-GFP tag at an MOI of 5. 4 days after infection, monocytes were sorted for GFP positive cells, yielding around 5% of total cells. Thesec cells were sent to Dr Katja Spiess in the laboratory of Professor Mette Rosenkilde at the Technical University of Denmark. There, approximately, 7x10^4 of these GFP-positive monocytes were seeded per well to 96 well culture plates coated with poly-D-lysine. 3 hours after seeding, cells were assayed by competition binding as described for COS-7 cells above. Determinations were made in duplicates.

2.4 Analysis of whole cell protein expression by mass spectrometry

2.4.1 Lysis, digestion and cleanup

Cells were harvested by centrifugation and washed 3x in cold PBS before being finally pelleted into a low adhesion microfuge tube. These pellets of THP-1 cells were dried and frozen at -80C for preparation and mass spectrometry analysis by Dr James Williamson, which performed all of the subsequent protocols, including data processing. Cell pellets were lysed in 2%/50mM Tetraethylammonium bromide (TEAB) pH 8.5. Samples were quantified by BCA assay and 50μg of each sample was taken and adjusted to the same volume with lysis buffer. Reduction and alkylation was achieved by addition of 10mM tris(2-carboxyethyl)phosphine and 20mM iodoacetamide for 20mins at room temperature in the dark followed by quenching with 10mM Dithiothreitol (DTT). Samples were cleaned up and digested using a modified Filter Aided Sample Preparation protocol. Briefly, samples were brought to 500uL volume with 8M urea/TEAB and loaded onto a 30kDa MWCO ultrafiltration device. Samples were then washed 3 times with 8M urea/TEAB followed by 3 times with 0.5% SDC/50mM TEAB. Samples were finally resuspended in 50uL of SDC/TEAB containing 1μg trypsin and incubated overnight at 37°C. After digestion, samples were recovered from the filter device by centrifugation, acidified to precipitate SDC and cleaned up by two-phase partitioning into 2x volumes of ethyl acetate (repeated twice) before drying in a vacuum centrifuge.

2.4.2 High pH Reversed Phase Fractionation

This was performed by Dr James Williamson. Sample was injected onto an Ultimate 3000 RSLC UHPLC system (Thermo Fisher Scientific) equipped with a 2.1 i.d x25cm, 1.7μm particle Kinetix Evo C18 column (Phenomenex). Mobile phase consisted of A: 3% ACN, B:ACN and C: 200mM ammonium formate pH 10. Isocratic conditions were 90% A/10%C and C was maintained at 10% throughout the gradient elution. Separations were carried out at 45°C. UV absorbance was monitored at 280nm and 15s fractions were collected into 96 well microplates using the integrated fraction collector. Peptide containing fractions were then orthogonally recombined into 24 fractions and dried in a vacuum centrifuge and resuspended in 10μL 5% DMSO 0.5% TFA for analysis.

2.4.3 LC-MS analysis

This was performed by Dr James Williamson. All samples were injected onto an Ultimate 3000 RSLC nano UHPLC equipped with a 300μm i.d. x 5mm Acclaim PepMap μ-Precolumn (Thermo Fisher Scientific) and a 75μm i.d. x50cm 2.1μm particle Acclaim PepMap RSLC analytical column. Loading solvent was 0.1% TFA, analytical solvent A: 0.1% FA and B: ACN+0.1% FA. All separations are carried out at 55°C. Samples were loaded at 10μL/min for 5 mins in loading solvent before beginning the analytical gradient.For High pH RP fractions a gradient of 3-5.6% B over 4 mins, 5.6-32%B over 162mins, followed by a 5 minute wash at 80%B and a 5 minute wash at 90%B and equilibration at 3%B for 5 mins.

2.4.4 Data Processing

This was performed by Dr James Williamson. All Raw files were searched by Mascot within Proteome Discoverer 2.1 (Thermo Fisher Scientific) against the Swissprot Human database and a database of common contaminants. For unlabelled samples the search parameters were as follows. Enzyme: Trypsin. MS1 tol: 10ppm. MS2 tol: 0.6Da. Mascot Percolator was used to calculate PSM FDR. Search results were further processed and filtered as follows: Peptides below a percolator FDR of 0.01% and proteins below the 0.01% protein FDR (calculated from a built in decoy database search) were rejected. Protein groups were then generated using the strict parsimony principle. Peptides both unique and razor with a co-isolation threshold of 50 and an average s/n threshold of 10 were used for quantification and a normalisation of these values to the total peptide amount in each channel was applied. Instances where a protein was identified but not quantified in all channels were rejected from further analysis. "Scaled" abundances of proteins provided by Proteome Discoverer were used to derive ratios of abundance.

Chapter 3

Detecting US28 protein expression during latency

3.1 Introduction

HCMV latency in early myeloid lineage cells is characterised by limited viral gene expression, which results in no production of infectious virus. Currently, latency-associated HCMV gene expression is confirmed by RT-qPCR detection of viral mRNA in early myeloid lineage cells from naturally latently infected individuals [391]. This is because expression of HCMV-encoded proteins during latent infection is likely to be at relatively low levels, and because HCMV establishes latent infection in relatively few cells, around 1 in 1,000 in naturally latently infected individuals [398] or roughly 10% of cells in experimentally latently infected monocytes [208, 217]. This means it has been difficult to detect HCMV-encoded proteins, which are being expressed during latency, due to this low level of latent carriage in the myeloid lineage.

The aim of this chapter was to detect the expression of US28 protein in a model of latent infection. Whilst US28 mRNA has been detectable in both natural and experimental models of latency [27, 78, 137, 161, 208], its expression has never been confirmed at the protein level. This aim became feasible after the publication of a new viral isolate SV40-GFP-TB40E [293], which has a cassette consisting of the GFP gene, controlled by the SV40 promotor, which is expressed during latency. This means that latently infected cells appear green by both fluorescence microscopy and flow cytometry. I therefore planned to exploit this by optimising a protocol to infect monocytes with SV40-GFP-TB40E, sort the latently infected, GFP-positive monocytes by fluorescence activated cell sorting (FACS), and then immunoblot for viral US28 using a commercial antibody. There were therefore many parameters which

needed to be optimised in order to establish a protocol for sorting latently infected CD14+ monocytes from healthy blood donors, as well as confirming the specificity of the commercial antibody to US28.

I wanted to detect US28 protein in latently infected cells because it would provide a rational basis for other projects, such as the ability to target the latent reservoir using an anti-US28 fusion toxin protein, F49A-FTP (see Chapter 5). Developing a protocol to isolate latently infected cells would also be useful in general; it would allow researchers to isolate and probe changes in latently infected cells, specifically.

In this chapter, I will show the optimisation of a protocol for enriching primary blood monocytes latently infected with SV40-GFP-TB40E. This protocol has now been used in two publications and will hopefully be helpful for further future studies [208, 217]. I will also demonstrate that, although the commercial anti-US28 antibody is able to detect US28 expression by transfected cells and lytically infected fibroblasts, it does not bind with sufficient sensitivity to detect US28 protein expressed during latency. I will also propose that other, newer, HCMV recombinant viruses may allow the successful detection of US28 protein during latency.

3.2 Results

3.2.1 GFP expression from latently infected monocytes is strongest between three and four days post infection

I reasoned that in order to immunoblot for US28, during experimental latent infection, the highest yield of latently infected cells possible would be required. GFP expression, from monocytes latently infected with SV40-GFP-TB40E not only dissipates, but fluctuates over time (personal observations), but that time course was not known. As it was important to sort infected monocytes when the maximum number of cells were expressing GFP, in order to maximise the yield of latently infected cells, I therefore sought to plot the time course of GFP expression in latently infected monocytes. To this aim, I infected monocytes with SV40-GFP-TB40E and counted the number of green cells per well of a 96 well plate, each day for eight days. My observation (Figure 3.1) was that GFP is expressed by the most monocytes between three and four days post infection. I, therefore, decided to sort cells either on day three or day four after infection, to maximise the yield of latently infected cells.

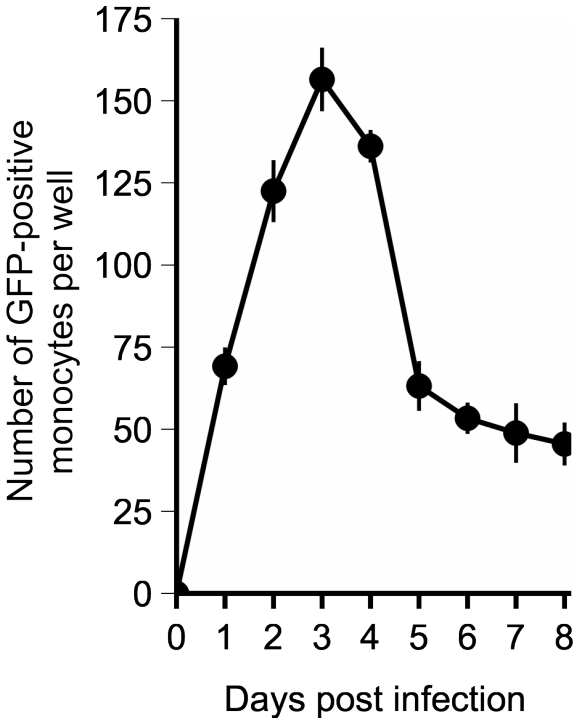

Figure 3.1 Maximum expression of GFP from monocytes infected with SV40-GFP-TB40E occurs between three and four days post infection. CD14+ monocytes from peripheral blood donations were isolated, plated, and then experimentally infected with SV40-GFP-TB40E at an MOI of 5. Each subsequent day post infection, the number of monocytes was counted in each of three separate experiments, with 10,000 cells per experiment. Means and error bars (showing standard deviations) were generated from these three independent experiments.

3.2.2 Monocytes should be infected and cultured in suspension in order to maximise survival and yield of GFP-positive cells

Monocytes are routinely experimentally infected with HCMV as an adherent monolayer or in suspension [146, 325]. However, it is not known whether virus infection is more efficient by one or the other method. As FACS can only be performed on suspended cells, cells that are adherent must be lifted from the plate, using EDTA buffer and cold temperatures, which can result in cell death. This meant that it was not clear whether the yield of green, latently infected monocytes, that could be isolated, would be greater if infection was performed while cells were plated or when in suspension. Consequently, I compared the two methods by taking equal numbers of monocytes and either plating them or leaving them in suspension, and then infecting both cultures with the same amount of SV40-GFP-TB40E in the same volume of media. After three days of incubation, cell survival and GFP expression were analysed using flow cytometry directly on suspension cells or after removal of adherent cells from the tissue culture plate. Data shows similar percentages of GFP-positive monocytes between those infected in suspension and those infected while adhered to a tissue culture plate (Figure 3.2A); this indicates that infection of monocytes is roughly as efficient for plated monocytes as for monocytes in suspension. We also measured cell survival, however, which was significantly improved in monocytes that were infected in suspension (Figure 3.2B), compared to those that had been plated (Figure 3.2C) and subsequently lifted from the plate. I therefore decided to infect monocytes in suspension whenever I sorted latently infected monocytes by FACS.

3.2.3 A commercial anti-US28 antibody binds US28 but with low affinity

I next wanted to confirm that the commercial antibody against US28 (TA314635), for which we could find no references of successful use, was able to selectively bind to US28 protein by immunoblotting, and that the antibody could detect US28 being expressed by SV40-GFP-TB40E. Consequently, I infected HFFs with SV40-GFP-TB40E, and immunoblotted for US28 using this antibody, with uninfected cells as a control. Pleasingly, I was able to detect US28 by immunoblot with this antibody, although the detection was poor which lead to high background staining (Figure 3.3A). However, as HCMV expresses other GPCRs, which run at similar masses on electrophoresis gels, I also wanted to confirm that this band was indeed US28 and not another protein expressed by HCMV during lytic infection [75, 242, 485]. To do this, I used an isolate of HCMV Titan, which is a wild type, low passage isolate of HCMV

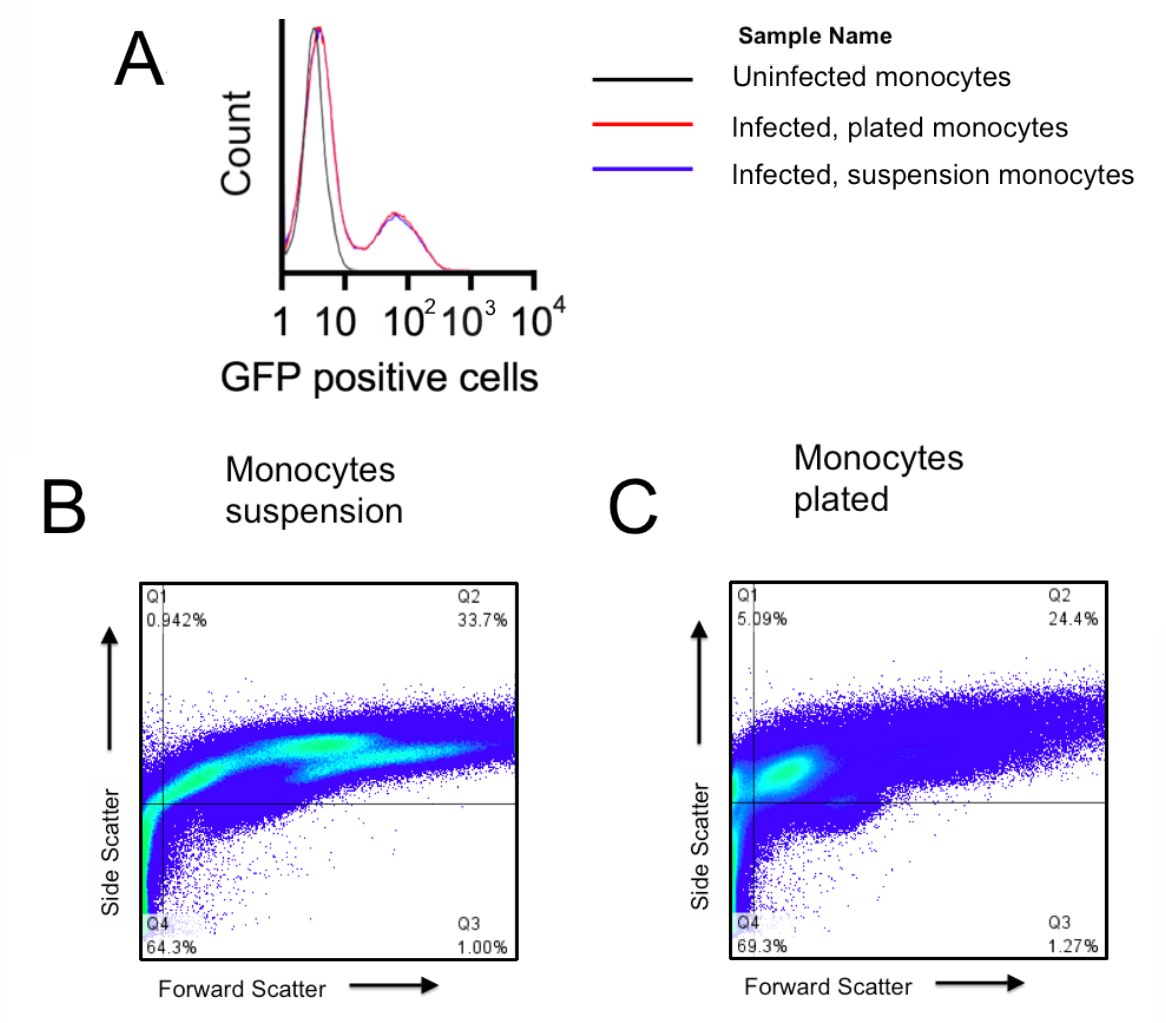

Figure 3.2 Infection efficiency is equal between monocytes plated or in suspension, but monocytes survival is better if kept in suspension. CD14+ monocytes from peripheral blood donations were isolated and either plated or left in suspension. Both sets of monocyte cultures were infected with SV40-GFP-TB40E at an MOI of 5, and three days post infection, monocytes in suspension were directly analysed by flow cytometry for survival and GFP expression, or adherent monocytes were lifted from the culture plate and similarly analysed (B and C, measured by forward scatter and side scatter) and surviving cells were measured for GFP expression (A).

with a UL32-GFP tag (Titan-WT) as well as a corresponding mutant which has a deletion in the US28 gene region (Titan-ΔUS28) [252]. Firstly, I confirmed that Titan-ΔUS28 did indeed lack the US28 gene by PCR amplification of the US28 gene region, followed by gel electrophoresis. This showed that the viral isolate, Titan-ΔUS28, was indeed missing around 1000 bps between US27 and US29, consistent with a deletion in the US28 gene (Figure 3.3B). I then performed a second immunoblot against US28, this time using proteins from HEK cells (transfected with US28 or mock transfected) as well as HFFs infected with Titan-WT, or Titan-ΔUS28. I was able to detect US28 from HEK cells transfected with US28 plasmid, as well as HFFs infected with Titan-WT but not Titan-ΔUS28 (Figure 3.3C). I therefore concluded that this antibody detects US28, exclusively, and not other proteins expressed by HCMV. The antibody would therefore be suitable for detecting US28 protein from sorted latently infected cells. I will discuss the varying masses of US28 in the discussion section.

3.2.4 US28 mRNA is expressed throughout latent infection

Previous analysis of latency-associated gene expression using RNA microarrays have shown conflicting results regarding when US28 is expressed after latent infection [78, 137]. However, these analyses were not strictly comparable; one analysed infection of granulocyte macrophage progenitors (GMPs) after infection with TOWNE virus [78], and the other analysed CD34+ progenitors infected with Toledo virus [137]. Consequently, I wanted to confirm the time course of expression of US28 RNA in latently infected CD14+ monocytes. To analyse when US28 mRNA is expressed after latent infection of CD14+ monocytes in my hands, I performed RT-qPCR analysis over several time points post infection, measuring US28 mRNA levels relative to input mRNA from incoming virus (Figure 3.4). I also infected monocytes with Titan-Δ-US28 to demonstrate that my primers only detect US28 mRNA (Figure 3.4). This showed that US28 is expressed from one day post-infection in our model of latency, and is still expressed at four days post infection, when I propose to sort cells and harvest protein.

3.2.5 US28 could not be detected in sorted, latently infected monocytes by immunoblot

I now had confidence that I could detect US28 by immunoblotting, at least in lytic infection, using this commercial antibody, and that I could also sort for GFP positive cells to a high yield. I therefore infected monocytes with SV40-GFP-TB40E, in suspension, and (in collaboration with the Department of Medicine flow cytometry core facility) sorted these cells for GFP

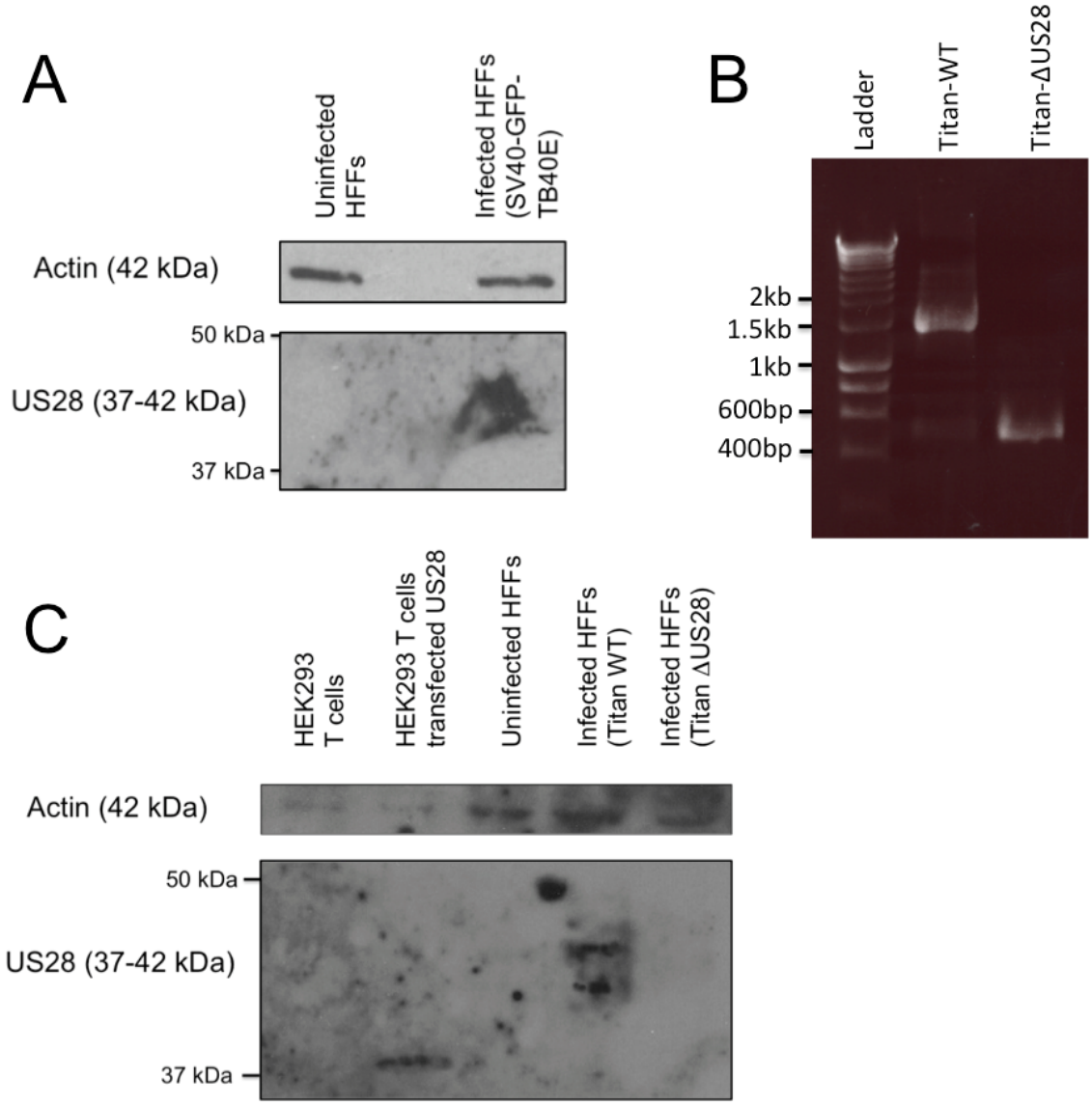

Figure 3.3 US28 protein is detectable in infected HFFs by immunoblotting. A) HFFs were infected with SV40-GFP-TB40E at an MOI of 1, or mock infected. Four days post infection, cells were lysed and lysates separated by SDS-PAGE followed by immunoblotting with a commercial anti-US28 antibody. B) HFFs were infected with Titan-WT or Titan-ΔUS28 at an MOI of 1 and three days post infection, cells were lysed and DNA was harvested. PCR amplification was performed, using primers against the US28 region, and the PCR products were separated by DNA gel electrophoresis. C) HEK cells were transfected with the expression vector pHRSIN containing US28, or mock transfected. HFFs were infected with Titan-WT, Titan-ΔUS28 at an MOI of 1, or mock infected. Three days post transfection or five days post infection, cells were lysed and lysates separated by protein electrophoresis followed by immunoblotting with a commercial anti-US28 antibody.

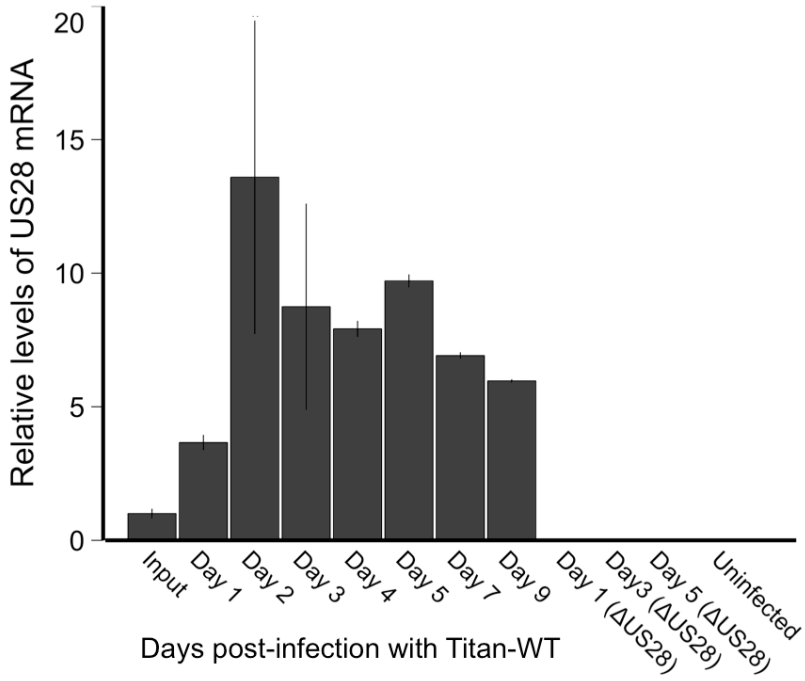

Figure 3.4 RT-qPCR analysis of US28 mRNA expression in infected monocytes over time. CD14+ peripheral blood monocytes were latently infected at an MOI of 5, with SV40-GFP-TB40E, and RNA from one culture was harvested immediately after infection as an input control. Each subsequent day post infection, for nine days, RNA was harvested from another culture. Additionally, CD14+ peripheral blood monocytes were latently infected at an MOI of 5 with Titan-ΔUS28 virus, as a negative control, and RNA was harvested on the days indicated, post infection. One monocyte culture was left uninfected and harvested two days later. US28 mRNA was quantified, relative to the input control sample, by RT-qPCR analysis. Means and standard deviations from three measurements and normalised to GAPDH.

positive monocytes four days post infection. The results from this FACS analysis are shown in Figure 3.5; we were able to collect around 3% of the total cells (GFP positive), which totalled 2 million GFP-positive, latently infected cells. I then performed immunoblotting analysis to detect the US28 protein. However, I was unable to detect any US28 protein using the same immunoblotting protocol as before (Figure 3.5D).

Although I was unable to detect US28 protein in latently infected cells, after FACS sorting, I recognised the potential uses for this protocol for other applications. I was concerned that FACS may insult sorted cells in such a way that could cause viral reactivation, or at least perturb latent infection. I, therefore, confirmed by RT-qPCR analysis of HCMV gene expression that these cells remained latent (Figure 3.6). RT-qPCR measurement of UL138 mRNA expression, from monocytes either before or after FACS, showed that latency-associated transcript UL138 mRNA expression remained high, with only low levels of the lytic transcripts (IE and UL99) in both samples, indicating that the virus did not reactivate due to sorting (Figure 3.6A). I also measured, in parallel, mRNA from infected monocytes which had been differentiated into dendritic cells to reactivate virus in order to demonstrate *bona fide* viral gene expression during lytic infection; reactivated virus in dendritic cells showed significant IE and UL99 mRNA expression (Figure 3.6A).

I was concerned about the fact that, in my hands, I was able to detect low levels of IE and UL99 expression in latently infected cells, which have been undetectable in previous studies of latency [136, 309, 347] which had used end-point PCR analysis instead of RT-qPCR. To confirm that our "latency-associated gene expression profile" was consistent with latency, I also used IF staining on latently infected monocytes in parallel with reactivated virus from monocyte-derived dendritic cells, to detect IE and UL99 proteins (Figure 3.6B). I only detected viral IE and UL99 protein expression in reactivated virus from infected dendritic cells and observed no IE or UL99 protein expression in latently infected monocytes. These observations are consistent with the view that HCMV does remain latent in CD14+ monocytes after FACS sorting.

3.2.6 The commercial anti-US28 antibody is not sufficiently specific to detect US28 by immunofluorescent staining

As I could not detect US28 protein by immunblot of sorted, latently infected monocytes, I also tried to detect US28 by immunofluorescent staining of latently infected cells. To do this, I used SV40-GFP-TB40E, so that I could correlate GFP-positive cells with US28 staining in red. Although I could see correlation between red staining of US28 with GFP expression in

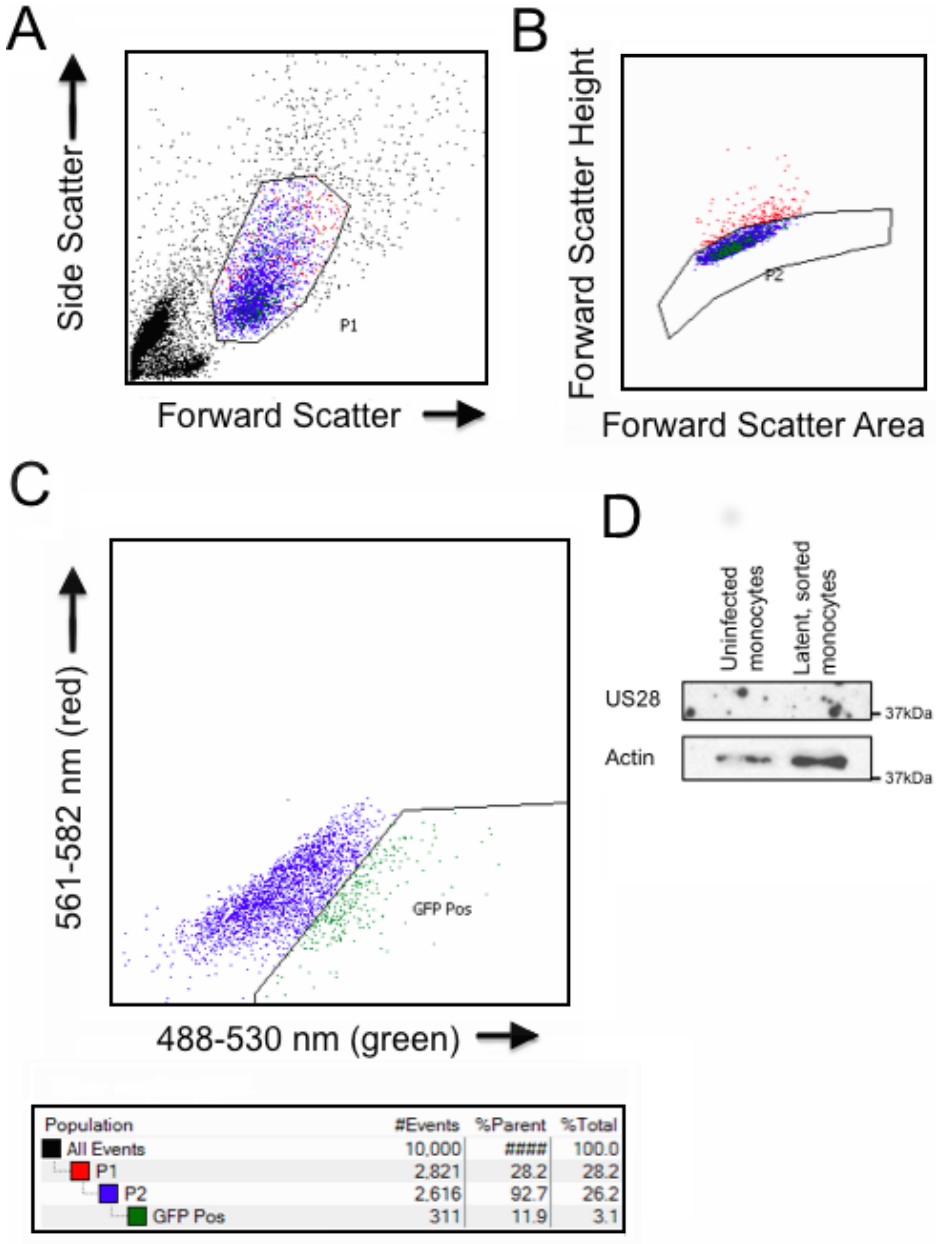

Figure 3.5 US28 expression in GFP positive, latently infected monocytes, was undetectable at the protein level. Monocytes were latently infected, in suspension, with SV40-GFP-TB40E at an MOI of 5, and isolated by fluorescence activated cell sorting four days post infection. A), B) and C) show the live cells, singlets and GFP positive cells that we isolated. D) After sorting, latently infected monocytes were lysed and lysates separated by protein electrophoresis followed by immunoblotting with a commercial anti-US28 antibody.

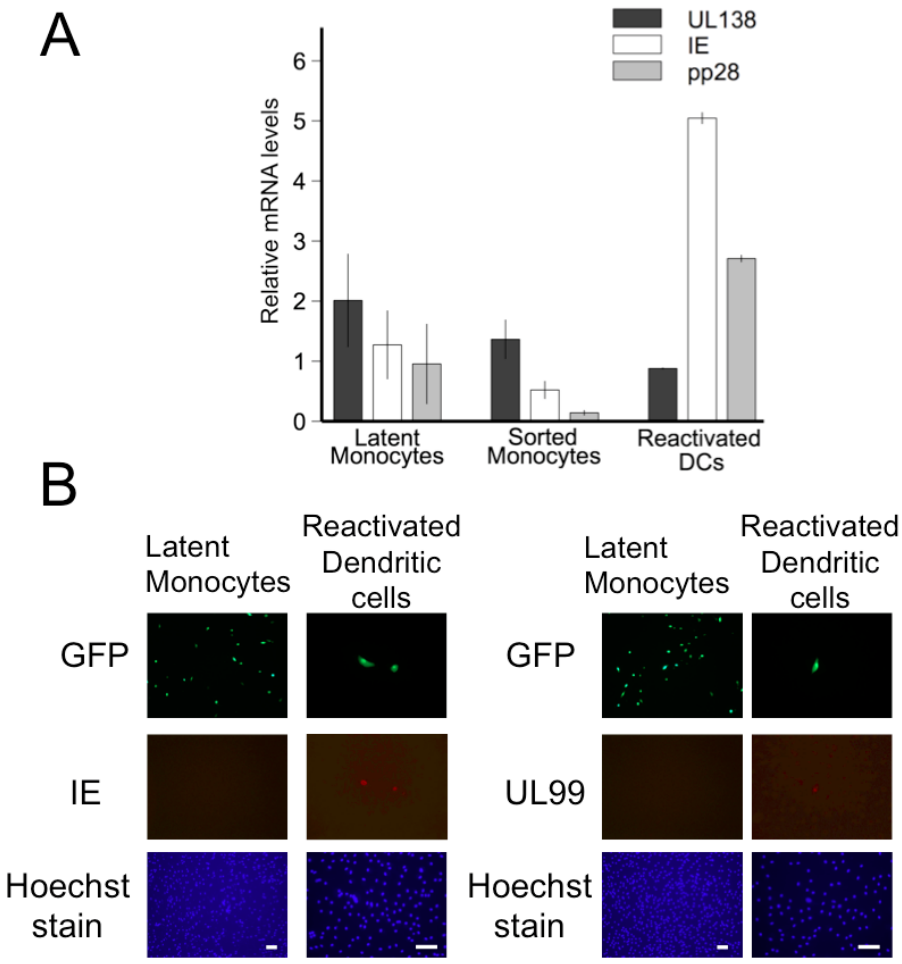

Figure 3.6 GFP-expressing, latently infected CD14+ monocytes, still display all the hallmarks of latent infection. Monocytes were latently infected, in suspension, with SV40-GFP-TB40E at an MOI of 5, and isolated by fluorescence activated cell sorting four days post infection. RNA was harvested immediately after sorting. RT-qPCR analysis of this RNA demonstrated that sorted monocytes express UL138 more so than the lytic genes: immediate early and UL99. Data was normalised to GAPDH RNA. This was compared to reactivated, monocyte-derived, mature dendritic cells, the RNA from these was harvested 4 days post terminal differentiation by LPS treatment, as a positive control for a lytic transcription profile. Means and error bars (showing standard deviations) were generated from three measurements. B) To demonstrate that low level detection of IE and UL99 by RT-qPCR in monocytes corresponded to limited protein expression, monocytes and reactivated dendritic cells were stained for IE and UL99 (pp28) and detected with a red fluorescent secondary antibody, either 4 days post infection or 4 days post terminal differentiation by LPS treatment. This demonstrated that UL99 and IE are not detectable in infected monocytes, but are expressed after differentiation and reactivation of these latently infected cells. White bars indicate 50μM scale.

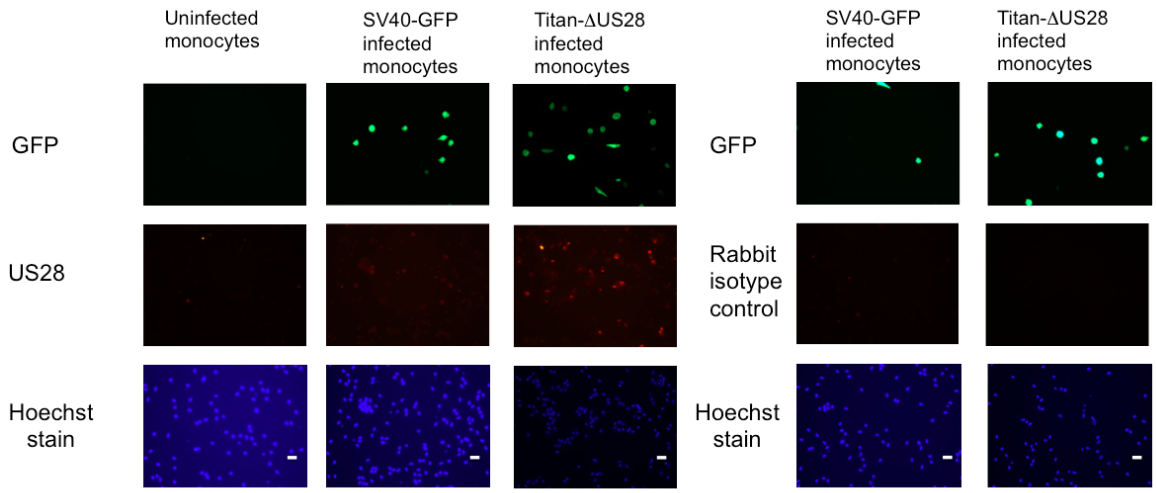

Figure 3.7 US28 was undetectable by indirect immunofluorescent staining in latently infected cells. CD14+ monocytes from peripheral blood donations were isolated and experimentally infected with SV40-GFP-TB40E, or Titan-ΔUS28 at an MOI of 5. Three days post infection, monocytes were fixed and stained, by immunofluorescent labelling, for US28 protein, or using a rabbit isotype control. Hoerchst staining (blue) was used to visualise nuclei. White bars indicate 50μM scale.

monocytes infected with SV40-GFP-TB40E, I could also see significant, and indeed more intense, staining of monocytes infected with Titan-ΔUS28 (Figure 3.7). This suggested that the anti-US28 commercial antibody may bind other targets by IF, and is not suitable for IF staining of monocytes.

3.3 Discussion

HCMV latency, in early myeloid lineage cells, is characterised by expression of very few viral genes, one of which is *US28* [391]. I aimed to exploit a new viral isolate, SV40-GFP-TB40E, which expresses GFP in latently infected monocytes, to sort monocytes and isolate the GFP-positive, latently infected cells. I optimised a protocol to sort for SV40-GFP-TB40E infected monocytes, and then sorted these cells and harvested RNA and protein from this enriched population of latently infected monocytes. Although I was unable to detect US28 protein in latently infected monocytes, I was able to isolate cells and show that they were still latently infected by RT-qPCR. This protocol has proven to be extremely useful and has now been used in other projects, as detailed in Chapter 5 and also published work [208, 217].

Additionally, we are currently using this sorting protocol to isolate single, latently infected monocytes and CD34+ progenitor cells in order to determine the individual gene expression profile of single, latently infected cells by RNA sequencing in collaboration with Dr Noam Stern-Ginossar at the Weitzmann Institute of Science (Israel). Finally, I also verified that the Titan isolates correctly encoded the *US28* gene region in the Titan wild type virus or had a *US28* gene deletion for Titan-ΔUS28, which is important as these two isolates will be used extensively in Chapter 4.

It seems most likely that I was unable to detect US28 protein in latently infected cells due to the low levels of US28 protein expression in latently infected monocytes (coupled with a relatively poor antibody, the only such commercially antibody available at the time). In Chapter 4, however, I show that US28 protein with an N-terminal HA-tag was being expressed in THP-1 cells, using an anti-HA antibody, with relative ease. This leads me to believe that tagging US28 in the viral genome may be the only way to detect US28 protein expression during latency. As an aside, it didn't escape my notice that US28 protein appears to run at slightly different masses, (between 37 kDa and 42 kDa), depending on the expression system (Figure 3.3). However, varying masses of US28 have been seen before and are likely due to differences in post translational modifications [161, 264, 291].

The data I will describe in Chapters 4 and 5 indicate that US28 is being expressed, and so it is likely that the detection of the US28 protein itself likely requires more sensitive assays. Humby et. al, were able to detect US28 protein from virions using an isolate of TB40E which has a triple FLAG-tagged (3XFLAG) variant of the US28 gene [161]. Anti-FLAG antibodies bind very strongly to 3XFLAG, and yet Humby et. al, still had to perform protein immunoprecipitation of 3XFLAG-US28, followed by immunoblotting, to detect US28 protein [161]. This indicates that US28 expression is indeed low, and suggests that detection of US28 using an anti-US28 antibody alone, without enriching for the protein by immunoprecipitation prior to immunoblotting is universally difficult. Similarly, Wu et. al, were able to detect US28 protein in latently infected THP-1 cells, again by isolating US28 protein by immunoprecipitation before performing immunoblot analysis [497].

There are a number of approaches that could be taken to detect US28 protein in latently infected monocytes. One such method is to use the TB40E, 3XFLAG-US28 isolate from Humby et. al, and immunoprecipitate US28 protein using anti-FLAG antibodies, before immunoblotting [161]. This isolate of TB40E has an SV40-mCherry tag, which would allow for FACS based on mCherry expression. Another method may be to use a TB40E isolate with a GFP-tagged variant of US28 [291]. It may be possible to see GFP expression in latently infected monocytes by fluorescence microscopy, and enhance the signal by using

an anti-GFP antibody conjugated to FITC. This method has an added bonus as if we could detect US28-GFP expression in cells by fluorescence microscopy, we would also be able to visualise how US28 is trafficked in latently infected monocytes as well as generate a time course of US28 protein expression during establishment and maintenance of latency. Another option may also be to subclone this US28-GFP construct into a lentivirus vector, and transduce expression in THP-1 cells, which could provide insights into US28 trafficking in monocytic cells.

In summary, in this chapter, I have demonstrated that I am able to isolate latently infected cells from monocyte cultures infected with SV40-GFP-TB40E and that two viral isolates, Titan-WT and Titan-ΔUS28, could be used for experiments requiring the deletion of the US28 gene. Although unable to detect US28 protein by the method proposed, future analysis will use viral isolates with tagged US28 genes to detect US28 protein expression during latency. Two viral isolates, TB40E with US28-3XFLAG and TB40E with US28-GFP are now in our possession and I plan to try to detect US28 protein, using the approaches discussed above, in latently infected monocytes, using these isolates.

Chapter 4

The role of US28 during HCMV latency

4.1 Introduction

HCMV is a ubiquitious pathogen which maintains a lifelong infection in its host [53, 356]. This is due largely to latent infection, which occurs in early myeloid lineage cells, and is only partly detected but not cleared by the immune system of a seropositive host [391, 493]. HCMV persistence in the host is likely maintained after primary infection by a cycle of subclinical reactivation events, which are generally controlled by cellular and antibody immune responses [39, 325, 341, 346, 405, 408, 439]. This reactivation from latency, which occurs when latently infected early myeloid lineage cells differentiate into macrophages and dendritic cells, is a clinical threat for transplant recipients, patients with AIDs and other immunocompromised individuals. Unfortunately, as yet, there is no clinical treatment to target latent HCMV infection.

Latent infection is characterised by a restricted viral transcription program, which includes the expression of only a small number of latency-associated transcripts, including *US28*. Latency is characterised by repression of the MIEP, which prevents expression of the immediate early proteins IE72 and IE86. This repression of IE protein expression stops the initiation of the lytic transcription program and the production of infectious virions [18, 207, 282, 322, 346, 408, 440, 496]. Though it is known that myeloid differentiation triggers reactivation of latent virus, the exact signals associated with myeloid differentiation, which induce HCMV reactivation, are not clear. It is established that an orchestrated set of events occur, mediated by both viral and cellular factors, which signal to the MIEP and trigger reactivation, [49, 161, 221, 228, 336, 496]. In particular, these effects are likely to involve ERK-MAP kinase signalling [189]. Consequently, these pathways, which are activated

during myeloid cell differentiation and maturation, are likely to play a prominent role in the differentiation-dependent activation of the MIEP [49, 221, 238, 255, 314, 336, 340, 445].

Latency-associated viral gene products are known to have profound effects on the latently infected cell, including modifying the cellular microRNAome [217, 218, 320], the cellular secretome [207, 248] and cell surface protein expression [483]. One viral latency-associated gene has been implicated as being pivotal in maintaining MIEP repression, the *US28* gene [161].

The *US28* gene encodes a CCR homologue and its mRNA has been detected during HCMV latency, which indicates that US28 protein is likely being expressed during latent infection [27, 78, 137, 326]. Deletion of the US28 gene has profound effects on latent infection in CD34+ progenitor cells and leads to lytic infection in these undifferentiated myeloid cells, due to a lack of MIEP repression [161]. The other HCMV-encoded CCR homologues, UL33, UL78, and US27 are not expressed during latent infection and, perhaps as expected, deletion of these genes does not affect establishment of HCMV latency [75, 161, 242, 485].

US28 is also the best characterised of the CCR homologues encoded by HCMV. It can signal via multiple different G-alpha proteins and this signalling is modulated by cell type and chemokine binding [59, 264, 272, 468, 471] to activate a number of different signalling pathways [32, 214, 251, 264, 265, 426, 470, 497]. US28 signalling can be modulated by binding of either CC or CX3C chemokines [32, 61, 127, 196] and high affinity chemokine binding to US28 is known to be mediated by its N-terminal domain [471]; one point mutation to US28, US28-Y16F, greatly reduces chemokine binding for RANTES and fractalkine [61]. This ligand binding phenotype of US28 has been proposed to contribute to chemokine scavenging [33, 472], although the *in vivo* relevance of this is not clear [41].

During lytic infection, US28 is known to promote proliferative signals including MAP kinase and NFκB in certain cell types [214, 264, 272, 397], both of which are known to activate the MIEP [42, 488]. The expression of US28 and its strong signalling activation has also been linked to vascular disease and oncomodulation [426, 427, 469]. This signalling by US28 during lytic infection requires G protein binding, via the highly conserved DRY motif of US28 that is found in most GPCRs, and all CCRs [366] and, consistent with this, a point mutation in this DRY motif of US28 (US28-R129A) greatly reduces G-protein binding and ablates US28 signalling capability [251, 252, 264, 473]. Similarly, the US28 C-terminus is also heavily phosphorylated; this phosphorlyation triggers beta-arrestin binding and internalisation of US28, which is known to modulate US28 signalling [263, 273, 383]. All these known activatory functions of US28 during lytic infection, however, appear to be

totally inconsistent with the observations that, during latency, US28 is required to enforce MIEP silencing [161] and suggests very different functions of US28 during latent and lytic infection. Humby and O'Connor also demonstrated that US28 protein - carried in *trans* by UV-inactivated wild type virus, cannot rescue the inability of a US28 deletion virus to repress the MIEP of CD34+ cells three days post infection [161].

A number of inhibitors against US28 have also been noted in the literature [469], the most well-studied of these is VUF2274, which is an inverse agonist of US28 signalling [60, 159, 160, 257, 467], that will be used extensively in this chapter.

In this chapter, I will demonstrate one mechanism by which US28 maintains HCMV latency in monocytes and, in particular, I will also describe a solution to the paradox of how US28 could be involved in repressing the MIEP and maintain latency while being a known MIEP activator during lytic infection.

Firstly, I will show that US28 expression is necessary for the maintenance of HCMV latency in CD14+ monocytes, which is in agreement with similar observations in CD34+ cells [161]. Secondly, I will show that this US28 activity is not dependent on US28 binding of chemokines but requires constitutive G protein-coupled signalling. I will also show that signalling by US28 has a completely different profile in undifferentiated monocytic (THP-1) cells compared to differentiated, macrophage-like cells. For instance, in THP-1 cells, US28 attenuates multiple different cell signalling pathways, (including MAP kinase and NFκB signalling), and it is the US28-mediated repression of these signalling pathways which help US28 to repress the MIEP, thereby maintaining latency. Consistent with this, treatment of latently infected cells with a small molecule inhibitor of US28 resulted in permissiveness of monocytes for HCMV lytic infection.

Thirdly, I will show that, despite this activity by US28, there is a secreted factor in latently infected cells which can prevent lytic infection of monocytes by a US28 deletion virus. Finally, I will demonstrate that monocytes infected with HCMV lacking US28, which now express viral IE proteins, are recognised and killed by pre-existing CMV-specific immune cells from HCMV seropositive donors and I will propose that small molecule inhibition of US28 could be a novel immunotherapeutic, "shock-and-kill" approach towards targeting latent HCMV for existing host T cell responses.

4.2 Results: US28 expression is required for HCMV latency in monocytes

4.2.1 US28 is required for HCMV to establish latency in monocytes

Latent infection with HCMV is characterised by the expression of latency-associated genes, little concomitant lytic immediate early (IE) gene expression [136] and the absence of production of infectious virions. As expected, infection of monocytes with the Titan clinical isolate of HCMV (Titan-WT) which has a UL32-GFP tag that is only expressed at late times of lytic infection, resulted in a characteristically latent infection - high levels of expression of viral UL138, a known latency-associated transcript, with little accompanying IE72 or late UL99 RNA (Figure 4.1A). Similarly, these Titan-WT infected cells showed no IE72 protein or late UL32-GFP protein expression (Figure 4.1B, left panels) nor did they produce infectious virions when analysed by co-culture on indicator fibroblasts (Figure 4.1C). In contrast, infection of monocytes with a virus in which US28 had been deleted (Titan-ΔUS28) showed high levels of IE72 RNA and concomitant expression of UL99 RNA, a true late gene transcript (Figure 4.1A) when infected with similar amounts of virus (Figure 4.1D). To confirm the expression of IE72 and late proteins in these cells, I also stained the infected monocytes for IE72 protein and the late gene UL32-GFP protein at seven days post infection and clearly observed the expression of IE and UL32 proteins (Figure 4.1B, right panels). Finally, I co-cultured these monocytes with indicator fibroblasts to quantify any virus release and also observed the presence of infectious virus in monocytes infected with Titan-ΔUS28 virus but not Titan-WT virus (Figure 4.1C). Consistent with viral lytic replication occurring only in Titan-ΔUS28 infected monocytes, an increase in viral genome copies was detected in Titan-ΔUS28 but not Titan-WT infected monocytes seven days post infection (Figure 4.1D).

Taken together, these data argue for a requirement for US28 in either the establishment or maintenance of latent infection of CD14+ monocytes and show that, in the absence of US28, HCMV infection of monocytes results in a full lytic infection.

4.2.2 Lytic infection of monocytes by Titan-ΔUS28 virus does not result from early induction of myeloid differentiation

Having established that infection of CD14+ monocytes with Titan-ΔUS28 resulted in a lytic rather than a latent infection seven days post infection, I wanted to rule out that US28 was simply maintaining a latent infection by actively suppressing myeloid cell differentiation. I

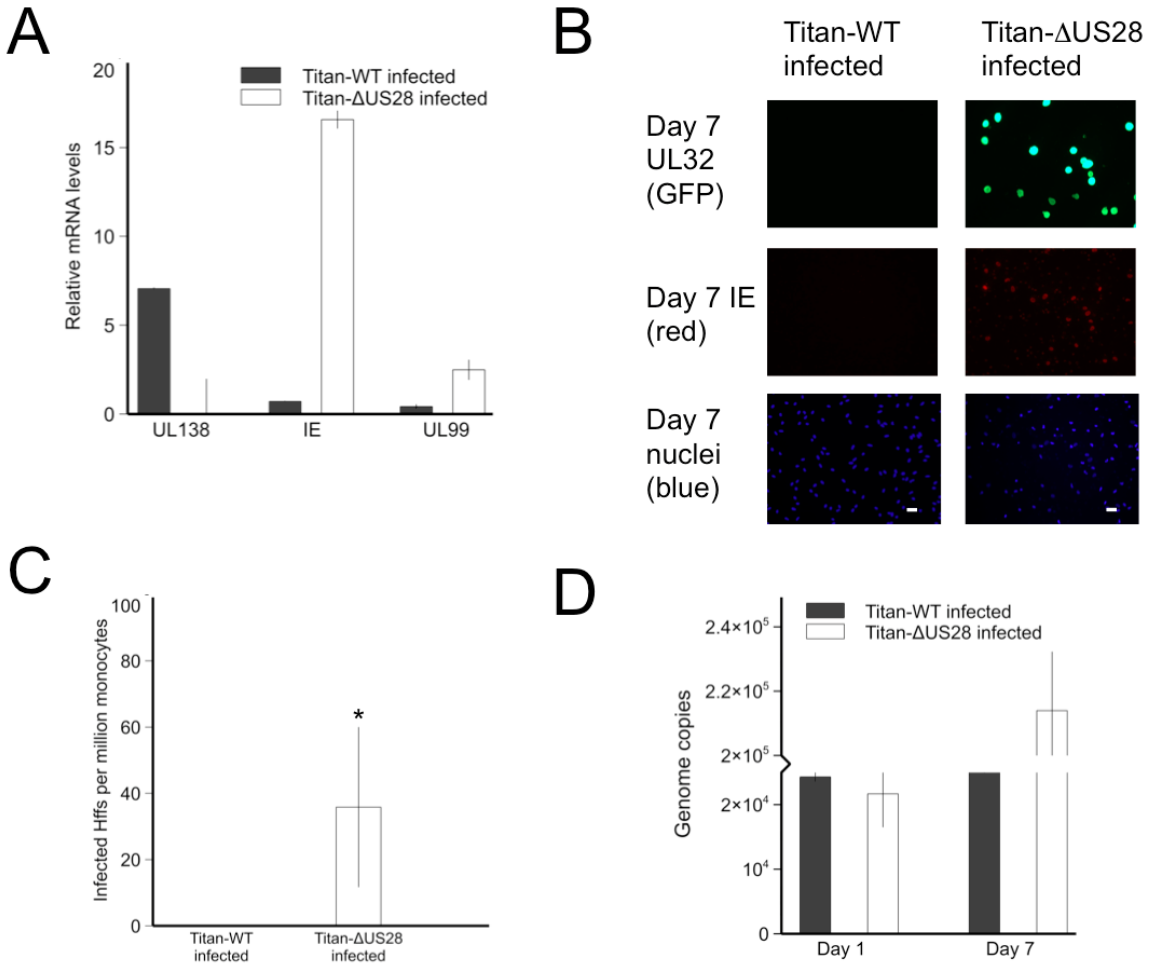

Figure 4.1 Infection of monocytes with Titan-ΔUS28 results in lytic infection. CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with HCMV Titan-WT or Titan-ΔUS28. A) Seven days post-infection, RNA from these cultures was harvested and analysed for expression of the latency-associated gene UL138, the major immediate early lytic gene IE1, and the major late gene UL99. Data were normalised to GAPDH RNA. B) Seven days post-infection, monocytes were also fixed and stained for IE protein and UL32-GFP (using an antibody against the GFP tag). C) These monocyte cultures were then co-cultured with HFFs and the number of infected HFFs was measured, by staining for cells expressing HCMV IE protein, 72 hours post-co-culture. D) Titan-ΔUS28 infected monocytes replicate viral genomes. CD14+ peripheral blood monocytes were isolated from the PBMCs of seropositive donors and experimentally infected at an MOI of 5 with Titan-WT or Titan-ΔUS28. Either 1 day, or 7 days post infection, DNA was harvested from cultures and genomes quantified by qPCR analysis against the MIEP region. All data points show means from at least four independent experiments, error bars show standard deviations and p-values * (p=0.05) were considered significant. White bars indicate 50μM scale.

reasoned that, if it were the case that an absence of US28 simply induces infected monocytes to differentiate and become fully permissive for HCMV lytic infection, there ought to be a temporal delay in the induction of lytic gene expression in monocytes infected with Titan-ΔUS28, to allow time for differentiation to occur. Consequently, I assayed for IE mRNA and protein expression in monocytes infected with Titan-WT or Titan-ΔUS28, but at very early time points post-infection. These analyses clearly showed that substantial levels of lytic IE gene expression were observed as early as 12 hours post-infection of monocytes with Titan-ΔUS28 (Figure 4.2A). I was also able to detect IE protein expression by immunofluorescent (IF) microscopy from as early as 24 hours post-infection (Figure 4.2B) and UL32-GFP from 48 hours post infection (Figure 4.2C). Additionally, supernatants from Titan-ΔUS28 infected monocytes at 3 days post infection, showed the presence of infectious virus on indicator fibroblasts (Figure 4.2D). All these data were entirely consistent with the view that Titan-ΔUS28 infected monocytes immediately undergo a lytic infection with little or no temporal delay and make it unlikely that infection in the absence of US28 simply induced differentiation of monocytes to a cell phenotype that is permissive for lytic infection. In contrast, and as expected, monocytes infected with Titan-WT virus showed no such lytic gene expression profile (Figure 4.2A-C) nor production of infectious virus (Figure 4.2D).

4.3 US28 expression in monocytes attenuates MAP kinase and NFκB signalling

4.3.1 US28 signalling maintains latency independently of chemokine binding

On the basis that US28 appeared to be having a profound effect on the outcome of infection of monocytes, at least in part supporting the establishment of latency, I next decided to assess the effects of US28 expression on monocytic cells in detail. To do this, I used lentiviral vectors to over-express an N-terminally HA-tagged US28 (HA-US28-WT) [208] in isolation in the monocytic THP-1 cell line which I and others have used as a model of latent HCMV infection [7, 27, 190]. At the same time, I also overexpressed two HA-tagged US28 mutants: HA-US28-R129A and HA-US28-Y16F, which have ablated signalling and chemokine binding function, respectively (Figure 4.3A).

I then infected these THP-1 cell lines stably expressing HA-US28-WT, HA-US28-R129A or HA-US28-Y16F proteins, with Titan-ΔUS28 virus, to assess if supplying these US28

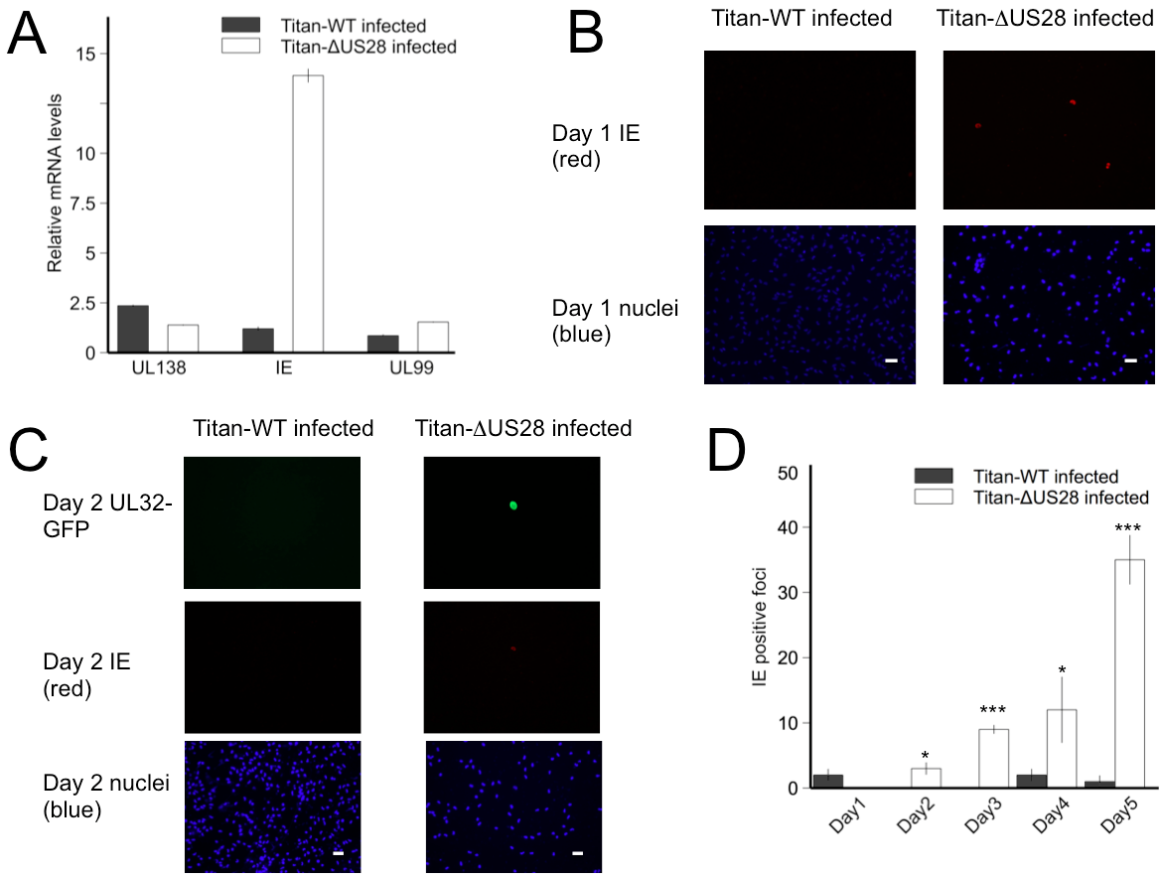

Figure 4.2 Titan-ΔUS28 virus initiates lytic infection immediately after infection of CD14+ monocytes. CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with HCMV Titan-WT or Titan-ΔUS28. A) 12 hours post infection, RNA from these cultures was harvested and analysed by RT-qPCR for the latent gene UL138 and the lytic genes, IE and UL99. Data were normalised to GAPDH RNA. B) One day post-infection, monocytes were fixed and stained for IE protein. C) Two days post-infection, monocytes were fixed and stained for IE or UL32-GFP protein. D) Each day post infection, media was harvested from monocytes infected with Titan-WT or Titan-ΔUS28 and titrated onto indicator HFFs. These were subsequently stained for HCMV IE protein, as a measure of viral titres, 24 hours post infection. All data points show means from at least four independent experiments, error bars show standard deviations and p-values * (p=0.05), *** (p=0.001) were considered significant. White bars indicate 50μM scale.

proteins in trans would affect the ability of Titan-ΔUS28 to undergo a lytic infection in these undifferentiated monocytic cells.

Figure 4.3B shows that, as expected, control THP-1 cells stably transduced with an empty vector underwent lytic infection when infected with Titan-ΔUS28 virus, in that IE and UL32-GFP protein were detectable. In contrast, cells expressing HA-US28-WT complemented the lack of US28 in Titan-ΔUS28 and this resulted in cells negative for IE and UL32-GFP expression - consistent with a latent infection. Interestingly, THP-1 cells expressing the HA-US28-R129A protein failed to complement the Titan-ΔUS28 mutation (these infected cells were IE and UL32-GFP positive), whereas infection of THP-1 cells stably expressing the HA-US28-Y16F mutant also complemented Titan-ΔUS28 and resulted in cells undergoing latent infection (as detected by a lack of IE and UL32-GFP expression) as measured by both IF staining (Figure 4.3B) and RT-qPCR analysis (Figure 4.3C). Also as expected, THP-1 cells infected with Titan-WT showed little lytic gene expression, regardless of expression of any HA-US28 construct (Figure 4.4B).

I also tested whether the failure of these HA-US28 constructs to complement Titan-ΔUS28, which resulted in lytic gene expression, also resulted in production of infectious virus. Figure 4.3D shows that cells in which IE lytic gene expression could be detected also produced infectious virions, as expected.

Finally, I confirmed that the ability of HA-US28-WT and HA-US28-Y16F to complement Titan-ΔUS28 to allow it to establish latent infection, resulted in cells from which HCMV could be reactivated (Figure 4.4A). Taken together, these data suggest that the ability of US28 to suppress lytic infection likely resides in its downstream signalling, via G protein activation, and that this signalling occurs independently from chemokine binding.

4.3.2 US28 suppresses or activates the MIEP depending on differentiation status of the monocytic cell

As US28 signalling appeared to be necessary for the establishment of latency in monocytes, I hypothesised that US28 expression likely negatively regulates the MIEP in undifferentiated monocytic cells. To test this, I used THP-1 cell lines, that had been transduced with an MIEP-eGFP construct [461], and transfected these cells by nucleofection with three HA-US28 constructs, and the empty vector control. Two days post transfection, I measured eGFP expression in these cell lines by flow cytometry. Figure 4.5A shows that, consistent with a role for suppression of lytic infection in undifferentiated THP-1 cells, HA-US28-WT did, indeed, show a repression of MIEP activity as did the HA-US28-Y16F mutant. In

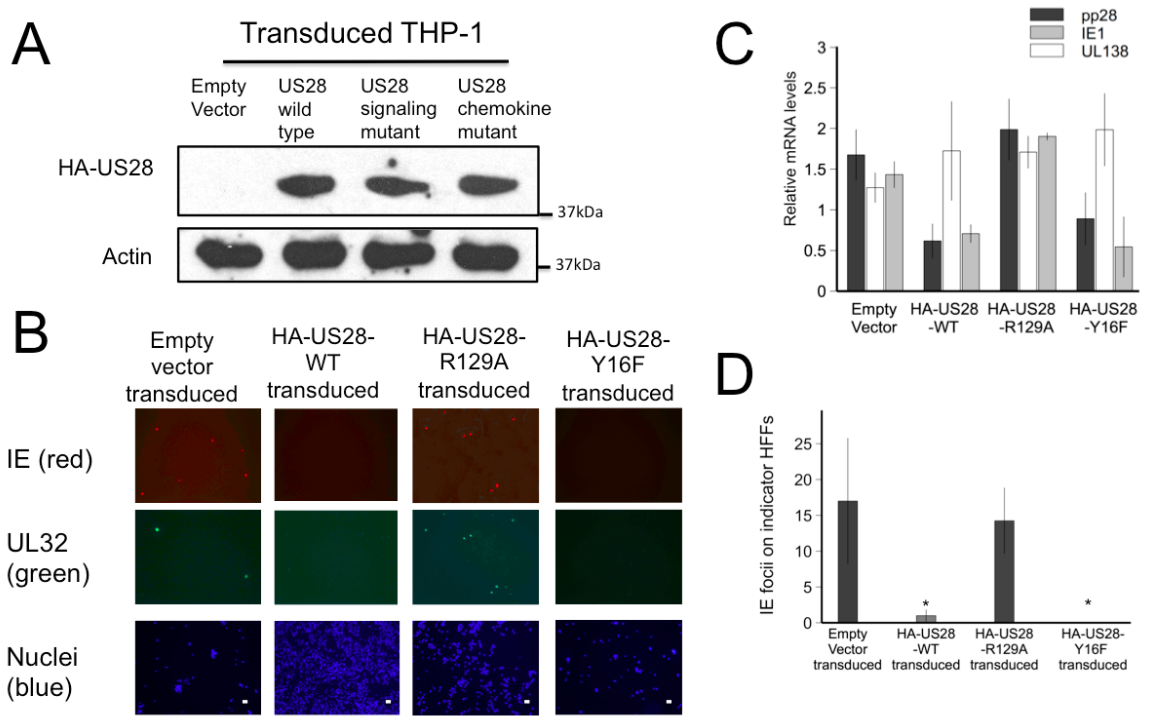

Figure 4.3 Ectopic US28 expression in THP-1 cells can complement for a deletion of US28 from the virus. THP-1 cells stably expressing an N-terminally HA- tagged US28 (HA-US28-WT), US28 with a disrupted G protein binding DRY motif (HA-US28-R129A) and US28 with a disrupted chemokine binding region (HA-US28-Y16F) were generated by lentiviral transduction and puromycin selection. A) Immunoblot analysis using an antibody against the N terminal HA tag was carried out on an empty vector transduced cell line and the three cell lines expressing HA-US28 constructs. B) These THP-1 cells, expressing different HA-US28 constructs and empty vector control cells, were infected with Titan-ΔUS28 at an MOI of 5 and fixed five days post infection. Fixed samples were stained for immediate early or UL32-GFP and nuclei were also stained. C) These THP-1 cells, expressing different HA-US28 constructs and empty vector control cells, were infected with Titan-ΔUS28 at an MOI of 5 and RNA harvested 5 days post infection. RNA was analysed by RT-qPCR for the latent gene UL138 and the lytic genes, IE and UL99. Data were normalised to GAPDH RNA. D) Media from these infected cells was titrated on indicator fibroblasts and the number of infectious virions quantified by IE staining. Data are means from at least three independent experiments, error bars show standard deviations and p-values * (p=0.05) were considered significant. White bars indicate 50μM scale.

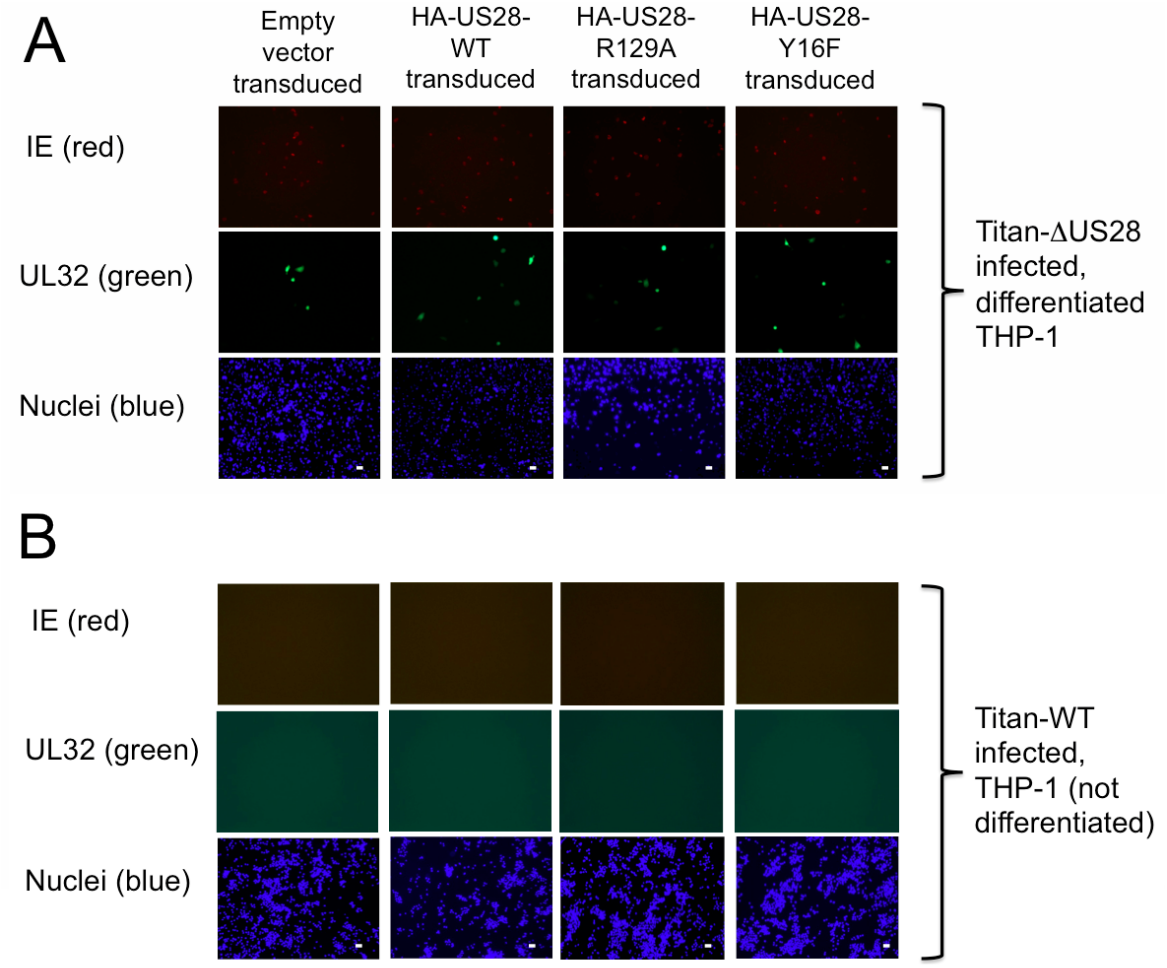

Figure 4.4 Ectopic US28 expression in undifferentiated THP-1 cells does not affect the establishment of latency when infected with Titan-WT virus or reactivation of Titan-ΔUS28 virus in differentiated THP-1 cells. THP-1 cells stably expressing HA-US28-WT, HA-US28-R129A or HA-US28-Y16F (see Figure 4.3), were infected with Titan-ΔUS28 before being differentiated by PMA treatment and fixed and stained five days post treatment (A), or were infected with Titan-WT at an MOI of 5 for five days before fixing and staining (B). Fixed samples were stained for immediate early (red) or UL32-GFP (green) and nuclei were also stained. White bars indicate 50μM scale.

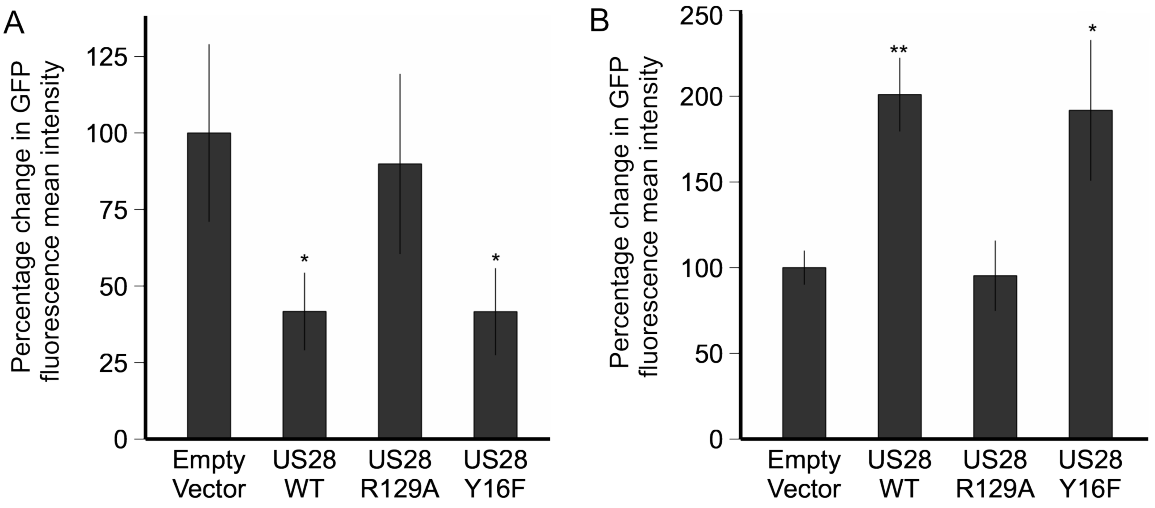

Figure 4.5 US28 represses the MIEP in undifferentiated myeloid cell lines, but activates it in differentiated myeloid cells. A) THP-1 cells which had been transduced with an MIEP-eGFP construct were then transfected by nucleofection with US28-WT, US28-R129A or US28-Y16F constructs. Three days after nucleofection, cells were analysed for eGFP expression by flow cytometry. B) Additionally, cells were treated with PMA two days after nucleofection and, two days after PMA treatment, analysed for eGFP expression by flow cytometry. Data shows percentage change in mean fluorescent intensities from four experimental replicates, after selecting for single cells and excluding dead cells using Zombie red dye. Error bars show standard deviations and p values * = 0.05 and ** =0.01 were calculated using Student's t-test and were considered significant.

contrast, the HA-US28-R129A signalling mutant showed no such repression. I also repeated this analysis but, two days after nucleofection with the HA-US28 constructs and empty vector control, I differentiated the THP-1 cells with PMA (Figure 4.5B). In contrast to the undifferentiated THP-1 cells, HA-US28-WT and the HA-US28-Y16F now activated the MIEP whereas HA-US28-R129A expression had no significant effect on MIEP activity. These data confirm that the effect of US28 on IE gene expression is differentiation-dependent; US28 appears to repress the MIEP in undifferentiated monocytic cells, consistent with a role of US28 in maintaining latency, but activates the MIEP after cellular differentiation, likely to promote lytic infection.

4.3.3 US28 attenuates MAP kinase and NFκB cell signalling pathways

US28 expression during lytic infection is known to activate a number of cell signalling pathways, including the NFκB and MAP kinase pathways, both of which are known to

activate the MIEP in fully permissive cells. To analyse the potential effect of US28 on such signalling during latent infection, I used phosphokinase antibody arrays to assess how US28-mediated changes in the levels of phosphorylation on an array of different cellular signalling proteins (Figure 4.6). Specifically, I compared THP-1 cells expressing either HA-US28-WT or the HA-US28-R129A signalling mutant; I reasoned that comparing HA-US28-WT to the HA-US28-R129A signalling mutant would control for potential non-specific effects of US28 protein overexpression. These analyses show that HA-US28-WT expression specifically decreased the phosphorylation levels of a number of cellular proteins, suggesting a general attenuation of cell signalling pathways by wild type US28 in undifferentiated monocytic cells. In particular, I noted significant reductions in phosphorylation levels of several signalling proteins, chief among them being ERK1/2 of the MAP kinase pathway (Figure 4.6).

My result, showing that US28 mediated suppression of MIEP activity and also inhibited ERK1/2, fits well with the view that MAP kinase signalling is likely involved in HCMV reactivation [189]. Consequently, I validated the results of the phosphokinase array by performing immunoblotting analysis on three cellular proteins that are key to the MAP kinase signalling pathway: ERK1/2, MSK-1 and CREB. Figure 4.7A and B (left hand panels), show that all three proteins were hypo-phosphorylated in THP-1 cells expressing HA-US28-WT compared to control cells expressing HA-US28-R129A. As US28 is associated with activation of MAP kinase during lytic infection, I also repeated this analysis in these THP-1 cells after they had been differentiated to a macrophage-like phenotype (which is permissive to HCMV lytic infection) by PMA treatment [322, 405, 408, 440]. Figure 4.7 shows that over expression of US28 in these differentiated cells now showed the opposite effect on ERK1/2, MSK-1 and CREB phosphorylation; HA-US28-WT protein resulted in their hyperphosphorylation, compared to cells expressing HA-US28-R129A (Figure 4.7A and B).

Finally, I performed nuclear/cytoplasmic fractionation followed by immunoblotting to analyse the effect of US28 on NFκB activation (Figure 4.7C). These analyses showed that the NFκB pathway is also attenuated by US28 in undifferentiated monocytic THP-1 cells in that, in contrast to HA-US28-R129A or control vector, US28 expression resulted in a relative lack of nuclear localisation of p65 (Figure 4.7C, left hand panels). Whereas, as predicted, this was reversed in differentiated THP-1 cells (Figure 4.7C, right hand panels).

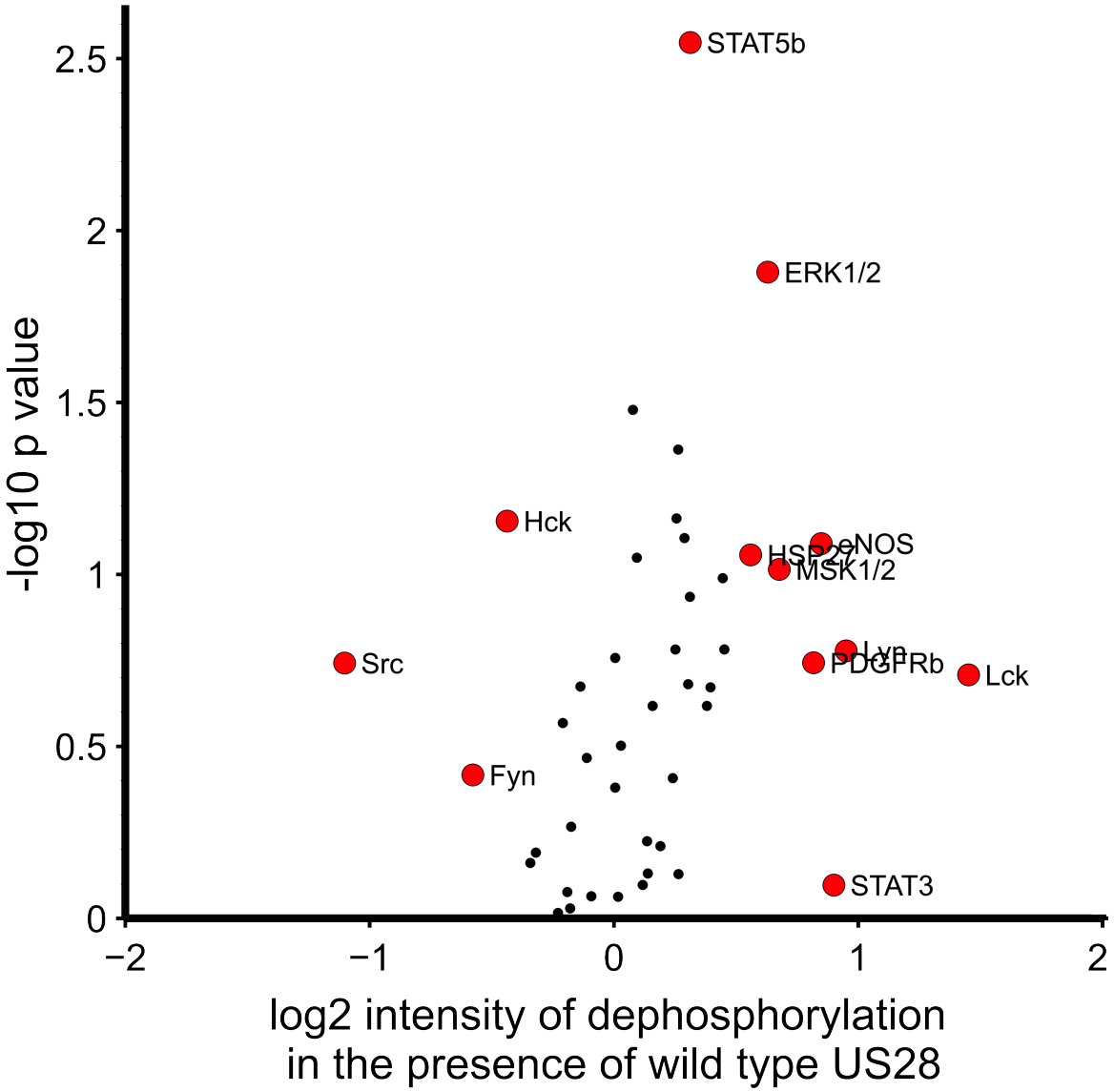

Figure 4.6 US28 expression, in isolation, in THP-1 cells attenuates cellular signalling. THP-1 cells which had been induced to express either US28-WT or US28-R129A (which cannot maintain latency) were lysed and analysed for changes in cellular kinase phosphorylation levels by antibody array. Data are represented as a volcano plot, with log2 fold change in dephosphorylation of each kinase from THP-1 cells expressing US28-WT over the levels induced by THP-1 cells expressing US28-R129A. Data points in red had a change in intensity of +/- log2(0.5) arbitrary units and/or a p value greater than log10(1.5).

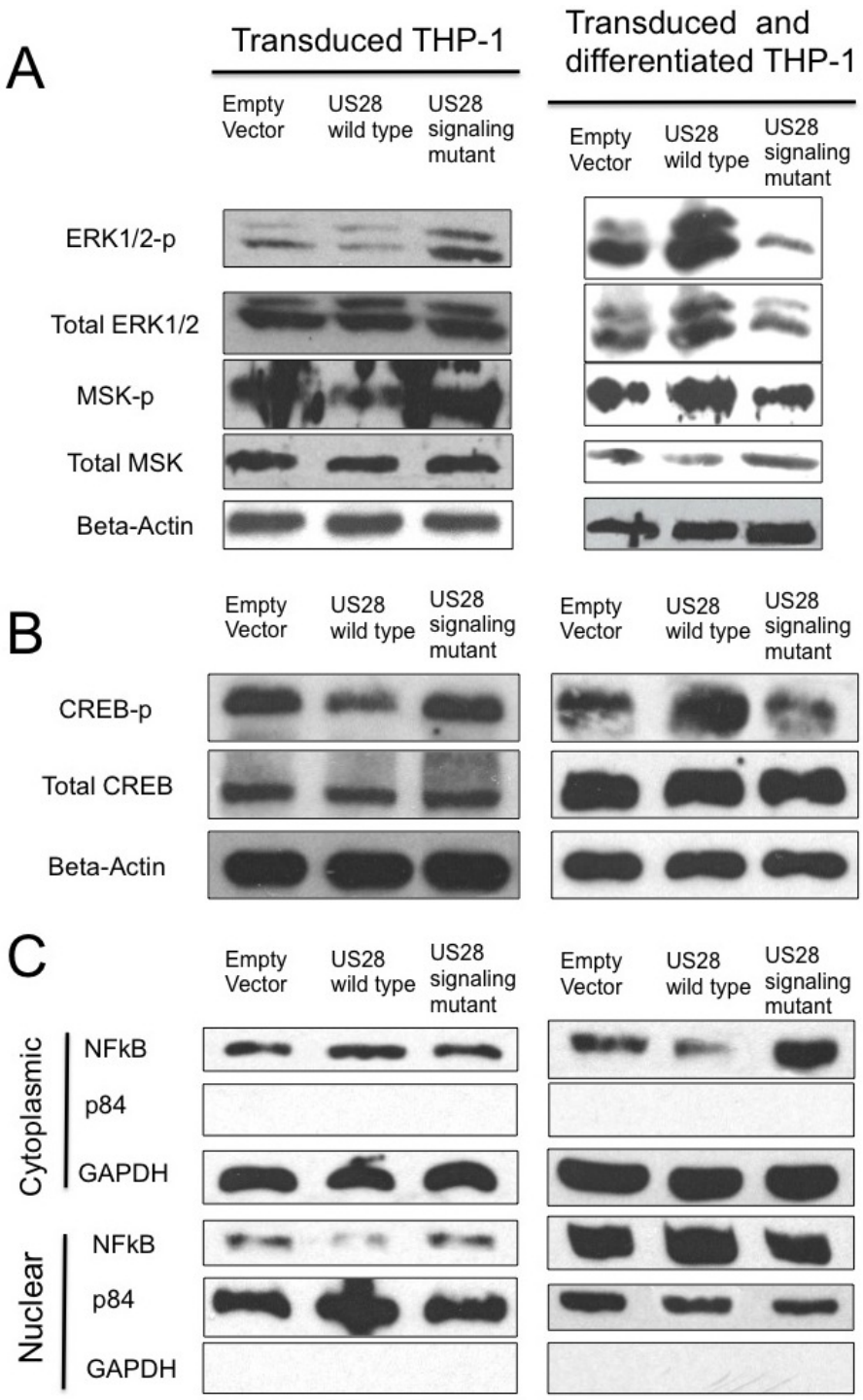

Figure 4.7 US28 expression, in THP-1 cells, in isolation, attenuates MAP kinase and NFκB cellular signalling.

Figure 4.7 US28 expression, in THP-1 cells, in isolation, attenuates MAP kinase and NFκB cellular signalling. A) THP-1 cells expressing either HA-US28-WT or HA-US28-R129A, (which cannot maintain latency) or an empty vector control were lysed and analysed by immunoblot for phospho- and total MSK-1; phospho- and total ERK1/2 and beta-actin (left panels). THP-1 cells were also differentiated with PMA treatment and, four days post treatment, were analysed by immunoblot for phospho- and total MSK-1; phospho- and total ERK1/2 and beta-actin (right panels). B) The same analysis was performed for phospho- and total CREB in undifferentiated cells (left panels) or four days after differentiation with PMA treatment (right panels). C) Cells were also fractionated into nuclear and cytoplasmic fractions and these fractions were analysed by immunoblot analysis for NFκB protein (p65). The nuclear protein p84 and the cytoplasmic protein GAPDH were used as loading and fractionation controls for the respective fractions. This analysis was performed in undifferentiated THP-1 cells (left panels) or four days after PMA-induced differentiation (right panels).

4.3.4 Inhibition of MAP kinase and NFκB cell signalling pathways can reduce lytic infection of monocytes by Titan-ΔUS28 virus

On the basis that US28 expression, in isolation in undifferentiated myeloid cells, attenuates MAP kinase and NFκB signalling pathways and that this correlates with the suppression of the MIEP and the ability of HCMV to establish latency, I reasoned that I could mimic the action of US28 in undifferentiated myeloid cells by inhibiting MAP kinase and/or NFκB signalling in blood-derived CD14+ monocytes, in the context of HCMV infection. I also reasoned that I could compel Titan-ΔUS28 virus to establish latency in monocytes by pretreating these monocytes with inhibitors of either MSK-1 or IKKα (H89 and BAY11-7082, respectively). To address this, I treated cells with an increasing concentation of inhibitors prior to infection with Titan-ΔUS28 and then measured the number of UL32-GFP-positive cells three days post infection, as an indicator of full, lytic infection. Figures 4.8A and B show that neither inhibitor alone was able to prevent Titan-ΔUS28 virus from undergoing lytic infection. However, infection in the presence of both inhibitors together did, indeed, lead to an absence of UL32-GFP gene expression in Titan-ΔUS28 infected monocytes in a dose-dependent manner and this could not be attributed to non-specific cell toxicity effects (Figure 4.8C).

My observations in Figure 4.2 that Titan-ΔUS28 virus initiated IE expression in infected monocytes 6-12 hours post infection also suggested that US28 may be required at very early times of HCMV infection of monocytes to suppress the MIEP and help establish latency. Therefore, I hypothesised that US28 protein expression likely blocks the signals activating

the MIEP in monocytes, during infection with Titan-ΔUS28, which could occur due to virus binding or internalisation. In order to test for this, I delayed inhibitor treatment of monocytes infected with Titan-ΔUS28 until either one hour or one day post infection, assuming that any delay in inhibition of MAP kinase and NFκB, would lead to the activation of the MIEP as a result of virus binding (for example) and/or entry, and that this MIEP activation would no longer be inhibitable if the MIEP activity was already fully established. Treatment of monocytes with H89 and BAY11-7082, one hour post infection, was still effective at blocking lytic infection by Titan-ΔUS28. However, if drug treatment was delayed until one day post infection, these inhibitors were no longer as effective at blocking lytic infection (Figures 4.8D and E). These data suggest that signals triggered within the first 24 hours of infection of monocytes by HCMV activate MIEP activity but that US28 then attenuates these signals to suppress IE expression in order to establish latent infection.

I was, however, concerned that treatment with these two inhibitors of MAP kinase and NFκB was not forcing Titan-ΔUS28 virus into latent infection, but simply repressing lytic gene expression leading to a lack of any infection at all. To test this, I measured UL138 expression from monocytes infected with Titan-ΔUS28 but treated with MAP kinase and NFκB inhibitors, as UL138 is expressed during latency. Figure 4.8F, shows that UL138 is expressed and, therefore, that treatment with these inhibitors did, indeed, induce *bona fide* latent infection.

4.3.5 The US28 inhibitor VUF2274 can induce lytic infection in monocytes infected with wild type HCMV

VUF2274 (BX 513 hydrochloride) is an antagonist of CCR1 and an inverse agonist of US28 [60]. Given that my results, so far, had shown that US28 signalling was required to help establish latency in monocytes, I predicted that treatment of monocytes, infected with wild-type HCMV, with VUF2274 would trigger viral lytic gene expression and, possibly, virus reactivation. To test this, I treated monocytes, which I had latently infected for three days with SV40-GFP-TB40E, with VUF2274 and quantified IE protein expression, by IF staining, two days after drug treatment (Figure 4.9A). I also quantified any production of infectious virus in cultures treated with VUF2274 by removing media from monocytes three days post drug treatment and titrating this media onto indicator fibroblasts (Figure 4.9B). As predicted, VUF2274 did induce IE gene expression (Figure 4.9A), and resulted in measurable release of virus from these reactivated cells (Figure 4.9B). Consistent with this, equivalent experiments using infection with Titan-WT, which has a UL32-GFP tag, also confirmed late

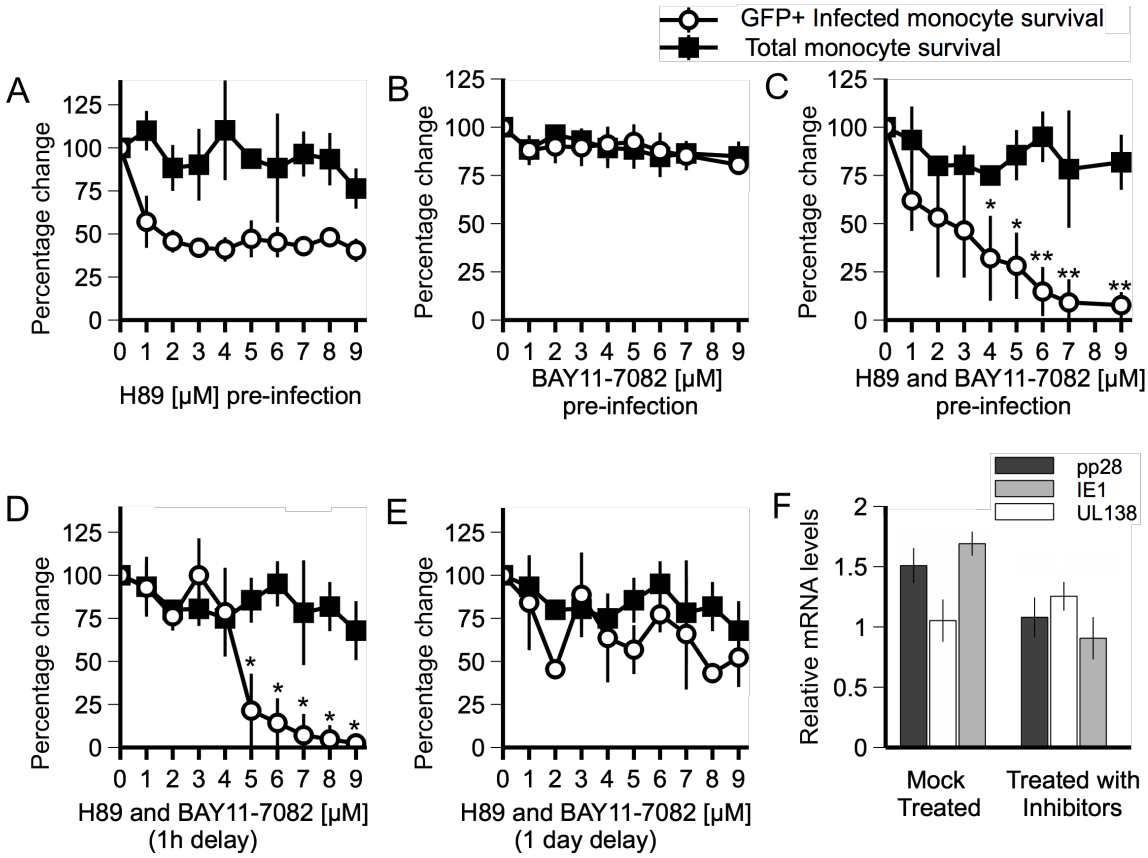

Figure 4.8 Inhibition of MAP kinase and NFκB pathways can prevent lytic infection of monocytes by Titan-ΔUS28. A) CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-ΔUS28 in the presence of an increasing concentration of H89 (an inhibitor of MSK-1). Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. B) Monocytes were infected with Titan-ΔUS28 in the presence of BAY11-7082 (an inhibitor of IKKα). Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. C) Monocytes were treated with both H89 and BAY11-7082 and then infected with Titan-ΔUS28 in the presence of both inhibitors. Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. D) Monocytes were infected with Titan-ΔUS28 but treatment with H89 and BAY11-7082 was delayed until one hour post-infection. Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining.

Figure 4.8 Inhibition of MAP kinase and NFκB pathways can prevent lytic infection of monocytes by Titan-ΔUS28. E) Monocytes were infected with Titan-ΔUS28 but treatment with H89 and BAY11-7082 was delayed until one day post-infection. Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. F) CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-ΔUS28 in the presence of 5μM H89 and 5μM BAY11-7082, three days post infection, RNA was harvested from these cells and analysed for expression of the latency-associated gene UL138, the major immediate early lytic gene IE1, and the major late gene UL99. Data were normalised to GAPDH RNA. All data points show means from at least three independent experiments, error bars show standard deviations, data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, and p-values * (p=0.05) and ** (p=0.01) were considered significant.

gene expression (UL32) in these VUF2274 treated cells (Figure 4.9C). Taken together, these data argue that inhibition of US28 signalling by VUF2274 appears to reactivate full lytic gene expression and lytic infection in monocytes latently infected with wild type HCMV. It should be pointed out, however, that VUF2274 did show significant toxicity (as measured by trypan blue staining) towards primary blood monocytes (Figure 4.9D), even at concentrations below an approximate Ki value of 10μM.

4.4 The HCMV latent secretome can repress lytic infection by Titan-ΔUS28 in CD14+ monocytes

As well as modulating cellular signalling, US28 is also known to modulate cellular chemokine secretion, in particular inducing secretion of VEGF and IL-6 during lytic infection [251, 252, 397]; the latter of which has been linked to HCMV reactivation from latency [217, 344]. Analysis of the HCMV latency-associated secretome from CD34+ cells has also previously highlighted TGF-β as a cellular factor that is secreted during latency and promotes suppression of the MIEP [207, 248]. I, therefore, wanted to see if there were any secreted factors associated with US28 expression during latency which could have helped to maintain latent infection in monocytes, or conversely, any factors that induced lytic infection were repressed by US28 expression.

To test this, I infected monocytes with either Titan-WT or Titan-ΔUS28. Three days post infection, I then took the media from these infected monocytes and used it to culture fresh monocytes. I then infected these fresh monocytes with Titan-WT or Titan-ΔUS28,

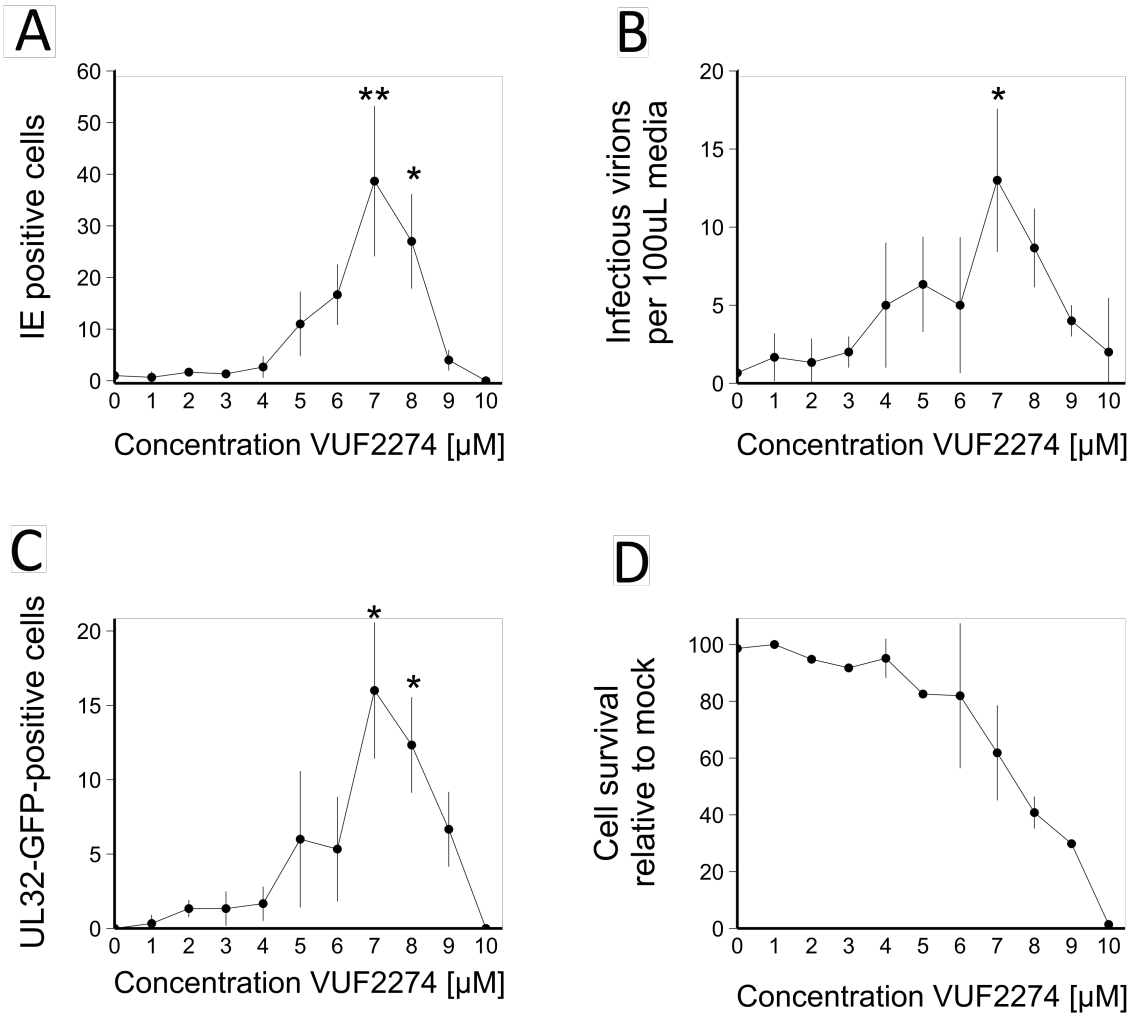

Figure 4.9 VUF2274 is able to induce reactivation of HCMV from latently infected monocytes. CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with SV40-GFP-TB40E. Three days post infection, an increasing concentration of the US28 inhibitor, VUF2274 was added to cells. A) Cells were stained, 48 hours post treatment, for IE protein, and IE positive cells were counted by immunofluorescent microscopy. B) 3 days post drug treatment, media was removed from these cells and titrated onto HFFs. 24 hours post infection, HFFs were fixed and stained for IE protein, and IE positive cells were counted by immunofluorescence microscopy. C) CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-WT. Three days post infection, an increasing concentration of the US28 inhibitor, VUF2274 was added to cells. Three days post-drug treatment, UL32-GFP positive cells were counted by fluorescence microscopy. D) Cell survival of monocytes in the presence of VUF2274 was measured using trypan blue exclusion staining. All data points show means from at least three independent experiments and error bars show standard deviations, data were analysed by ANOVA followed by Tukey's post hoc test, and p-values * (p=0.05) and ** (p=0.01) were considered significant.

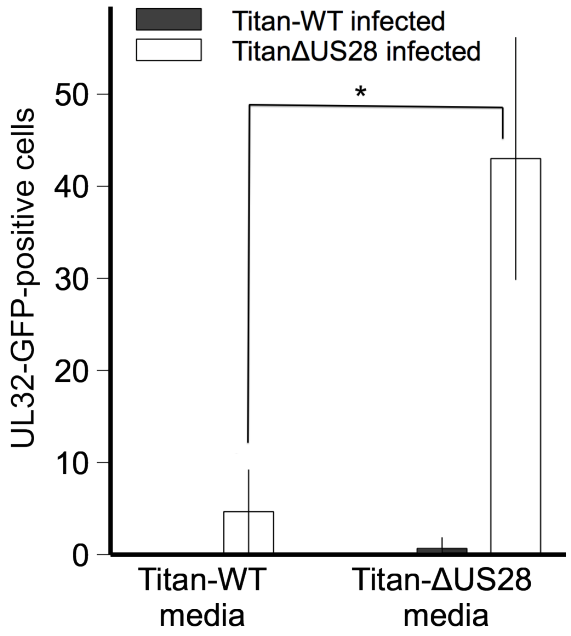

Figure 4.10 Media from monocytes infected with Titan-WT can repress lytic infection of monocytes by Titan-ΔUS28. CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-WT or Titan-ΔUS28. Three days post infection, this media was harvested and fresh CD14+ monocytes were infected, at an MOI of 5, with Titan-WT or Titan-ΔUS28, in the presence of this media (detailed on the x-axis). Three days after this second infection, UL32-GFP positive cells were counted as a measure of lytic infection. All data points show means from three independent experiments, error bars show standard deviations and p-values * (p=0.05) were considered significant.

maintaining the presence of media from the original cultures, throughout and after infection. Three days after this second infection, I counted the number of UL32-GFP positive cells, as a measure of lytic infection. I reasoned that any factors secreted by Titan-WT, to maintain latency, would therefore block lytic infection of monocytes by Titan-ΔUS28, or vice versa. As Figure 4.10 shows, media from Titan-WT infected monocytes was able to greatly reduce the number of UL32-GFP-positive monocytes after infection with Titan-ΔUS28, however media from Titan-ΔUS28 infected monocytes had no effect towards promoting lytic infection in monocytes infected with Titan-WT. I, therefore, concluded that there is likely to be a factor in the media secreted from monocytes latently infected with Titan-WT that promotes latency and is able to suppress lytic infection of monocytes by Titan-ΔUS28.

Given that I had identified that media from monocytes, infected with Titan-WT, can suppress lytic infection of monocytes with Titan-ΔUS28, I wanted to identify the secreted factor(s) that mediate this effect. To do so, I infected CD14+ monocytes with Titan-WT or

Titan-ΔUS28, and seven days post infection I harvested the media from these monocytes and performed a chemokine array in order to identify changes in factors that are secreted by monocytes latently infected with Titan-WT or lytically infected with Titan-ΔUS28. As a positive control, I also included monocytes treated with UV-inactivated virus which is known to have a different cytokine expression profile to latently infected monocytes [248]. As expected, latent infection of monocytes with Titan-WT virus initiated secretion of MCP-1, which agreed with analysis in latency associated with the secretome of CD34+ cells (Figure 4.11A) [248]. However, surprisingly, I was unable to detect any significant changes between the chemokines secreted by monocytes latently infected with Titan-WT and lytically infected with Titan-ΔUS28 (Figure 4.11B).

As I was surprised not to find significant changes in cytokine secretion between monocytes latently infected with Titan-WT and lytically infected with Titan-ΔUS28, I also compared cytokine secretion between THP-1 cells expressing HA-US28-WT, HA-US28-R129A and HA-US28-Y16F, in order to compare the effects of US28 expression in isolation in monocytic cells, on cytokine secretion. Comparing cells expressing HA-US28-WT and its chemokine binding mutant HA-US28-Y16F, I was able to measure changes in cytokine levels that were likely due to US28 binding and internalisation; reassuringly, RANTES and fractalkine, two known ligands of US28, were among the chemokines that showed major differences between HA-US28-WT and HA-US28-Y16F expressing THP-1 cells (Figure 4.11C). I then compared media from THP-1 cells expressing HA-US28-WT and HA-US28-R129A, and excluded any chemokines which changed between HA-US28-WT and HA-US28-Y16F, as these were likely ligands of US28. After doing this, there remained five chemokines which changed greater than two-fold between HA-US28-WT and HA-US28-R129A expressing THP-1 cells, and with p-values below p=0.05 (Figure 4.11D). These were: interleukin 1-β, hepatocyte growth factor (HGF), Granulocyte colony-stimulating factor (G-CSF), Osteoprotegerin (OPG) and a soluble form of IL6 receptor (IL6-R). Due to time constraints, these chemokines have not yet been pursued further.

4.5 Monocytes infected with Titan-ΔUS28 virus are targets for killing by pre-existing HCMV-specific donor cytotoxic T cells

After primary infection, it is well established that healthy HCMV carriers maintain extremely high frequencies of HCMV-specific CD8+ cytotoxic T cell (CTLs) in their peripheral blood,

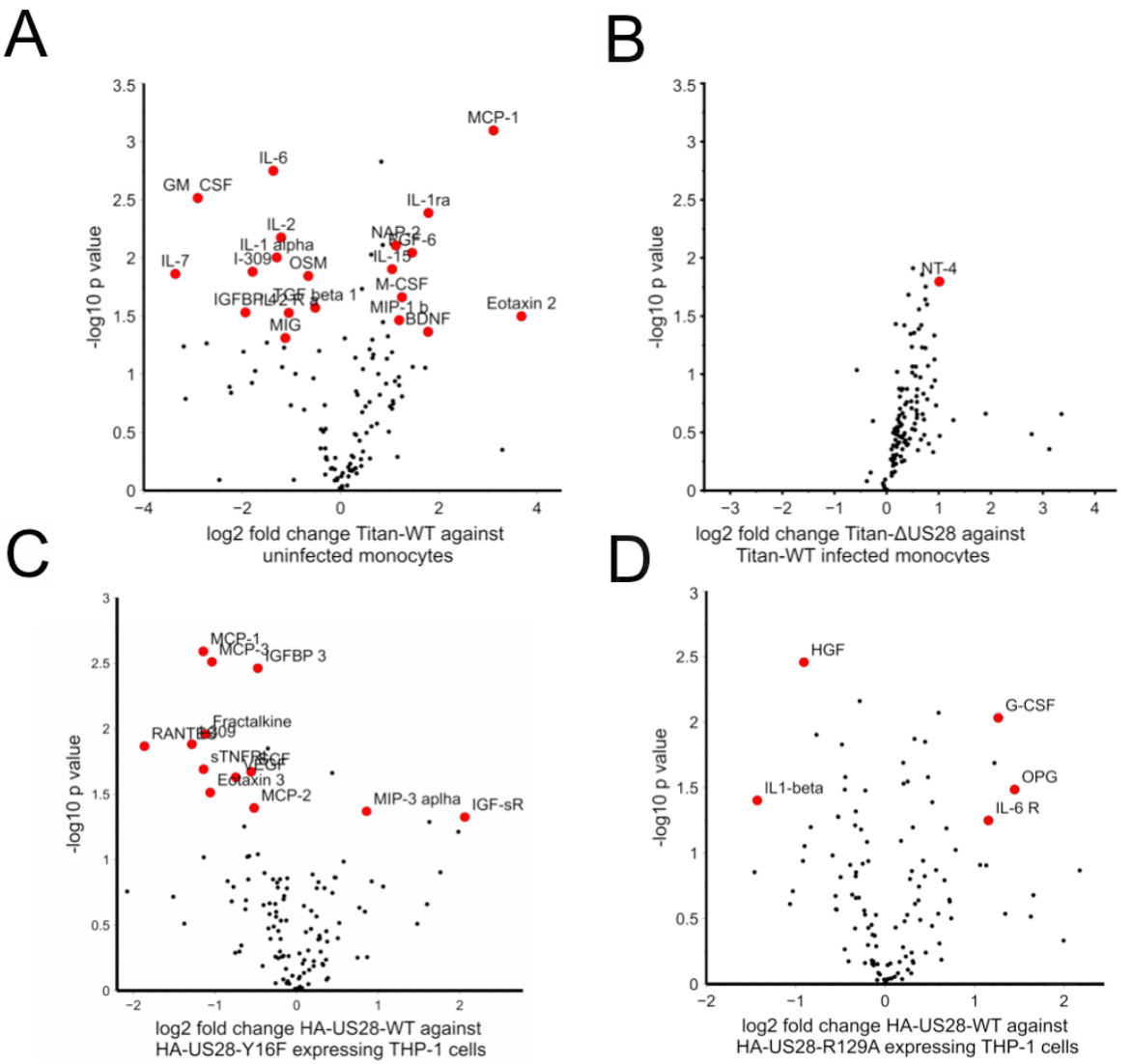

Figure 4.11 Although US28 expression in isolation induced changes in THP-1 cytokine secretion, there was no significant change in cytokine secretion between Titan-WT and Titan-ΔUS28 infected monocytes. A and B) CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-WT or Titan-ΔUS28. Six days post infection, these cultures were washed in PBS and then the following day, the media from these cells was harvested, clarified by centrifugation, and then analysed for secreted cytokines using an antibody array against human cytokines. Titan-WT infected monocytes were compared to monocytes treated with UV-inactivated virus (A), or monocytes infected with Titan-WT were compared to monocytes infected with Titan-ΔUS28 (B). C and D) THP-1 cells expressing HA-US28-WT, HA-US28-R129A or HA-US28-Y16F were generated by lentiviral transduction (see Figure 4.3). Cells were washed as before and the next day media from these THP-1 cells was harvested, clarified by centrifugation, and then analysed for secreted cytokines using an antibody array against human cytokines.

Figure 4.11 Although US28 expression in isolation induced changes in THP-1 cytokine secretion, there was no significant change in cytokine secretion between Titan-WT and Titan-ΔUS28 infected monocytes. C) THP-1 cells expressing HA-US28-WT compared to the levels for THP-1 cells expressing HA-US28-Y16F, a chemokine binding mutant. D) THP-1 cells expressing HA-US28-WT compared to the levels induced by THP-1 cells expressing HA-US28-R129A, a signalling mutant. For each scatter plot, data on the x-axis represent mean change in intensity of each cytokine blot on the array and the y-axis shows the p-values generated by two technical repeats.

which are often dominated by CTLs which recognise IE antigens; up to 10% of effector memory CD8+ CTLs can recognise IE72 in some donors [167, 191, 432]. However, because latently infected cells do not express these lytic antigens, latently infected cells likely escape these HCMV-specific CTL responses. I, therefore, reasoned that the inability of Titan-ΔUS28 infected monocytes to undergo latent infection, and their resulting high level of lytic gene expression, should make them targets for pre-existing HCMV specific CTLs in the peripheral blood of healthy HCMV carriers. Similarly, the same would be observed in monocytes latently infected with wild-type virus if US28 were inhibited by VUF2274. Figure 4.12A, shows that co-culture of Titan-ΔUS28-infected monocytes with donor-matched IE72-specific T cell clones resulted in a reduction in the frequency of reactivation of latently infected cells from these infected monocytes after their differentiation and maturation to mature dendritic cells (mDCs). As expected, I also found that treatment with IE-specific T cells resulted in a reduction in virus release from monocytes infected with Titan-ΔUS28, due to killing of these lytically infected cells in the absence of differentiation and maturation (Figure 4.12B). I also repeated this analysis with donor-matched total PBMCs, instead of IE72-specific T cell clones, and saw similar reductions in virus production from monocytes infected with Titan-ΔUS28 compared to those infected with Titan-WT after co-culture with HCMV specific T cells and subsequent differentiation and maturation to reactivate any surviving latently infected cells (Figure 4.12C).

To confirm that these observations were a result of T cell killing rather than, for instance, repression of virus production, I also incubated monocytes infected with Titan-WT or Titan-ΔUS28 with isolated CD8+ T cells from matched donors and Dr Sarah Jackson, in Dr Mark Wills' laboratory, analysed T cell specific increases in two markers of T cell activation, CD69 and 4-1BB, by flow cytometry, to assay for CMV specific CD8+ T cell responses. The CD8+ T cells exposed to Titan-ΔUS28 infected monocytes were more activated than those exposed to monocytes latently infected with Titan-WT (Figure 4.12D). As a read

out of the functional capacity of these CMV specific CD8+ T cells, we also analysed their production of the chemokines IFN-γ and TNF-α as well as upregulation of the degranulation marker CD107a and expression of Granzymes A, B and K. Figure 4.12E shows that, using these functional effector markers, CD8+ T cells clearly recognised Titan-ΔUS28 infected monocytes but not Titan-WT infected monocytes, and that the expression of CD107a as well as the production of Granzymes, IFN-γ and TNF-α by CMV specific CD8+ T cells in response to the Titan-ΔUS28 virus were equivalent to levels seen in response to the positive control monocytes which had been infected with Titan-WT virus and pulsed with IE1/2 peptides as positive control targets for CMV specific CD8+ T cell responses. These data, taken together, argue that monocytes infected with Titan-ΔUS28 are robustly detected by pre-existing CD8+ T cells in HCMV seropositive donors.

Finally, I tested whether treatment of latently infected cells with VUF2274 also made them targets for IE72-specific CTLs. Figure 4.12F shows that, consistent with previous analyses [208, 217, 293], monocytes latently infected with SV40-GFP-TB40E were detectable as GFP+ cells, and their numbers remained relatively constant when cultured with IE72-specific CTLs. In contrast, when these latently infected monocytes (GFP+ cells) were treated with VUF2274 and IE72-specific CTLs, a steady loss of latently infected cell number was observed (Figure 4.12F). Unfortunately, due to the long-term toxicity of VUF2274, I was not able to show that this also resulted in a subsequent reduction in reactivation events after monocyte differentiation and maturation to mDCs.

Taken together, these data suggest that the pre-existing CTL response to HCMV, in healthy carriers, is able to target and kill monocytes infected with Titan-ΔUS28 and the treatment of latently infected monocytes with the US28 inhibitor, VUF2274, also makes them novel CTL targets. On this basis, I suggest that inhibition of US28 with, for instance, small molecule inhibitors could result in untimely reactivation of latent virus and allow their targeting by pre-existing HCMV-specific host T cell responses.

4.6 Discussion

HCMV latency and reactivation pose a significant clinical threat to immunosuppressed transplant recipients and other immunocompromised individuals [434]. However, currently, there are only a few published strategies to treat HCMV latency [207, 208, 483]. HCMV establishes latent infection in early myeloid lineage cells [391], where its latent life-cycle is characterised by expression of only a small subset of viral genes, independent of viral IE gene expression. This includes expression of the viral chemokine receptor homologue *US28*

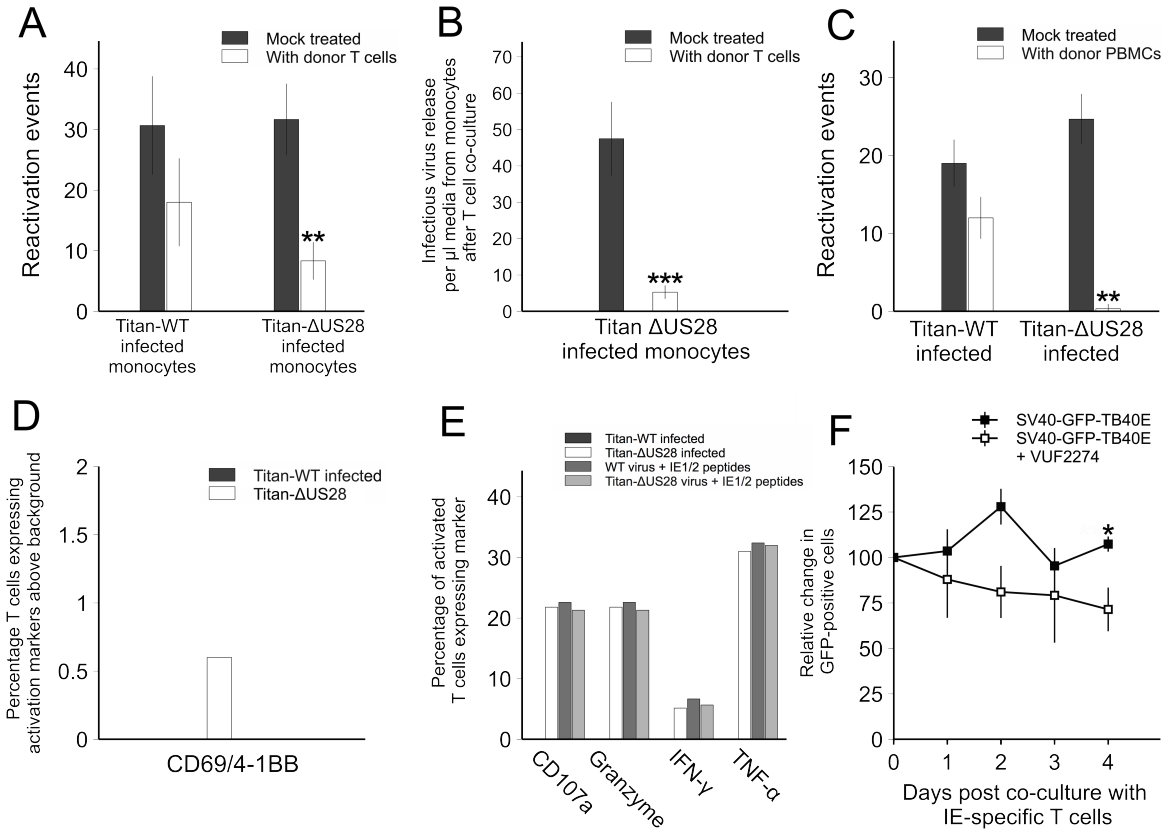

Figure 4.12 Monocytes infected with Titan-ΔUS28, or monocytes infected with SV40-GFP-TB40E in the presence of US28 inhibitors, are targets for HCMV-specific T cell responses. A) CD14+ peripheral blood monocytes from an HLA-A2-positive donor were infected with either Titan-WT or Titan-ΔUS28 at an MOI of 5. Three days post infection, monocytes were co-cultured with IE72-specific T cells for a further three days. After this, monocytes were washed to remove T cells and were then differentiated and matured into dendritic cells by cytokine treatment to trigger virus reactivation. After this, monocytes were co-cultured on indicator fibroblasts for 7 days and then stained for IE protein-positive foci to quantify reactivation events. All data points show means from at least three independent experiments and error bars show standard deviations. B) Monocytes from an HLA-A2-positive donor were infected with either Titan-WT or Titan-ΔUS28 at an MOI of 5. Three days post infection monocytes were co-cultured with IE72-specific T cells for three days. After this, media was removed from all cultures and titrated onto HFFs to quantify virus release from these infected monocytes in the presence or absence of IE-specific T cells. All data points show means from at least three independent experiments, error bars show standard deviations.

Figure 4.12 Monocytes infected with Titan-ΔUS28, or monocytes infected with SV40-GFP-TB40E in the presence of US28 inhibitors, are targets for HCMV-specific T cell responses. C) PBMCs from seropositive donors were sorted into CD14+ monocytes and non-monocyte populations of cells. The isolated adherent monocytes were then experimentally infected at an MOI of 5 with Titan-WT or Titan-ΔUS28. Three days post infection, the non-monocyte PBMCs were added back to the infected monocytes for four days and then removed by washing. The remaining adherent monocytes were then differentiated and matured to induce virus reactivation. Reactivated virus was quantified by fibroblast co-culture and staining for IE foci. Standard deviations are shown and statistical significance was determined using the Student's t-test, where p-values ** (p=0.01), *** (p=0.001) were considered significant. All data points show means from at least three independent experiments, error bars show standard deviations. D) CD14+ peripheral blood monocytes and CD8+ T cells were isolated from the PBMCs of seropositive donors and the monocytes were experimentally infected at an MOI of 5 with Titan-WT or Titan-ΔUS28. Five days post infection, latent monocytes were left untreated or co-cultured overnight with isolated CD8+ T cells from the same donor and anti-CD107a antibody. The CD8+ T cells were then analysed for expression of the activation markers CD69 and 4-1BB, CD107a, Granzymes A, B and K, TNFα and IFN-γ expression. D) shows the percentage of CD8+ T cells expressing both CD69 and 4-1BB, above background stimulation, in response to Titan-WT infected monocytes compared to Titan-ΔUS28 infected monocytes. E) Of these activated monocytes, the proportion of HCMV-specific CD8+ T cells expressing CD107a, and producing Granzymes (A, B and K), IFN-γ and TNFα are shown, as are CD8+ T cells stimulated with virus infected monocytes and pulsed with IE1/2 peptides as antigen specific positive controls. F) Monocytes from an HLA-A2-positive donor were latently infected with SV40-GFP-TB40E and three days post infection were left untreated or treated with VUF2274. Two days post treatment with drug, both sets of monocytes were co-cultured with IE72-specific T cells, following which, monocytes expressing GFP were counted over the next four days. All data points show means from at least three independent experiments, and error bars show standard deviations. Statistical significance was determined using the Student's t-test, where p-values * (p=0.05) was considered significant.

[326, 388]. *US28* expression during HCMV lytic infection is well established to activate multiple cell signalling pathways which can activate the viral MIEP [32, 214, 251, 264, 265, 426, 470]. These signals have previously been linked to vascular diseases as well as oncomodulation [426, 427, 469]. However, this powerful signal activation appears contrary to the recently identified requirement for US28 expression to establish a latent infection in CD34+ stem cells, likely by repressing viral IE gene expression [161].

Here, I confirm this important function of US28 during latent infection in CD14+ monocytes and, in part, solve this paradox by showing that US28 appears to have very different effects on cell signalling between undifferentiated and differentiated myeloid cells. In undifferentiated myeloid cells, US28 attenuates cellular signalling of MAP kinase and NFκB which supports suppression of the MIEP to prevent lytic infection. In contrast, in differentiated myeloid cells, US28 activates these same signalling pathways to help drive IE expression and virus reactivation. My analysis, using a viral isolate with a deletion for the *US28* gene, showed that US28 was necessary for the maintenance of HCMV latency in monocytes; monocytes infected with Titan-ΔUS28 underwent full lytic infection and produced infectious virus.

These initial analyses were made seven days post infection. Interestingly, repeating this analysis at early times post-infection indicated that this ability of Titan-ΔUS28 to initiate a lytic infection in monocytes was immediate and did not require time for monocytes to become differentiated to a macrophage/DC phenotype, arguing that US28 does not function by suppressing myeloid differentiation and subsequent viral reactivation. I observed IE mRNA expression at 12 hours post-infection, IE protein expression 24 hours post-infection and UL32-GFP late protein expression 48 hours post-infection, indicating that infection of monocytes by Titan-ΔUS28 undergoes a similar time course of gene expression as lytic infection of fibroblasts. This suggests that, in the absence of US28 protein, monocytes themselves can support lytic HCMV infection and that differentiation of monocytes is not necessary for lytic infection under these conditions. In the future, I would like to use flow cytometry to analyse cell surface markers for signs of differentiation, for example, a decrease in cell surface CD14 and increases in CD83, when monocytes are infected with Titan-ΔUS28, would help to confirm that monocytes do not differentiate under these conditions.

Complementation analyses in THP-1 cells stably expressing US28 functional mutants gave us substantial insight into mechanisms by which US28 suppressed lytic infection of monocytes. Infection of THP-1 cell lines stably expressing different US28 mutants, including wild type US28 protein (HA-US28-WT), US28 protein which cannot signal (HA-US28-R129A) and US28 protein which cannot bind chemokines (HA-US28-Y16F), with

Titan-ΔUS28 shows that US28 maintains latency by G protein-mediated signalling and that this signalling occurs in a constitutive manner, independently of chemokine binding. These observations fitted well with analyses of the effect of US28 on the MIEP in transfection assays. Nucleofection of the US28 constructs into THP-1 cells which had been transduced with an MIEP-eGFP construct [461] demonstrated that HA-US28-WT repressed the MIEP in a signalling-dependent manner and further showed that in differentiated THP-1 cells, the activity of US28 switched from a repressor, as seen in undifferentiated monocytic cells, to an activator of the MIEP and this, too, was dependent on the signalling capacity of US28. This was entirely consistent with data in other permissive cell types which have consistently shown the ability of US28 to activate IE expression [42, 488].

My view, that the regulation of IE expression by US28 was likely signalling dependent, was confirmed by my observations that US28 profoundly affected the level of a number of cellular phosphokinases. In undifferentiated monocytic cells, HA-US28-WT significantly attenuated the MAP kinase pathway; in particular, ERK1/2, MSK1 and CREB were all less phosphorylated. Similarly, consistent with the observation that in cells that are permissive for HCMV lytic infection [32, 42, 488], US28 has an activatory effect on the MIEP and in differentiated monocytic cells, US28 activated MAP kinase signalling. This is diametrically opposed to its suppressive effect that US28 has on the MIEP in undifferentiated monocytic cells. US28 also differentially affected NFκB localisation in undifferentiated and differentiated monocytic cells; in undifferentiated cells, US28 resulted in increased cytoplasmic NFκB localisation but in differentiated cells it enhanced NFκB nuclear localisation. This, again, is consistent with the known NFκB-mediated activation of the MIEP [42] and helps explain the differentiation-dependent reversal of US28 activity on the MIEP and IE gene expression in undifferentiated or differentiated monocytic cells which suppress or support IE gene expression, respectively. Taken together, it appears that US28 activity in early myeloid lineage cells serves to maintain latency by attenuating reactivation signals, such as MAP kinase [189] and NFκB, which are both known to activate the MIEP [42, 59, 100, 186]. This radical differentiation-dependent reversal of US28 activity helps resolve the problem of why US28, considered to be a strong activator of cellular signalling in lytic infection, is also expressed during latency, when lytic infection is known to be actively suppressed.

How US28 apparently reverses its signalling properties, in undifferentiated and differentiated myeloid cells, in such a significant manner remains unclear. US28 has been investigated in a range of different cell types, however, and its effects between different cell types have not always been consistent [469, 470]. My observations appear to be the first evidence that US28 can attenuate cell signalling, independently from other viral GPCRs, in a constitutive manner,

at least in undifferentiated myeloid cells. Two models may explain these observations: firstly, phosphorylation of the C terminal tail of US28 is known to modify its signalling properties [263, 273, 383] and US28 is especially known to be phosphorylated by PKC [273], which is known to change isoform expression during myeloid cell differentiation [72]. It is, therefore, possible that changes in cellular kinase expression during myeloid differentiation alter US28 signalling by phosphorylation. Alternatively, US28 is known to interact promiscuously with a range of different G-alpha proteins [59, 272, 468], and similarly, changes in cellular G- alpha protein expression are known to occur during myeloid differentiation [9, 442]. Consequently, such differentiation-specific changes in the G-alpha protein interaction partners available to US28, could lead to changes the signalling properties of US28. The method to investigate this intriguing change in US28 activity would be using BioID (proximity-dependent biotin identification), which would require the addition of a biotin ligase fusion protein to the C terminus of US28 [365]. This biotin ligase will then biotinylate any proteins which are in close proximity to US28, which will mostly consist of the proteins with which US28 interacts. These proteins can then be isolated by affinity purification (as they will bind a streptavidin column) followed by mass spectrometry analysis. This technique may be preferable to immunprecipitation of US28 followed by mass spectrometry of interacting proteins, as BioID allows the identification of proteins which only interact briefly - such as the G-alpha proteins which bind US28 [193, 254, 364, 365].

More recent information on additional functions of US28 include studies which have shown that US28 activates PLC-β in monocytes [497], via G protein-coupling in a chemokine-independent manner, and suggests that attenuation of the MAP kinase and NFκB signalling pathways may not be the only mechanism by which US28 may affect IE expression differently in undifferentiated and differentiated monocytic cells.

My attempts to reproduce US28-mediated attenuation of lytic cycle in undifferentiated cells, using small molecule inhibitors to either MAP kinase or NFκB in isolation, had only a limited effect on the ability of Titan-ΔUS28 to establish lytic infection of monocytes. However, inhibition of both MAP kinase and NFκB signalling pathways, concomitantly, profoundly reduced lytic gene expression in Titan-ΔUS28 infected monocytes. My view is that lytic infection, via activation of the MIEP, can be stimulated by either MAP kinase or NFκB signalling but requires activation of at least one of these two pathways. I was also able to show that this inhibition of MAP kinase and NFκB together was able to force Titan-ΔUS28 infected monocytes from lytic infection to latency, using RT-qPCR analysis of viral gene expression (Figure 4.8). My observation that delaying this treatment of inhibitors, until 24 hours post infection, no longer prevents lytic infection, demonstrates that US28 has

to act at a very early time point post infection to suppress activatory signals to the MIEP and thus prevent lytic infection. My view is that these activatory signals are likely triggered by viral binding and/or entry into the cell, perhaps triggering innate immune responses, which could lead to the activation of the MIEP [116, 504] and that US28 may serve to attenuate this response and thereby stifle IE activation and induce viral latency. Unfortunately, the long-term toxicity of inhibiting both MAP kinase and NFκB meant that I could not reactivate virus from treated cultures.

In addition to my observation that US28 attenuates cell signalling to maintain latency, there appears to be an additional mechanism by which HCMV maintains a latent infection in CD14+ monocytes, by apparently secreting a factor which represses lytic infection. This factor, secreted by monocytes latently infected with Titan-WT, was able to repress lytic gene expression in monocytes infected with Titan-ΔUS28. In this analysis, I measured the loss of UL32-GFP expression (a late lytic gene) as a proxy for the inability to establish latency. However, I accept that it will be necessary to use RT-qPCR analysis to confirm that IE gene expression is also greatly reduced in these analyses as well as using monocyte co-culture with fibroblasts to measure virus output (which we would expect to be greatly reduced in the presence of the latent secretome). To attempt to identify potential secreted cellular factors from latent monocytes which could be responsible for repression of lytic infection. I performed an antibody array which detected cytokines, in order to measure changes in cytokine secretion between monocytes infected with Titan-WT, Titan-ΔUS28 and monocytes treated with UV inactivated virus. The secretome of latently infected CD34+ progenitor cells has already been published [248] and interestingly, my data seem to agree loosely with these observations. Eotaxin 2, MCP-1 and ILR-1a all increase during latent infection of CD14+ monocytes, similar to changes during latency in the CD34+ secretome [248]. In contrast, TGF-β appeared to decrease in the secretome of latently infected CD14+ monocytes and IL-10 does not appear to change significantly, despite these cytokines being shown to increase in the secretome of latently infected CD34+ cells [207, 248]. Indeed, TGF-β would be an ideal candidate for a factor secreted by latently infected cells which could inhibit lytic infection of monocytes, as TGF-β has been shown to repress the MIEP via HDAC-4 upregulation [207].

I also performed a cytokine array to measure changes in cytokine release from THP-1 cells, resulting from ectopic US28 expression and signalling. I first compared THP-1 cells expressing HA-US28-WT with those expressing HA-US28-Y16F (which cannot bind chemokines) in order to rule out any chemokines that US28 may bind and internalise, thereby removing them from the media. This analysis was generally successful, as I was able to

identify most chemokines that are known to bind US28; these included MCP-1, MCP-3, fractalkine, RANTES and Eotaxin-3 - all are known ligands of US28. However, I did not detect changes in three known US28 ligands: eotaxin-1, MIP1α or β which could be because expression of these ligands was too low to detect. More interestingly, IGFBP3, sTNFR, MCP-2, and I109 are not known ligands of US28 but did change significantly between THP-1 cells expressing HA-US28-WT and those expressing HA-US28-Y16F, which may point to several new US28 ligands; these hits need to be confirmed by ELISA analysis. More importantly, I detected five cytokines which appear to change in secretion levels when HA-US28-WT is expressed compared to HA-US28-R129A: HGF, IL1-β, G-CSF, OPG and IL-6r. Of these, G-CSF is interesting as this cytokine induces differentiation. However, its increased expression when HA-US28-WT is expressed seems at odds with maintaining latency. The soluble IL6-receptor, however, may play a role in diverting IL-6 [359] from the media and could prevent HCMV reactivation [344]; again, this should be validated and further investigated.

Finally, based on my findings that US28 is crucial to establish HCMV latency in monocytes, by suppressing IE gene expression and subsequent lytic infection of monocytes, I predicted that inhibition of US28 activity, using the inverse agonist VUF2274, would stimulate lytic gene expression in normally latently infected monocytes. This was, indeed, the case and led to proof of principle that inducing lytic infection in monocytes could lead to their targeting by pre-existing host HCMV-specific CTL responses. Firstly, IE72-specific CD8+ T cell clones reduced viral reactivation from monocytes infected with Titan-ΔUS28 compared to monocytes infected with Titan-WT virus and, similarly, treatment of experimentally latent monocytes with VUF2274 also made latently infected cells targetable by these IE72-specific CD8+ T cell clones. I also demonstrated that monocytes infected with Titan-ΔUS28 are targets for PBMCs from healthy HCMV-positive donors and confirmed that this was mediated by classical CTL killing on the basis of staining for markers of T cell activation and degranulation.

Although this approach of "shock and kill", using HDAC inhibitors, has already been demonstrated to be effective against latent HCMV [207], HDACs have a wide range of biological functions and the inhibition of HDACs could have significant off-target effects. Consequently, using a specific small molecule inhibitor of US28 to "reactivate" IE expression is an attractive alternative, particularly in healthy seropositive tissue donors where reactivation events are thought to be subclinical [39, 325]. I do note, however, that VUF2274 did show some cytotoxicity, likely due to off-target effects, which possibly include the inhibition of CCR1. It would also be helpful to demonstrate that VUF2274 does indeed inhibit US28-

mediated repression of cellular signalling pathways by immunoblot of ERK1/2 and other such phosphoproteins in the MAP kinase pathway, and also show that VUF2274 lifts US28-mediated repression of the MIEP, for example, by luciferase-reporter assays. As the structure of the US28 protein has recently been solved [50], this could aid the development of more specific small-molecule inverse agonists of US28.

Taken together, my observations point to a crucial role for viral US28 in the establishment of HCMV latency in monocytes which is mediated by differentiation-dependent US28 signalling and that inhibition of US28, resulting in the induction of IE expression in normally latently infected cells, could aid in novel immunotherapeutic strategies to target and clear the HCMV latent reservoir.

Chapter 5

Targeting latently infected cells with a novel antiviral fusion toxin protein

5.1 Introduction

HCMV poses a significant clinical threat to transplant recipients, especially seronegative transplant recipients who may have to receive organs or grafts from seropositive donors. In a system as complicated as a transplanted solid organ, routinely from cadavers, it is likely that HCMV resides in tissues in both latent and lytic states. In contrast, however, in allogeneic hematopoeitic stem cell transplants (allo-HSCTs), which consist of CD34+ progenitor stem cells from a G-CSF mobilised donor, HCMV resides in a latent state in these cells but reactivates upon cellular differentiation and maturation. These allo-HSCTs are used as a treatment for patients with high-risk leukaemias, and other non-malignant diseases of the immune system, where the immune system has to be ablated and replaced by the allo-HSCT. As such, these patients are immunosupressed and at risk of complications from reactivated HCMV.

HCMV causes several different complications for these allo-HSCT transplant recipients. Firstly, reactivation of HCMV, which is poorly controlled in these patients at early times post-transplant, causes CMV disease which has a high mortality rate if left untreated [35]. To avoid this problem, prophylactic treatment with antivirals, ganciclovir and foscarnet, reduces CMV disease incidence [434], but ganciclovir-mediated neutropenia can lead to increased mortality from bacterial and fungal infections [287]. Secondly, inclusion of donor CD4+ and CD8+ T cells in the HCMV seropositive graft reduces the incidence of CMV disease, as this provides donor cellular immunity to the transplant recipient's own reactivating virus [230], but the inclusion of T cells can increase the risk of graft verses host disease [287]. To avoid

these complications, wherever possible, seronegative allo-HSCT recipients are matched to seronegative donors. However, this reduces the pool of potential donor-recipient matches.

As indicated earlier, there would be significant clinical benefits if the latent load of HCMV could be reduced in CD34+ progenitor stem cells and CD14+ monocytes. A significant reduction in the levels of HCMV reactivation would be likely to improve donor survival by reducing the subsequent risks of CMV disease, ganciclovir-mediated neutropenia, T cell-mediated graft verses host disease and also improve the chances of donor-recipient matching by removing HCMV serostatus as a factor [34–36, 230–232].

Fusion toxin proteins (FTPs) are proteins which act as ligands, normally based on cytokines or chemokines, which have been modified by fusion to a toxic protein from a pathogen [10, 495]. They are designed to exploit high-affinity, and highly selective receptor-ligand interactions, to direct the cytotoxic proteins to target cells and have shown success as novel cancer therapies [81, 482]. Moreover, the approach has untapped potential as a treatment for other conditions, such as infectious diseases, where pathogen-encoded receptors provide unique targets, which could allow better specificity [411]. Recently, Spiess et. al, exploited the high-affinity binding of fractalkine to the HCMV-encoded US28 receptor to generate fusion toxin proteins with antiviral activity against cells which were lytically infected with HCMV [412]. They began by fusing a *Pseudomonas* exotoxin-A motif to the soluble fractalkine domain which generated CX3CL1-FTP, which showed strong antiviral properties. The principle is that CX3CL1-FTP binds US28, which internalises upon ligand binding, pulling the exotoxin-A protein into the HCMV-infected cell. A KDEL motif at the C terminus of CX3CL1-FTP then mediates localisation of the FTP to the endoplasmic reticulum (ER) via KDEL receptors, where exotoxin-A catalyses ADP ribosylation of elongation factor 2 of the cellular translation machinery, and leads to inhibition of translation and cell death [305, 385]. Spiess et. al, recognised that the fractalkine receptor, CX3CR1 is expressed on many cell types, especially monocytes, NK cells and neurons [178], and therefore, that CX3CL1-FTP could show significant toxicity *in vivo*. To overcome this, they generated a point mutant in phenylalanine 49 of CX3CL1 (this residue is known to be key to fractalkine binding of CX3CR1 [270], and generated the FTP called F49A-FTP, which has significantly reduced binding affinity for CX3CR1 compared to US28, thus conferring US28-binding specificity [412].

Having demonstrated that US28 is expressed in our model of HCMV latency (see Chapter 3) and, indeed, is necessary for latent infection (Chapter 4), we wanted to demonstrate that we could use F49A-FTP to exploit the latency-associated expression of US28 in latently infected monocytes and CD34+ progenitor cells to target and kill cells that were latently

infected. In this chapter I show that F49A-FTP is able to kill latently infected cells, in a US28-dependent manner. As expected, the killing of latently infected cells reduces the frequency of virus reactivation from treated cultures and this killing is also effective against naturally latently infected CD14+ monocytes, with only marginal reduction in efficacy. I also show that F49A-FTP toxicity in monocytes is due to residual background binding to the fractalkine receptor - CX3CR1. Therefore, these observations provide a proof of principle that F49A-FTP can purge the latent load of HCMV in hematopoietic stem cell grafts which could form the basis for a novel approach to greatly reduce the clinical threat of HCMV positive grafts in stem cell transplants.

5.2 F49A-FTP kills cells in a US28-dependent manner.

Spiess et. al, have previously shown that F49A-FTP is able to kill fibroblast cells which were lytically infected with HCMV [412]. However, they did not show that this killing was dependent on specific expression of US28; indeed, F49A-FTP could have been binding to other HCMV-encoded CCR homologues, such as US27, which are not expressed during latency. It was, therefore, important that I demonstrated that this cytotoxity was due solely to US28 expression and not due to other factors associated with viral infection. To show this, I infected HFFs with two isolates of HCMV: Titan-WT or Titan-ΔUS28, described in Chapter 3, both of which have a green fluorescent protein (GFP) tagged UL32 gene. Cell cultures were then treated with F49A-FTP for 72 hours before infected cells were visualised by fluorescence microscopy. It was clear that F49A-FTP was able to kill HFFs infected with Titan-WT but not Titan-ΔUS28, demonstrating that this killing was dependent on US28 expression (Figure 5.1).

In addition to lytic infection, we also wanted to assess the ability of F49A-FTP to kill cells in which HCMV would normally establish latent infection. To do this, we used THP-1 cells which are a monocyte-like cell line and can be used as a model of HCMV latency [7, 27, 190, 218]. As stated in Chapter 4, I generated THP-1 cell lines using lentivirus which express different N-terminally HA-tagged US28 proteins; the wild type protein US28 (HA-US28-WT), the US28 signalling mutant (HA-US28-R129A) and chemokine binding mutant (HA-US28-Y16F). After puromycin selection and confirmation of HA-US28 expression by immunoblot analysis (Figure 5.2A), THP-1 cells were treated with F49A-FTP for 48 hours and cell survival was measured by trypan blue staining. Figure 5.2B showed that F49A-FTP efficiently killed THP-1 cells expressing HA-US28-WT and HA-US28-R129A, but not empty vector-transduced control cells or cells expressing the chemokine binding

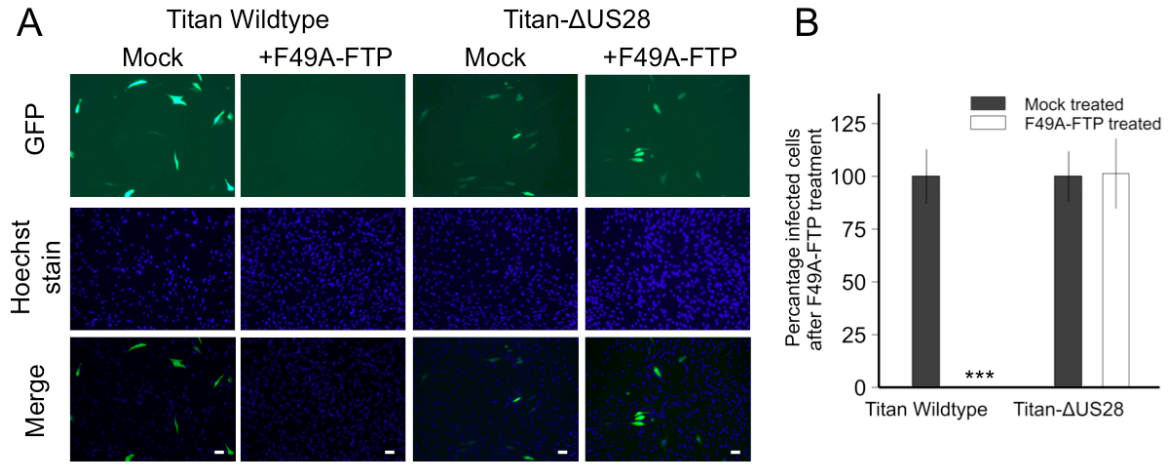

Figure 5.1 F49A-FTP kills lytically infected cells due to their expression of US28. Human foreskin fibroblast cells (HFFs) were infected with either HCMV Titan wild-type or HCMV Titan-ΔUS28 at an MOI of 0.1. Both viral isolates have a UL32-GFP tag, causing infected cells to appear green by fluorescence microscopy. Cultures were then either mock-treated with PBS or treated with $5x10^{-8}$ M F49A-FTP for 72 hours and quantified by fluorescence microscopy. A) representative images of the virally infected cultures with or without F49A-FTP. B) A graphical representation of these data. Cell numbers were quantified by Hoechst staining cell nuclei and the percentage of infected (green) cells is shown as a percentage of the control. Means and error bars (showing standard deviations) were generated from three independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values *** (p=0.001) were considered significant. White bars indicate 50μM scale.

mutant (HA-US28-Y16F). These data demonstrate that F49A-FTP kills monocytic cells expressing US28 in isolation, but that a mutation of the chemokine binding site at tyrosine 16, which is necessary for fractalkine binding by US28 [61] ablates killing by F49A-FTP.

5.3 Latently infected monocytes have a higher affinity for CX3CL1, indicating cell surface expression of US28.

On the basis that F49A-FTP was able to kill myeloid cells expressing US28 in isolation, and that US28 is expressed during latent infection (see Chapter 3, and [27, 78, 137, 326]), we reasoned that F49A-FTP could enable us to target and kill latently infected myeloid cells. US28 is trafficked to the cell surface during lytic infection [123], making it a target for F49A-FTP. However, my attempts to detect US28 expression in latently infected cells were unsuccessful by immunofluorescent staining. Consequently, I had no data to confirm that US28 is trafficked to the cell surface during latency. To overcome this, in collaboration with Dr Katja Speiss in the lab of Mette Rosenkilde (University of Copenhagen), we used radiolabelling assays to demonstrate that US28 protein is present on the cell surface of latently infected cells. To do this, I latently infected CD14+ monocytes with SV40-GFP-TB40E and sorted these monocytes to enrich for GFP-positive, latently infected monocytes, using the FACS protocol that I optimised in Chapter 3.

Dr Spiess then measured the affinity of sorted, latently infected cells for ^{125}iodine-radiolabelled fractalkine, a high-affinity ligand of both US28 and the endogenous receptor CX3CR1, that is expressed on monocytes. The population of enriched, latently infected monocytes showed a higher binding affinity for fractalkine compared to sorted, GFP-low, uninfected monocytes (Figure 5.3A). This increase in binding affinity for latently infected monocytes compared to uninfected monocytes was similar to the increase observed in COS-7 expressing US28 compared to COS-7 cells expressing CX3CR1 (Figure 5.3B).

However, whilst this increase in affinity for fractalkine could have resulted from latency-associated expression of US28, it was also possible that, it could also have been due to increased expression of CX3CR1, perhaps induced by latent infection. To differentiate between these two possibilities, I performed RT-qPCR analysis over several time points post infection, measuring relative CX3CR1 expression, to determine whether CX3CR1 mRNA expression is induced by HCMV infection in monocytes. Figure 5.3C shows that infection by UV-inactivated virus does cause an initial increase in CX3CR1 expression, whilst latent infection by live virus resulted in no significant CX3CR1 increase, especially by five days

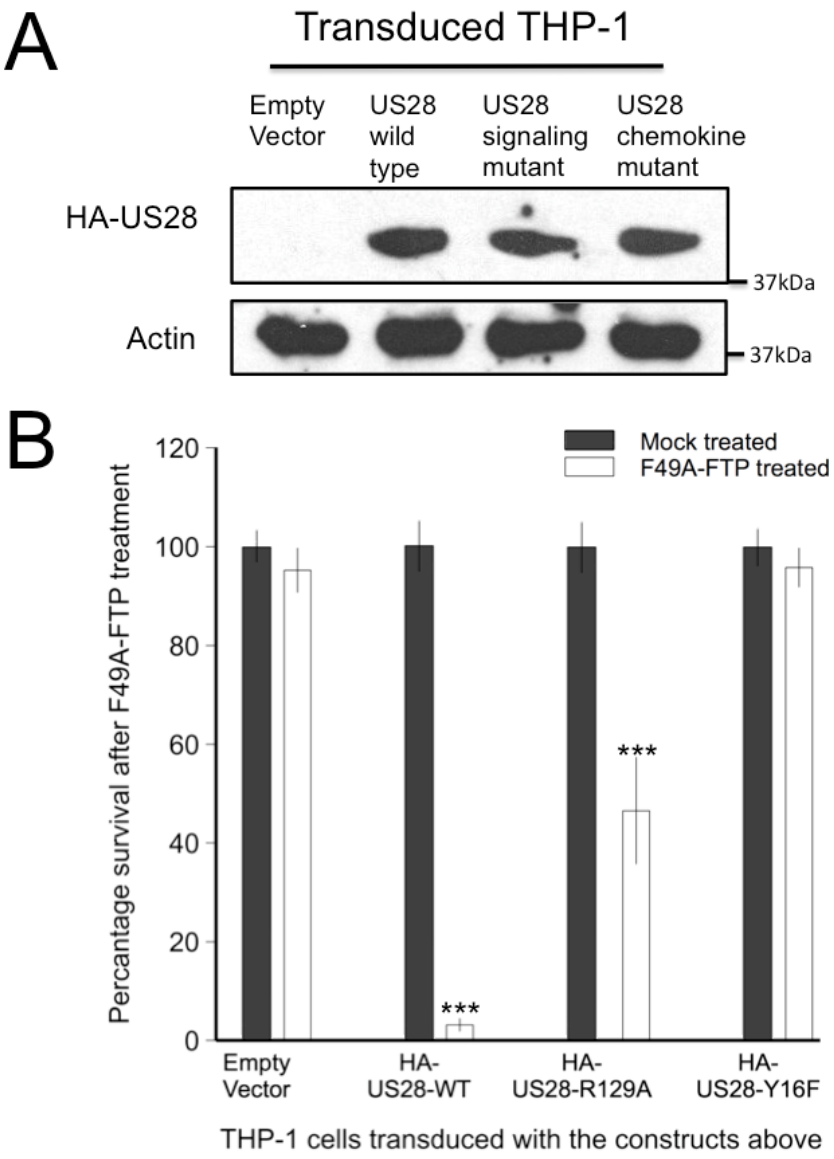

Figure 5.2 F49A-FTP kills monocyte-like THP-1 cells which express US28. THP-1 cells expressing HA-US28-WT, HA-US28-R129A or HA-US28-Y16F were generated by lentiviral transduction and puromycin selection, while control cells were transduced with empty vector. A) Immunoblots are shown of cell lysates from transduced THP-1 cells using an anti-HA antibody against the N-terminal HA tag on all the HA-US28 constructs. B) THP-1 cells expressing HA-US28 constructs, or control cells, were treated with $5x10^{-8}$ M F49A-FTP for 48 hours. Cell death was then quantified by trypan blue staining. Means and error bars (showing standard deviations) were generated from seven independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values *** (p=0.001) were considered significant.

post infection when the binding assays were carried out in Figure 5.3A. Taken together, these data are consistent with the view that US28 is expressed on the cell surface of latently infected monocytes.

5.4 Optimisation of F49A-FTP to kill experimentally latently infected monocytes.

Knowing that US28 expression during latent infection of cells did, indeed, increase binding affinity to fractalkine, I next analysed whether F49A-FTP could kill latently infected cells. To start, I needed to optimise two key parameters for this protocol; the concentration of F49A-FTP to use, as well as the duration of treatment needed. To do this, I experimentally infected CD14+ monocytes with SV40-GFP-TB40E and treated cultures with three different concentrations of F49A-FTP (10^{-8} M, 10^{-7} M and 10^{-6} M). I then counted the number of GFP-positive monocytes, as a measure of latent cell killing, each day for seven days. Figure 5.4 shows that an F49A-FTP concentration of 10^{-8} M was not sufficient to clear the majority of GFP-positive monocytes but that 10^{-7} M was as effective as 10^{-6} M F49A-FTP. These data also suggested that three days of treatment with F49A-FTP was sufficient to mediate significant killing of latently infected cells. Treatment of uninfected monocytes with F49A-FTP, at the same concentrations as above, also showed that 10^{-8} M F49A-FTP showed low levels of cell death, but 10^{-7} M showed significant levels of cell death (Figure 5.4B). Consequently, the ideal concentration of F49A-FTP to use, in order to kill latently infected cells with low level background cell death, would be between 10^{-8} M and 10^{-7} M.

To further define the optimum concentration of F49A-FTP to use, I titrated F49A-FTP (between 10^{-8} M and 10^{-6} M), on CD14+ monocytes experimentally infected with SV40-GFP-TB40E. I also decided that it would be best to measure the number of reactivation events from monocytes treated with F49A-FTP, by monocyte differentiation, as this could be considered to be a more informative measure of latent load than positive monocyte numbers, since it is these reactivation events which cause disease in immunocompromised transplant recipients. To do this, I treated infected monocyte cultures with F49A-FTP for three days (as three days was sufficient time to greatly reduce GFP-positive monocytes in Figure 3.4), and then replaced the media with fresh media containing cytokines followed by lipopolysaccharide (LPS), in order to stimulate differentiation and maturation to mature dendritic cells (mDCs); thereby resulting in reactivation of any remaining latent viral genomes. I co-cultured these CD14+ monocyte-derived mDCs with HFFs, and quantified foci of viral IE expression, by

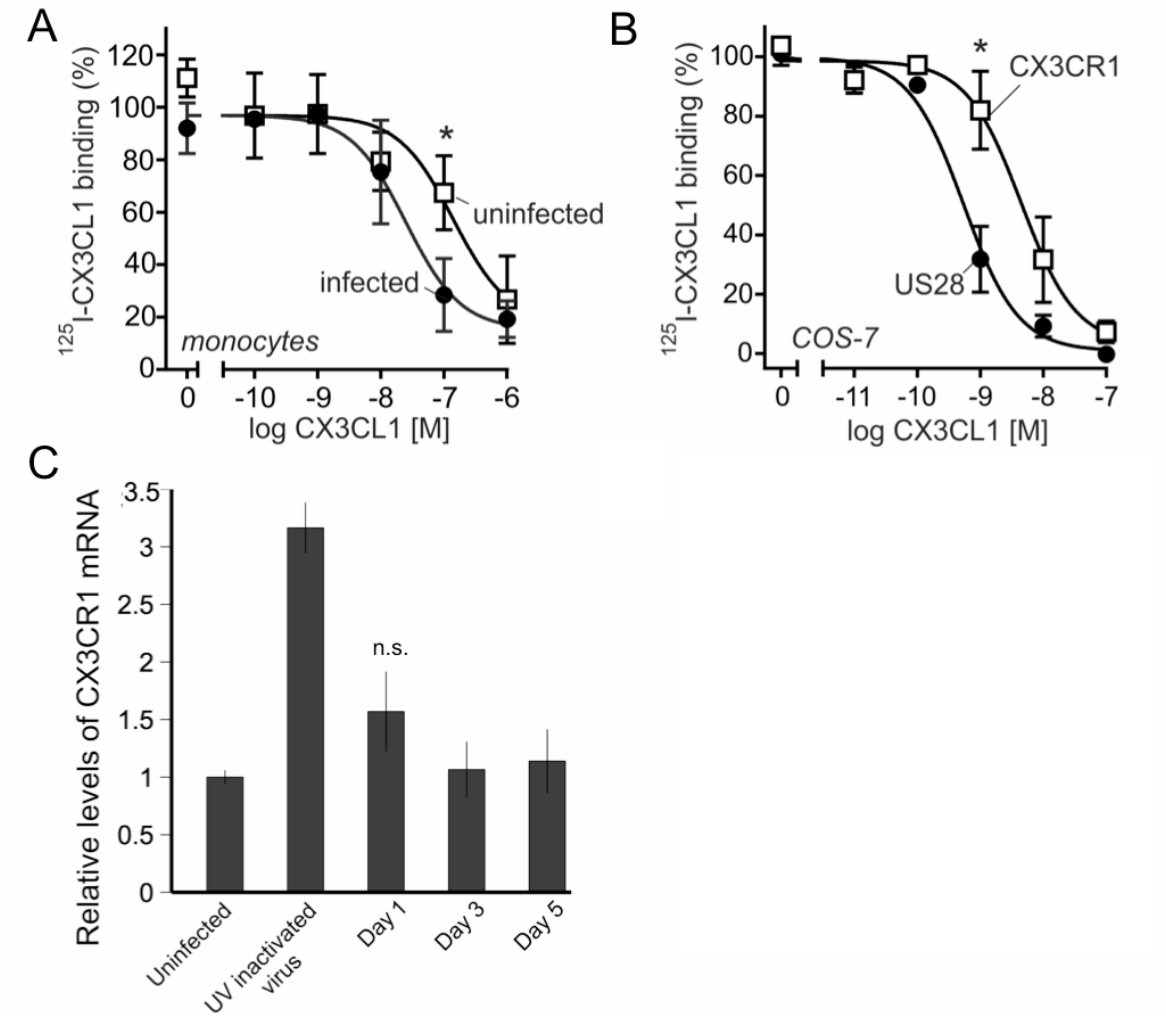

Figure 5.3 Latently infected monocytes have high affinity for CX3CL1, which indicates cell surface US28 expression. CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with SV40-GFP-TB40E, and then sent to Katja Spiess for homologous competition binding between COS-7 cells expressing US28 or CX3CR1 and latently infected, or uninfected monocytes. A) Binding of ^{125}I-CX3CL1 to sorted, GFP-low, uninfected monocytes (white symbols) and sorted, GFP-positive, latently infection monocytes (black symbols). The data are normalised to maximal binding on infected cells. Error bars indicate SEM for five independent biological replicates. B) Binding of 125I-CX3CL1 to transiently transfected COS-7 cells expressing US28 (black) or CX3CR1 (white symbols). The data are normalized to maximal specific binding on US28 expressing cells. Error bars indicate SEM for three independent biological replicates. For panels A and B, data acquisition and analysis was performed by Katja Spiess.

Figure 5.3 Latently infected monocytes have high affinity for CX3CL1, which indicates cell surface US28 expression. C) RT-qPCR analysis of CX3CR1 mRNA expression in monocytes latently infected at an MOI of 5, with SV40-GFP-TB40E, or monocytes treated with UV-inactivated virus. Values are relative to an uninfected control cells. UV inactivated control monocytes were harvested one day post infection. Means and standard deviations are from three measurements and normalised to GAPDH. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as * (p= 0.05) were considered significant.

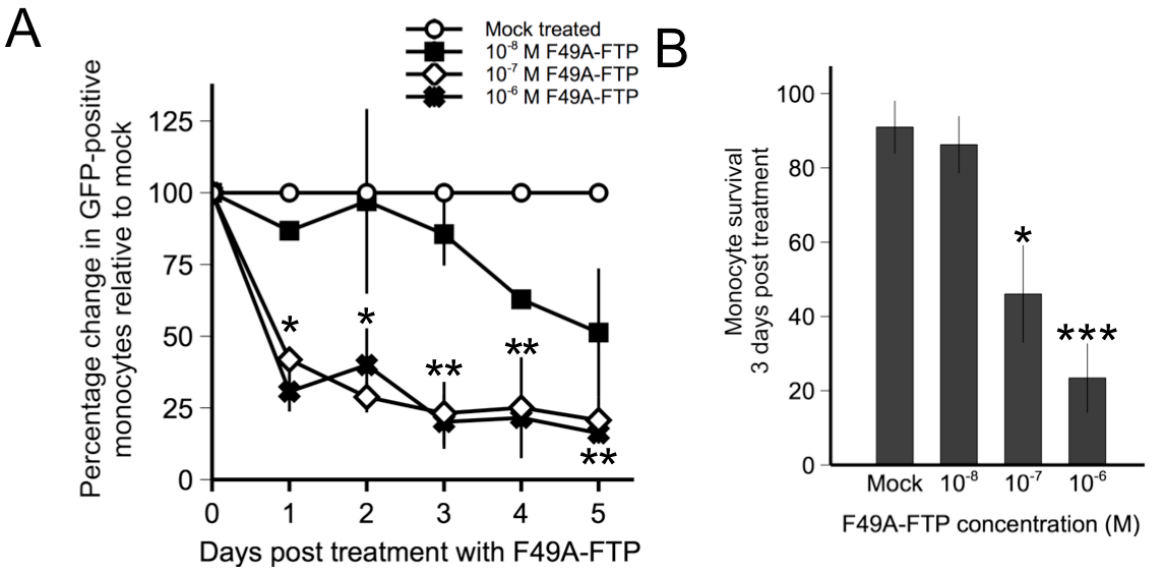

Figure 5.4 F49A-FTP kills experimentally latently infected monocytes, reducing the number of latently infected, GFP-positive cells. (A) CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with SV40-GFP-TB40E. After 24 hours, F49A-FTP was added to monocyte cultures at the indicated concentrations. Each day hence, the number of GFP-positive monocytes per well were quantified by fluorescence microscopy and compared to a mock-treated latently infected cell controls which was set at 100%. Means and error bars (showing standard deviations) were generated from two independent experiments, data were analysed by ANOVA followed by Tukey's post hoc test, and p-values * (p=0.05) and ** (p=0.01) were considered significant. B) Uninfected CD14+ peripheral blood monocytes were treated with F49A-FTP at the indicated concentrations. Three days after treatment monocytes were trypan blue stained to measure cell survival. Means and error bars (showing standard deviations) were generated from three independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as * or *** indicating p= 0.05 or p=0.001 respectively.

immunofluorescent staining, in the fibroblasts as a measure of the reactivation of infectious virus (Figure 5.5A, black circles). Additionally, I measured monocyte cell death by trypan blue staining after three days of F49A-FTP treatment in monocyte cultures treated in parallel with the same titration of F49A-FTP (Figure 5.5A, white squares). When analysed together, these data indicate that a concentration of F49A-FTP of $5x10^{-8}$ M showed an optimal balance of reducing reactivation events while preserving general monocyte cell survival. Consequently, I decided to use this concentration of F49A-FTP for future assays.

I also needed to determine the optimum length of time to treat monocytes with F49A-FTP. To do this, I treated monocyte cultures with $5x10^{-8}$ M F49A-FTP for increasing amounts of time and, at each time point, I replaced media with cytokines to induce differentiation and virus reactivation, as described above. I then measured the reduction in reactivation events by IE staining, again as above, against the duration of F49A-FTP treatment, and was able to show that a treatment duration of 72 hours showed convincing clearance of latent HCMV from experimentally infected monocytes (Figure 5.5B).

5.4.1 The F49A mutation confers greater selectivity to kill latently infected monocytes

As stated in the introduction, in addition to F49A-FTP, Spiess et. al, developed another FTP, which consisted of the fractalkine soluble domain and the PE endotoxin but which did not have the F49A mutation (CX3CL1-FTP). This showed reduced affinity to US28 over CX3CR1 [412]. I therefore wanted to test whether this FTP was less effective than F49A-FTP at killing latently infected cells, as this would confirm that binding of US28 is important for the killing of latently infected cells. It would also confirm that the correct FTP was being used to treat latently infected cells. If CX3CL1-FTP proved to be more effective, for whatever reason, then it would be sensible to switch FTPs for any further work. To show this, I titrated CX3CL1-FTP and F49A-FTP onto infected monocytes, and differentiated the monocytes, as before, to measure changes in the frequency of reactivation events. As expected, F49A-FTP, which has a higher selectivity for US28 over CX3CR1, showed improved selectivity towards killing latently infected cells and at a lower concentration (Figure 5.6).

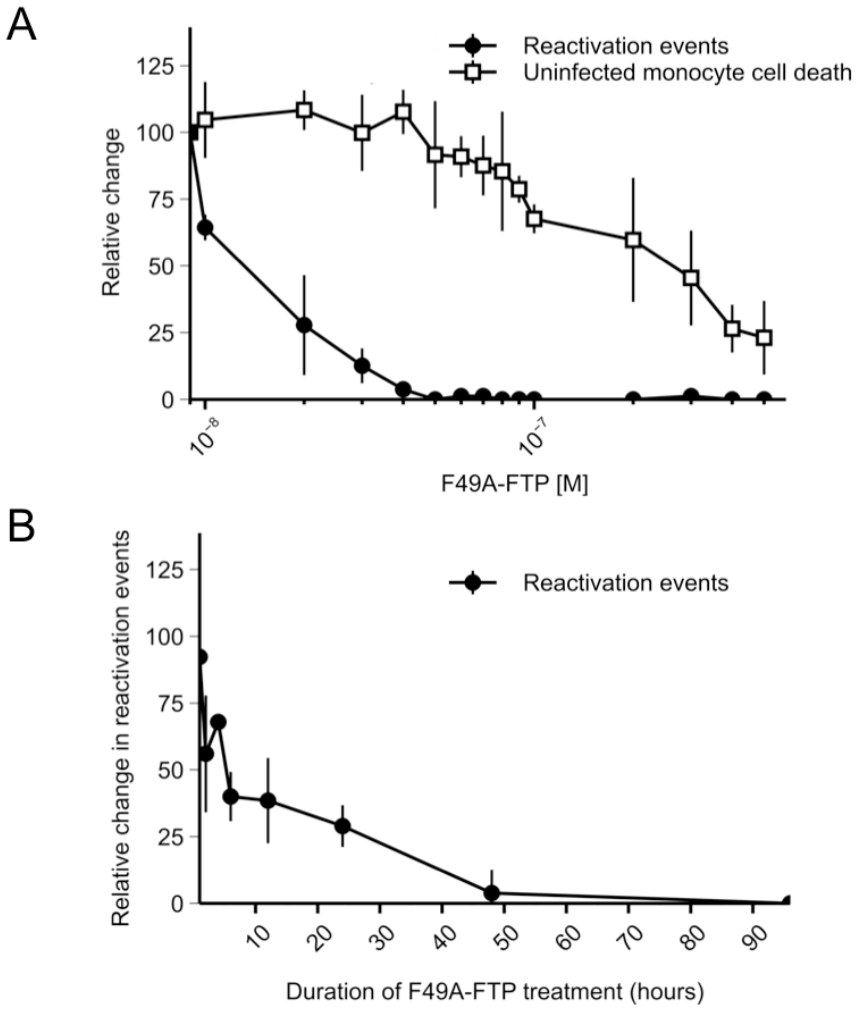

Figure 5.5 F49A-FTP kills latently infected monocytes specifically compared to uninfected monocytes. A) CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with SV40-GFP-TB40E. After 24 hours, F49A-FTP was added to monocyte cultures at the concentration gradient indicated. After 72 hours, media was changed and cytokines were added to induce monocyte differentiation and reactivation. These CD14+ monocyte-derived mDCs were then co-cultured with HFFs and foci of viral IE expression were quantified by immunofluorescent staining, as a measure of the reactivation of infectious virus (black circles). Additionally, CD14+ peripheral blood monocytes were isolated and, after 24 hours, F49A-FTP was added to monocyte cultures at the concentration gradient indicated. After 72 hours, monocyte cell death was analysed by trypan blue staining (white squares). Means and error bars (showing standard deviations) were generated from three independent experiments.

Figure 5.5 F49A-FTP kills latently infected monocytes specifically compared to uninfected monocytes. B) CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with SV40-GFP-TB40E. After 24 hours, F49A-FTP was added to monocyte cultures and then removed after the durations of time indicated. Media was changed and cytokines as well as LPS were added to induce monocyte differentiation and reactivation. These CD14+ monocyte-derived mDCs were co-cultured with HFFs and foci of viral IE expression were quantified by immunofluorescent staining, as a measure of the reactivation of infectious virus. Means and error bars (showing standard deviations) were generated from three independent experiments.

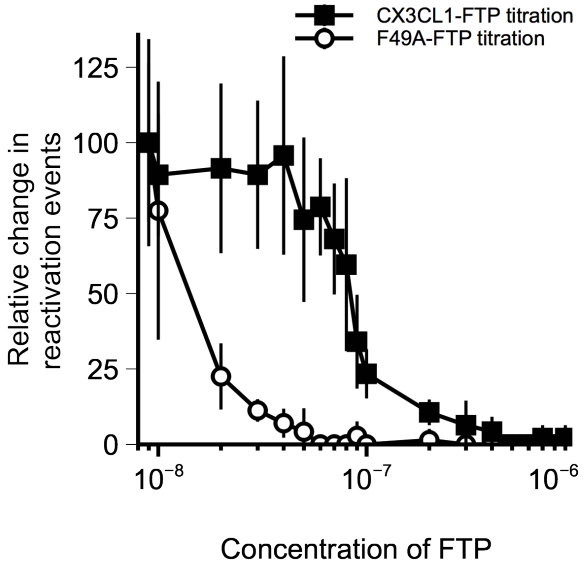

Figure 5.6 The F49A mutation confers selectivity for the FTP towards latently infected cells. CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with SV40-GFP-TB40E. After 24 hours, either CX3CL1-FTP or F49A-FTP was added to monocyte cultures, at the concentration indicated. After 72 hours, media was changed and cytokines as well as LPS were added to induce monocyte differentiation and reactivation. These CD14+ monocyte-derived mDCs were then co-cultured with HFFs and foci of viral IE expression were quantified by immunofluorescent staining, as a measure of the reactivation of infectious virus. Black squares indicate the change in reactivation events from monocytes treated with CX3CL1-FTP, while white circles indicate the change in reactivation events from monocytes treated with F49A-FTP. Means and error bars (showing standard deviations) were generated from at least four independent experiments.

5.4.2 HDAC inhibitors do not greatly improve F49A-FTP selectivity to kill latently infected monocytes

Previously, work has shown that HDAC inhibitors relieve repression of the MIEP, causing some lytic gene expression [207, 339]. As US28 is very likely to be expressed in greater amounts during lytic infection compared to latent infection, I also wanted to test whether the HDAC inhibitor MC1568 could induce more US28 expression, and whether this induction of US28 expression could confer increased selectivity of F49A-FTP towards killing latently infected cells. First, I infected CD14+ monocytes with SV40-GFP-TB40E, and harvested mRNA immediately after infection (as an mRNA input control) or each day post infection. Two days post infection, I also treated half of the cultures with MC1568 and then continued to harvest mRNA for a further three days. I then measured US28 mRNA expression by RT-qPCR, but found only a small increase in US28 mRNA expression, relative to the input control mRNA, after MC1568 treatment (Figure 5.7A). Despite this, I titrated F49A-FTP onto monocytes, as before, in the presence or absence of MC1568 but saw no noticeable change in the efficacy of F49A-FTP in the presence of MC1568 (Figure 5.7B). I concluded that MC1568 did not improve F49A-FTP efficacy and therefore did not to pursue this approach further.

5.5 F49A-FTP kills experimentally latently infected monocytes and greatly reduces reactivation events.

Having developed an optimised concentration and duration of treatment of F49A-FTP, for the killing of latently infected monocytes, and also having additionally ascertained that CX3CL1-FTP and MC1568 treatment did not improve killing of latently infected cells, I tested the efficacy of F49A-FTP against latently infected monocytes using this optimised protocol. To do this, I experimentally infected CD14+ monocytes with SV40-GFP-TB40E, and treated cultures for 72 hours with $5x10^{-8}$ M F49A-FTP. I then counted the number of GFP-positive monocytes between different cultures (Figure 5.8A) and then replaced the media with cytokine-containing media and LPS to induce monocyte differentiation and maturation, and triggered virus reactivation, as before. After full maturation of the latently infected monocytes, I counted the number of GFP-positive, CD14+ monocyte-derived mDCs (Figure 5.8B). Finally, I co-cultured these CD14+ monocyte-derived mDCs with HFFs and quantified foci of viral IE expression, in the fibroblasts, as a measure of the reactivation of

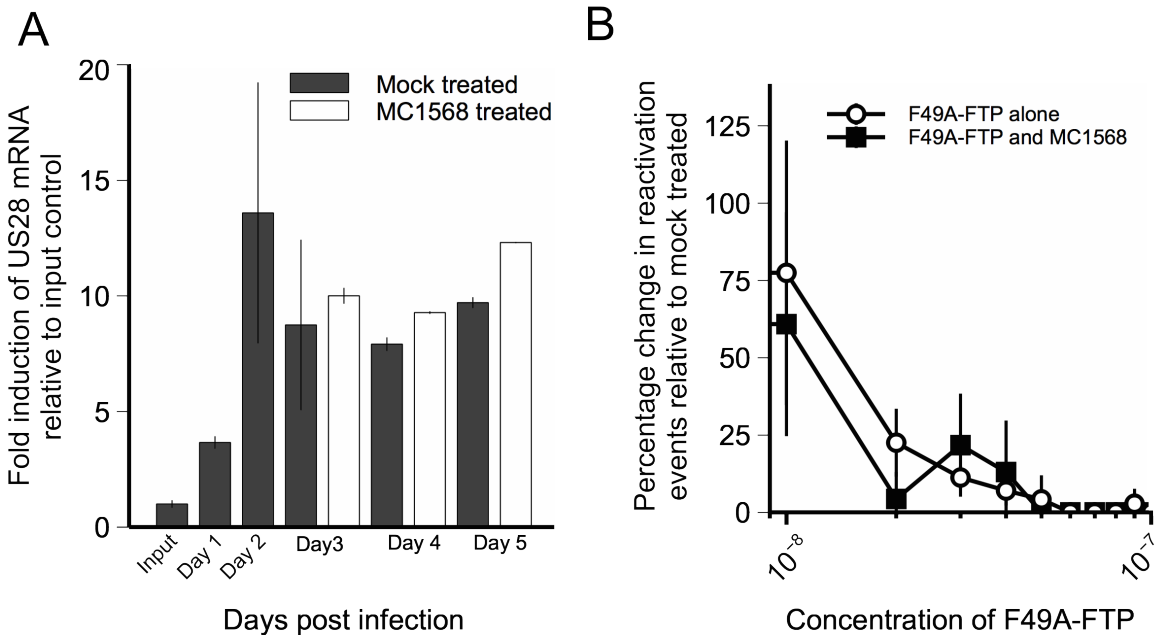

Figure 5.7 MC1568 induces only minor increases in US28 expression, which do not affect F49A-FTP efficacy. A) CD14+ monocytes were infected at an MOI of 5 with SV40-GFP-TB40E and mRNA was harvested immediately after infection, for use as an mRNA input control. Subsequently, each day post infection, mRNA was harvested. Two days post infection, I also treated half of the remaining cultures with 5μg/ml MC1568, and then continued to harvest mRNA for a further three days. I then measured US28 mRNA expression by RT-qPCR; means and standard deviations are from three measurements and normalised to GAPDH. B) CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with SV40-GFP-TB40E. After 24 hours, F49A-FTP was added to all monocyte cultures, at the concentration gradient indicated, in the presence or absence of 5μg/ml MC1568. After 72 hours, media was changed and cytokines as well as LPS were added to induce monocyte differentiation and reactivation. These CD14+ monocyte-derived mDCs were then co-cultured with HFFs and foci of viral IE expression were quantified by immunofluorescent staining, as a measure of the reactivation of infectious virus in the presence (black squares) or absence (white circles) of MC1568. Data points show means from at least four independent experiments, error bars show standard deviations.

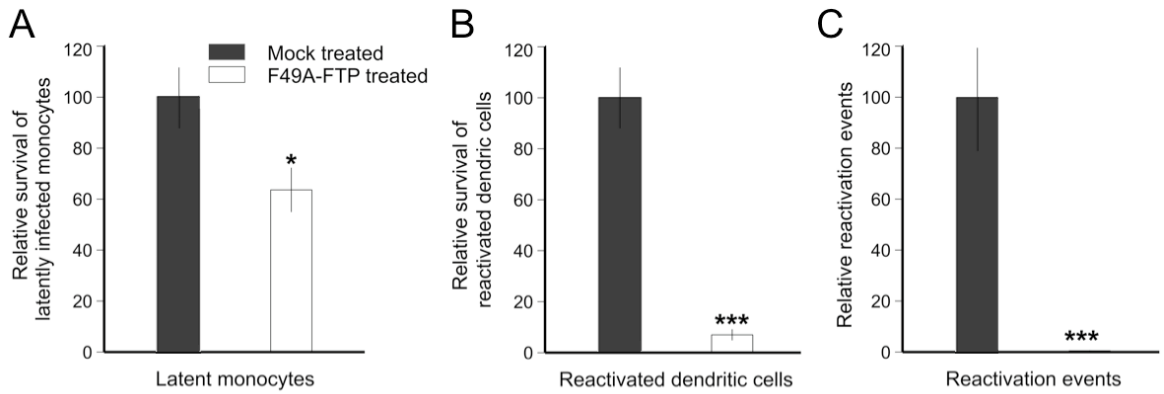

Figure 5.8 F49A-FTP kills experimentally latently infected monocytes, reducing HCMV reactivation events. CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with SV40-GFP-TB40E. After 24 hours, F49A-FTP was added to monocyte cultures, and incubated for 72 hours. Changes in the number of GFP-positive cells were quantified by fluorescence microscopy and compared to mock-treated latently infected cell controls which was set at 100% (A). These monocytes were then differentiated using GM-CSF and IL-4 stimulation followed by LPS treatment to generate mature dendritic cells (mDCs). Similarly, changes in the percentage of GFP-positive mDCs were also measured (B). Finally, the cells shown in (B) were co-cultured with fibroblasts for two weeks and the number of IE foci were counted (C). Means and error bars (showing standard deviations) were generated from five independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as * or *** indicating p= 0.05 or p=0.001 respectively.

infectious virus (Figure 5.8C). These data demonstrated that the protocol for treating latently infected monocytes with F49A-FTP was highly effective at clearing latently infected cells.

Intriguingly, in these analyses GFP-positive monocyte survival (62%) appeared significantly higher than GFP-positive mDC survival (12%), and these DCs showed no reactivation events. I reasoned that this difference could be due to the method of measuring latently infected monocyte survival, in that a GFP-positive monocyte could be in the process of dying, or indeed already dead, but residual GFP positivity would still be counted. As I could not use a live/dead stain without perturbing monocyte survival, I experimentally infected CD14+ monocytes with SV40-GFP-TB40E, and treated cultures for 72 hours with $5x10^{-8}$ M F49A-FTP. I then counted the number of GFP-positive monocytes between different cultures (Figure 5.9, "monocytes day 3") and then replaced the media. Following a further seven day culture (the time required to differentiate monocytes to immature DCs) either with or without cytokines, I then counted the difference in GFP-positive monocytes again, or the difference in GFP-positive monocyte-derived mDCs (Figure 5.9, "monocytes day 10 vs dendritic cells").

By comparing the difference in GFP-positive monocytes at three days post treatment, and the same cultures seven days later, I could see that this extra culture time resulted in more of a decrease in GFP-positive monocyte in the F49A-FTP treated cultures, as these dying cells stop appearing GFP-positive (Figure 5.9).

I next considered that, if this observed targeting of latently infected cells was specifically due to US28 expression, F49A-FTP should be less effective against monocytes infected with a US28 deletion virus. To address this, I used the same protocol as described above to measure F49A-FTP-killing of infected cells by counting reductions in GFP-positive monocytes; reductions in GFP-positive mDCs and reactivation events. For this experiment, I infected monocytes with Titan-WT or Titan-ΔUS28. These two HCMV isolates are GFP-tagged on the UL32 gene, which is only expressed during lytic infection, I could not observe UL32-GFP-expression in monocytes infected with Titan-WT, but I could observe UL32-GFP expression in monocytes infected with Titan-ΔUS28, because Titan-ΔUS28 established lytic infection in monocytes as described in Chapter 4. As Figure 5.10 shows, treatment of monocytes latently infected with Titan-WT virus with F49A-FTP clearly showed a reduction in the frequency of reactivating mDCs and reactivation of infectious virus from these cells, whilst treatment of monocytes infected with Titan-ΔUS28 with F49A-FTP showed no such decreases (Figure 5.10).

It should be noted that this control experiment is, necessarily, an imperfect one as US28 expression is known to be necessary for the maintenance of latency; this means that Titan-ΔUS28 infected monocytes were expressing the entire viral genome, and not just latency-associated genes. Nevertheless, we have already shown that lytically infected fibroblasts are killed by F49A-FTP in a US28-dependent manner (see Figure 5.1) and so the fact that monocytes infected with Titan-ΔUS28 are not killed by F49A-FTP still supports the notion that F49A-FTP kills latently infected monocytes in a US28 dependent manner.

Given that US28 is present on the envelope of HCMV virions, I considered that the efficacy of killing by F49A-FTP could have been enhanced by virion-delivered US28 protein [123, 161, 306, 315, 464]. If true, this could have been problematic as, in natural latency, most infected cells are unlikely to have been recently subjected to virions carrying US28. In order to show that US28 expressed by the latently infected cell was sufficient to target F49A-FTP for cell killing, I delayed F49A-FTP treatment of experimentally infected monocytes by 1, 3, 5 and 7 days post infection, to allow time for virion-delivered US28 to be degraded. I found that F49A-FTP treatment was as effective at clearing latently infected cells when applied seven days post infection compared to one day post infection (Figure 5.11A).

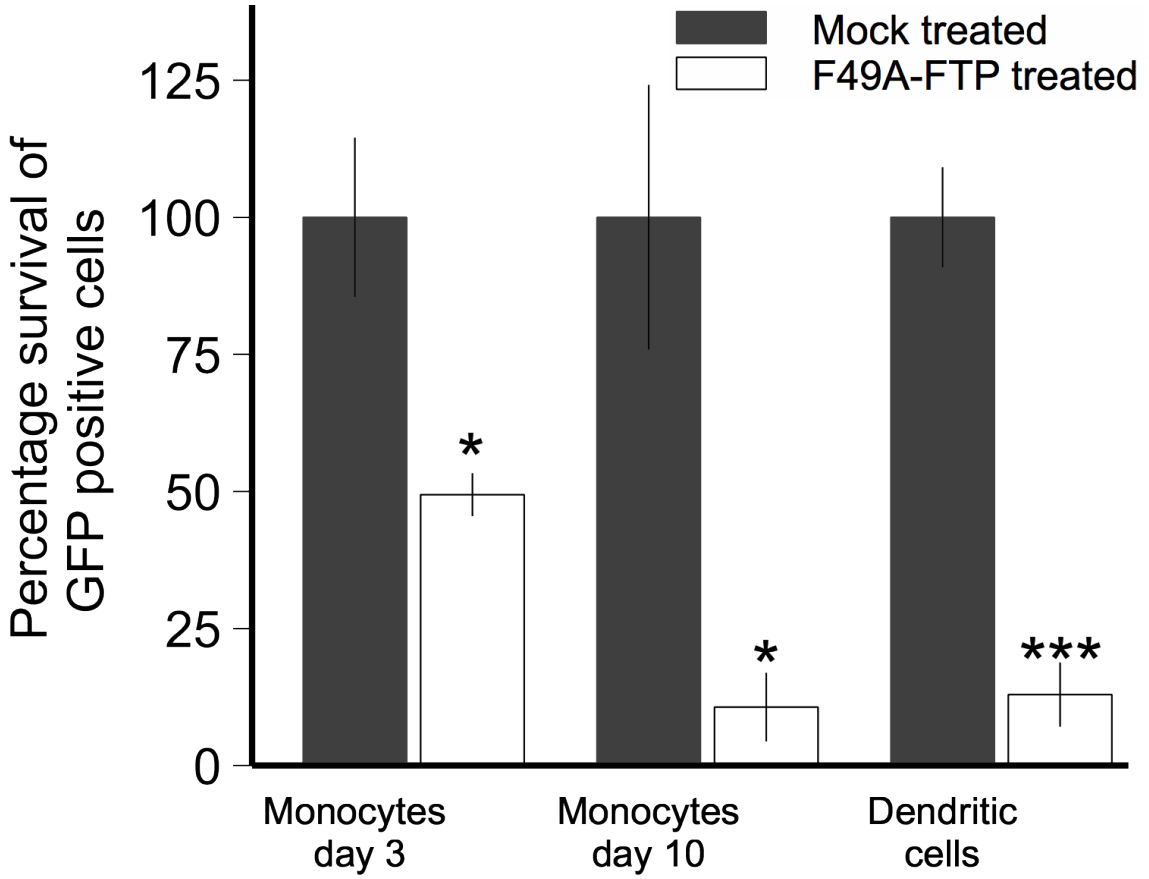

Figure 5.9 There is a time delay between F49A-FTP killing and latent monocytes losing their GFP-positivity. CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with SV40-GFP-TB40E. After 24 hours, F49A-FTP was added to monocyte cultures and incubated for 72 hours. Changes in the number of GFP-positive cells were quantified by fluorescence microscopy and compared to mock-treated latently infected cell controls which was set at 100% (monocytes day 3). These monocytes were then either treated with cytokines and LPS to induce differentiation and maturation to mDCs (dendritic cells) or mock treated (monocytes, 10 days). Similarly, changes in the percentage of GFP-positive mDCs or remaining GFP-positive monocytes were then measured. Means and error bars (showing standard deviations) were generated from five independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as *, *** indicating p= 0.05 or p=0.001, respectively.

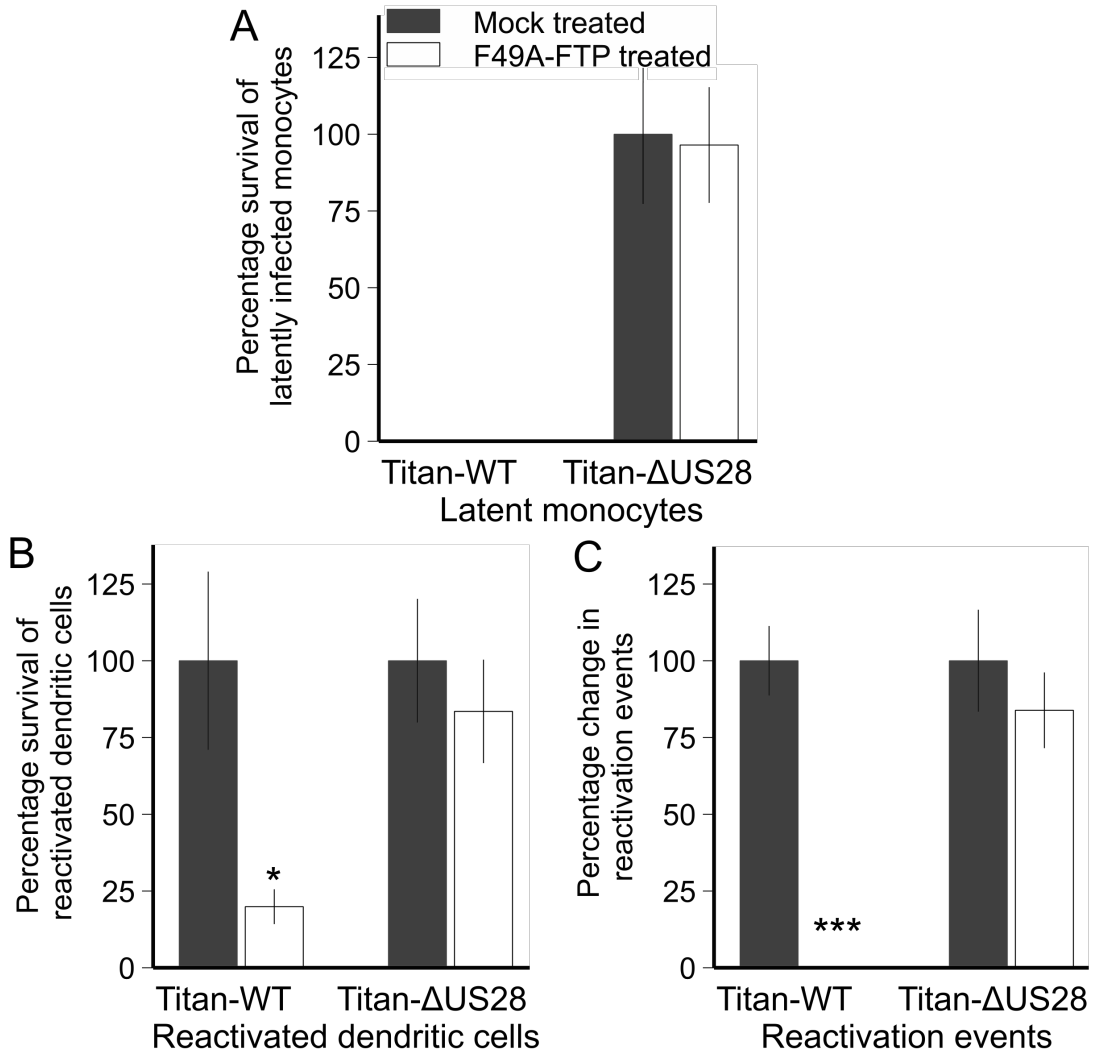

Figure 5.10 F49A-FTP cannot kill monocytes infected with Titan-ΔUS28. Monocytes were isolated and experimentally infected with either HCMV Titan wild-type or HCMV Titan with a US28 deletion at an MOI of 5. Cultures were then either mock-treated with PBS or treated with F49A-FTP for three days and changes in the number of GFP-positive cells were quantified by fluorescence microscopy and compared to a mock-treated latently infected cell controls which was set at 100% (A). Cultures were then reactivated by differentiation into mature dendritic cells, as before. Using the UL32-GFP tag on these viral isolates, reactivated dendritic cells were then counted and compared to levels of reactivation of monocytes infected with Titan wild-type virus in the absence of drug, which was set to 100% (B). After co-culture with reporter fibroblast cells, incubation for two weeks and finally staining for IE-positive foci, reactivation events were quantified (C). Means and error bars (showing standard deviations) were generated from three independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as *, *** indicating p= 0.05 or p=0.001, respectively.

Finally, as very few cells are likely to be infected in a naturally latently infected individual, I tested F49A-FTP efficacy against cultures which were experimentally infected at very low MOI; F49A-FTP only marginally lost its efficacy when treating cultures with very few latently infected monocytes (Figure 5.11B).

5.6 The F49A-FTP also kills experimentally infected CD34+ progenitor cells, thereby reducing reactivation events

As CD34+ progenitor cells, a major component of hematopoietic stem cell transplants, are also sites of latent carriage of HCMV *in vivo*, I repeated these analyses with experimentally latently infected CD34+ progenitor cells. I first confirmed that infected CD34+ cells established a latent infection and, consistent with previous studies [207], and work described in Chapter 3, infected CD34+ cells did, indeed, show a profile of viral transcription associated with latency; a low level of lytic IE RNA compared to much higher levels of latency-associated UL138 RNA (Figure 5.12A) and, importantly, a lack of production of infectious virus in these latently infected cells (Figure 5.12B). In contrast, differentiation and maturation of these latently infected cells to mature CD34+ derived DCs resulted in a characteristic reactivation of the lytic transcription profile with a switch to high levels of lytic IE compared to UL138 (Figure 5.12A), as well as efficient virus production (Figure 5.12E).

As before, I used the optimised protocol as determined for monocytes, for treating latently infected CD34+ cells with F49A-FTP and observed that F49A-FTP also reduced the number of GFP-expressing latently infected CD34+ cells (Figure 5.12C), reduced the number of GFP-expressing CD34+ derived mDCs resulting from reactivation of GFP-tagged virus (Figure 5.12D) and significantly reduced the amount of reactivated infectious virus capable of re-infecting HFFs in co-culture (Figure 5.12E).

5.6.1 The F49A-FTP toxicity towards uninfected cells is due to CX3CR1 expression and can be blocked with fractalkine treatment

As I had based the analysis of the effect of F49A-FTP on latently infected CD34+ cells on the optimum conditions for monocytes, I wanted to test the cytotoxicity of F49A-FTP, at our anti-virally active concentration of $5x10^{-8}$ M, on uninfected CD34+ progenitor cells. To do this, I used uninfected CD34+ cells for the analysis, as infected cells would be killed at a different rate and treated these uninfected CD34+ cells with F49A-FTP for 72 hours and

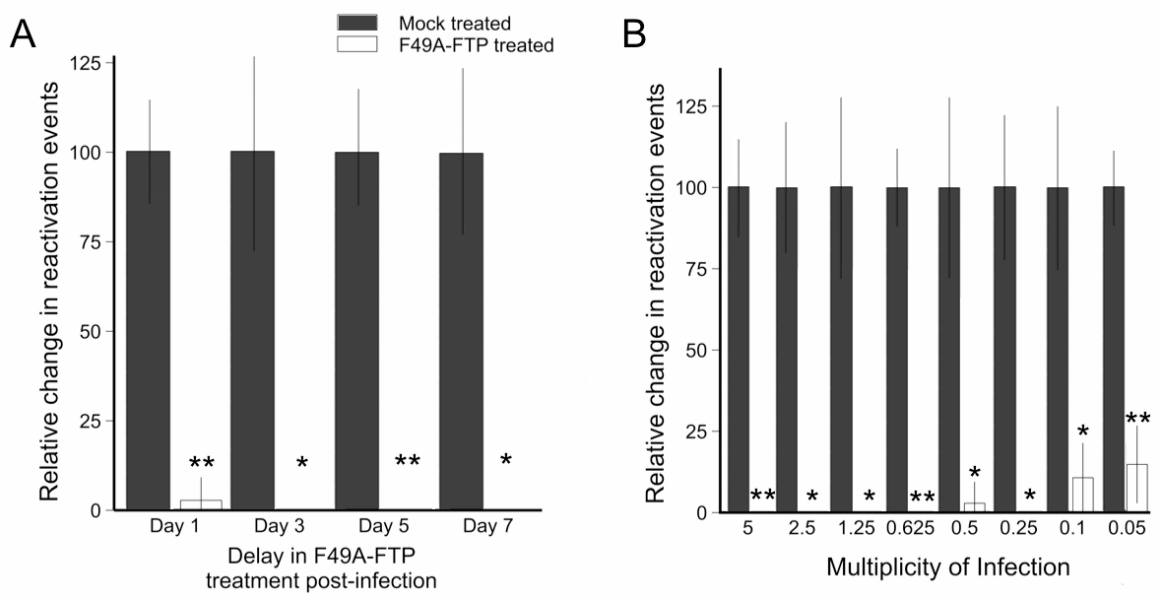

Figure 5.11 Delaying treatment of latently infected monocytes with F49A-FTP does not alter its efficacy at clearing latent HCMV; F49A-FTP is also effective at clearing virus at low multiplicities of infection. A) Monocytes were isolated and infected with SV40-GFP-TB40E at a predicted MOI of 5. Cultures were then treated with F49A-FTP between one and seven days post infection. After this, latently infected cells were reactivated by differentiation and maturation to mDCs. These monocyte-derived, mature dendritic cells were then co-cultured with fibroblasts for two weeks and the number of reactivated IE foci were detected by immunoflourescent staining. B) Monocytes were infected with increasing dilutions of a stock of SV40-GFP-TB40E virus (shown as multiplicities of infection between 5 - 0.05); this resulted in 9.1%, 4.8%, 3.2%, 1.3%, 0.76%, 0.47%, 0.15% and 0.08% latently infected cells, respectively, as estimated by counting the percentage of GFP-positive cells, against the total number of cells by fluorescence and light microscopy. These monocytes were then treated with F49A-FTP 24 hours post infection. After this, latently infected cells were reactivated by differentiation and maturation to mDCs. These monocyte-derived, mature dendritic cells were then co-cultured with fibroblasts for two weeks and the number of reactivated IE foci were detected by immunoflourescent staining. For lower MOIs, an increased number of monocytes were infected in order to keep absolute reactivation event numbers similar across all MOIs. The total reactivation events measured for the untreated monocyte derived dendritic cells, from left to right, were: 52, 48, 53, 43, 45, 47, 36, and 35 respectively. For F49A-FTP treated cultures infected with MOIs of 0.5, 0.1 and 0.05, the number of reactivation events was 1, 4 and 5, respectively. F49A-FTP was, therefore, still very effective at clearing latent virus, although a slight drop in efficacy was noticeable at MOIs of 0.1 and 0.05. Means and error bars (showing standard deviations) were generated from three independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as *, ** indicating p= 0.05 or p=0.01, respectively.

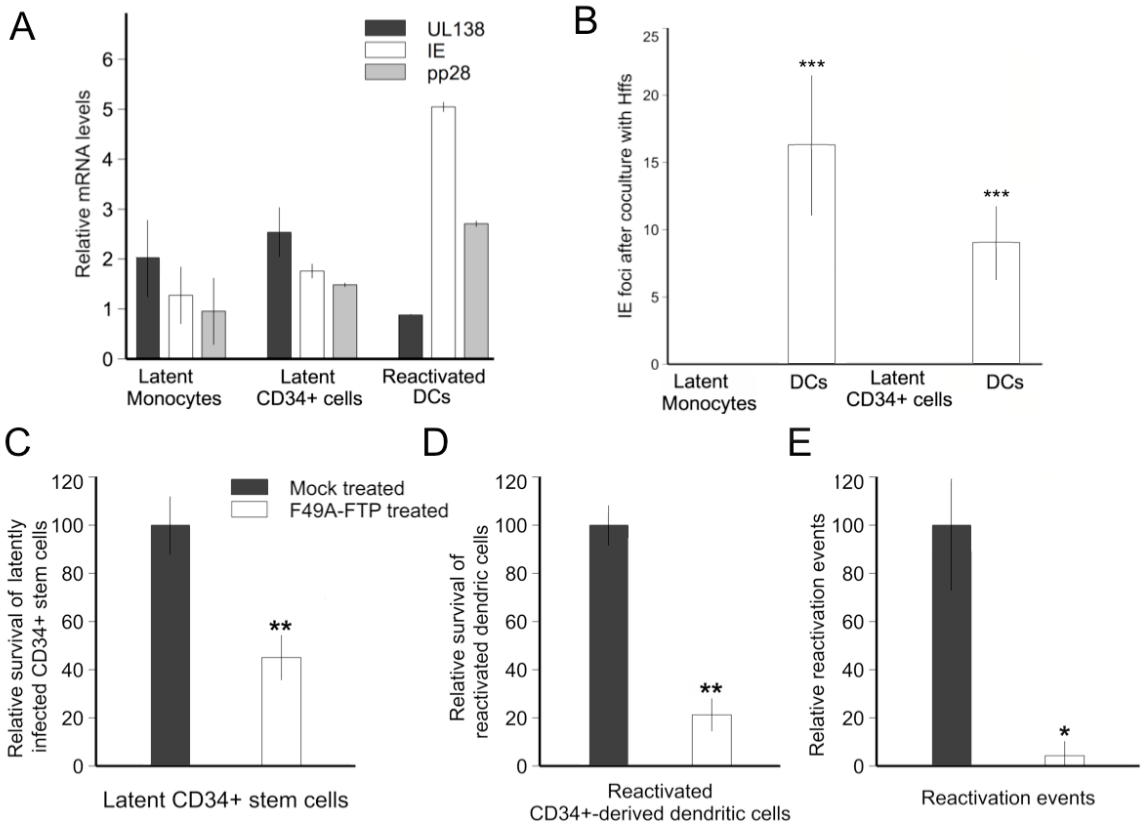

Figure 5.12 Infected CD34+ cells display hallmarks of latent infection and can be killed by F49A-FTP. A) CD34+ progenitor cells were latently infected with SV40-GFP-TB40E at an MOI of 5, RNA was then harvested at four days post infection and RT-qPCR analysis of this RNA demonstrated that sorted monocytes express UL138 (a latency associated gene) to much higher levels than the lytic genes: immediate early or UL99. Data were normalised to GAPDH RNA. This was compared to reactivated, monocyte-derived, mature dendritic cells, the RNA from these was harvested 4 days post terminal differentiation by LPS treatment, as a positive control for a lytic transcription profile. Means and error bars (showing standard deviations) were generated from three measurements. B) Monocytes or CD34+ stem cells were infected with SV40-GFP-TB40E and either differentiated with cytokine treatment and matured, or mock treated. After this, cells were co-cultured with HFFs and incubated for 2 weeks. Cultures were then stained for IE gene expression and IE positive foci counted. Data show mean and standard deviations from three independent experiments. C) CD34+ progenitor cells were experimentally infected with SV40-GFP-TB40E at an MOI of 5. After 24 hours, F49A-FTP was added to CD34+ cell cultures and incubated for 72 hours. Changes in the number of GFP-positive latent cells were analysed by fluorescence microscopy and compared to a mock-treated control, which was set at 100% (C).

Figure 5.12 Infected CD34+ cells display hallmarks of latent infection and can be killed by F49A-FTP. D) These CD34+ cells were then differentiated to mature dendritic cells and changes in the percentage of GFP-positive mature dendritic cells were measured (D). E) Finally, the cells shown in (D) were co-cultured with fibroblasts for two weeks and the number of IE foci were counted (E). Means and error bars (showing standard deviations) were generated from three independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as *, ** or *** indicating p= 0.05, p=0.01 or p=0.001 respectively.

then analysed cell death by trypan blue staining. I noticed that the cytotoxicity of F49A-FTP towards CD34+ cells was reduced compared to CD14+ monocytes (Figure 5.13A). In order to confirm that the cytotoxicity of F49A-FTP to the uninfected cells was due to background binding of F49A-FTP to low-affinity CX3CR1 receptors, I repeated this analysis but blocked CX3CR1 with its natural ligand, fractalkine, while treating cells with F49A-FTP. For CD34+ cells, I increased the concentration of F49A-FTP to 10^{-7} M, so that I could see a measurable killing of uninfected CD34+ progenitor cells by F49A-FTP. As expected, fractalkine treatment with F49A-FTP was able to reduce cytotoxicity, confirming that F49A-FTP does indeed kill uninfected cells via off-target binding of CX3CR1 (Figure 5.13B). Finally, I tested whether such treatment with fractalkine could block any off-target CX3CR1 binding, thereby improving F49A-FTP efficacy on latently infected CD34+ cells. To do this, I used the optimised protocol for F49A-FTP treatment, in the presence of fractalkine, and differentiated monocytes to dendritic cells in order to measure reactivation events by co-culture with indicator fibroblasts. However, I could see no improvement in efficacy of F49A-FTP when I also treated with fractalkine as well (Figure 5.13C).

5.7 F49A-FTP is able to kill naturally latently infected monocytes.

Finally, having optimised a protocol for killing experimentally latently infected cells with F49A-FTP, which in experimentally infected CD14+ monocytes or CD34+ progenitor cells, resulted in a profound reduction in virus reactivation events after differentiation and maturation of latently infected cells, I tested whether F49A-FTP could target naturally latently infected monocytes. Such analysis of naturally infected cells is the best approximation to an allo-HSCT and so would be the best demonstration of F49A-FTP efficacy. To do this, I isolated latently infected monocytes from healthy seropositive donors and treated

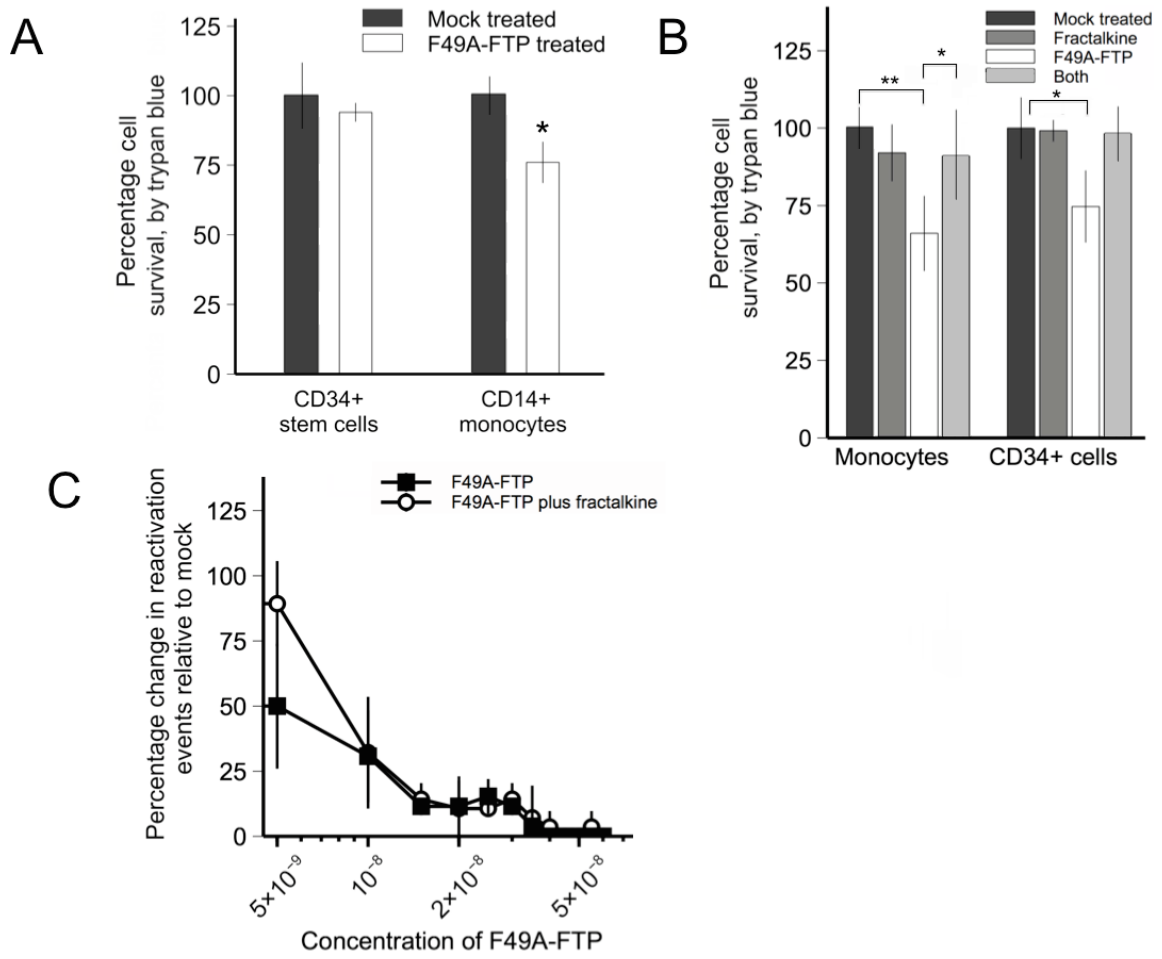

Figure 5.13 F49A-FTP kills uninfected cells by binding CX3CR1, which can be blocked by fractalkine. A) CD14+ peripheral blood monocytes, or CD34+ cells were isolated and treated with $5x10^{-8}$ M F49A-FTP. After 72 hours, cell death was quantified by trypan blue staining and light microscopy. B) CD14+ peripheral blood monocytes, or CD34+ cells were isolated and treated with either 10^{-7} M F49A-FTP alone, 10^{-7} M fractalkine, or both F49A-FTP with fractalkine. After 72 hours, cell death was quantified by trypan blue staining and light microscopy. C) CD14+ peripheral blood monocytes were isolated and experimentally infected with SV40-GFP-TB40E at an MOI of 5. After 24 hours, monocytes were treated with either $5x10^{-8}$ M F49A-FTP alone, or F49A-FTP with 10^{-7} M fractalkine. After 72 hours, media was changed and cytokines were added to induce monocyte differentiation and reactivation. These CD14+ monocyte-derived mDCs were co-cultured with HFFs, and foci of viral IE expression were quantified by immunofluorescent staining, as a measure of the reactivation of infectious virus. Black squares indicate the change in reactivation events from monocytes treated with F49A-FTP alone, while white circles indicate the change in reactivation events from monocytes treated with F49A-FTP with fractalkine. Means and error bars (showing standard deviations) were generated from three independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as *, ** indicating p= 0.05 or p=0.01, respectively.

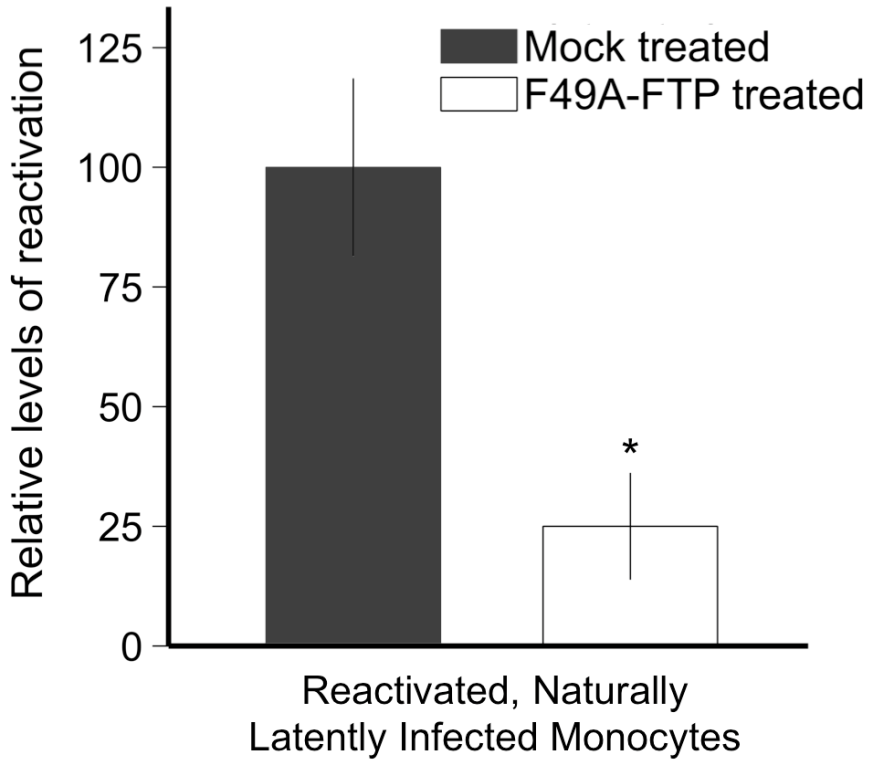

Figure 5.14 F49A-FTP kills naturally latently infected monocytes, reducing reactivation events. $1.2x10^8$ blood monocytes were isolated from three CMV-positive blood donors. Half of these cells were incubated with F49A-FTP for 72 hours. Monocytes were then differentiated using GM-CSF and IL-4 stimulation followed by LPS treatment. Monocyte-derived mDCs were then co-cultured with fibroblasts for three weeks, and the number of reactivated IE foci were detected by immunoflourescent staining. Means and error bars (showing standard deviations) were generated from three independent experiments. Statistical analyses were carried out using a paired two-tailed t-test and P-values expressed as * indicating p=0.05.

them with F49A-FTP, as before. To assess any reduction in naturally latent load in these cells, I then differentiated and matured the monocyte cultures, to mDCs, to induce virus reactivation. Subsequent to this, I measured reactivation of infectious virus by co-culture with HFF indicator cells, followed by immunofluorescent staining for IE-positive foci in these fibroblast co-cultures. Figure 5.14 clearly shows that treatment of naturally latently infected monocytes with F49A-FTP also causes a strong reduction in viral reactivation from these fusion-toxin treated, naturally latent cells.

5.8 Discussion

HCMV can establish latent infection in early myeloid lineage cells [391] which are a major cell type present in haemotopoetic stem cell transplants. The presence of latent HCMV in allogenic haematopoetic stem cell transplants can lead to serious medical complications [434], especially in seronegative recipients of grafts from seropositive donors. This disease is due to reactivation of latent cytomegalovirus after differentiation of these early myeloid cells to terminally differentiated macrophages and DCs, a process which is necessary for the restoration of immune system function in allo-HSCT recipients. Currently, there is only one example of a strategy to selectively kill naturally latently infected CD34+ cells by drug treatment [483], as well as one immunotherapeutic strategy to target latently infected monocytes [207, 493], (and see Chapter 4). None of these therapies are currently used clinically, and the potential toxicity of the vinca alkaloid vincristine [483] as well as undesirable side effects of valproate treatment [207] mean that other therapies against HCMV latency are likely to be needed.

Recently, an antiviral FTP, F49A-FTP, was published by Spiess et, al. This FTP exploits cell surface expression of US28 during lytic infection to kill lytically infected fibroblast cells [412]. I demonstrated in this chapter that other viral factors, expressed during lytic infection, do not play a role in the selectivity of F49A-FTP for killing cells lytically infected with HCMV by showing that F49A-FTP cannot kill HFFs infected with a US28-deletion virus (Titan-ΔUS28) but is able to clear HFFs lytically infected with Titan-WT which expresses US28. In addition to this, I used THP-1 cells expressing various US28 constructs to show that F49A-FTP kills monocytic cells expressing US28 in isolation and that the Y16F mutation in US28, which is known to abrogate US28 binding of fractalkine, also abrogates binding of F49A-FTP. This information is useful for hypothesising how US28 could become mutated during HCMV lytic infection to escape F49A-FTP treatment. This is important as in Chapter 4, I showed that US28-Y16F protein signals just as well as US28-WT, including being able to signal to establish latency, as such escape mutations in the N terminal region of US28 would suffer only minor fitness costs, and so the emergence of these mutants could be problematic after F49A-FTP treatment. I also noted that US28-R129A, which is a US28 mutant that cannot perform G-protein coupled signalling, appears to allow less killing by F49A-FTP than US28-WT. Expression of US28-WT and US28-R129A in COS-7 cells suggested that the two constructs are recycled at the same rate, and US28-R129A does not have abrogated binding to fractalkine [472]. As such, the reason for this lower efficacy remains unknown.

We and others have shown that US28 mRNA is also expressed in latently infected early myeloid lineage cells and that US28 expression is necessary for the establishment of latent infection in these cells [27, 78, 137, 161, 326] (and see Chapters 3 and 4). However to date, there were no data confirming the expression of US28 protein on the cell surface.

Using radiolabelling affinity assays, we have confirmed that US28 protein is, indeed, present on the cell surface of latently infected CD14+ monocytes and that CX3CR1 expression is not significantly modified by viral infection. With this information, I began to optimise a protocol for killing latently infected monocytes with F49A-FTP. I could use this protocol to measure the efficacy of F49A-FTP killing of latently infected monocytes, either by decreases in GFP-positive monocytes, GFP-positive mDCs or changes in IE-positive foci. This showed that treatment with $5x10^{-8}$ M F49A-FTP, for 72 hours, was sufficient to kill most latently infected monocytes, and thereby essentially clear reactivation events from treated cultures. Increases in this concentration led to increased killing of uninfected cells, by binding of CX3CR1, to which F49A-FTP has residual affinity. As expected, another FTP, CX3CL1-FTP, which has lower specificity for US28 over CX3CR1, was less effective at killing latently infected cells.

Once I was convinced that I had an optimised protocol for killing latently infected monocytes with F49A-FTP, I further demonstrated the efficacy of F49A-FTP, by showing that F49A-FTP treatment of monocytes and CD34+ progenitor cells led to a reduction in GFP-positive monocytes, a reduction in GFP-positive CD34+ cells, subsequent reductions in GFP-positive mDCs and corresponding reductions in IE-positive foci of reactivation. I also showed in monocytes that F49A-FTP is still effective when monocytes are infected with a low MOI of SV40-GFP-TB40E and that delaying treatment did not effect F49A-FTP killing of latently infected cells, ruling out a role of incoming virion US28 in this killing.

Finally, recognising that the aim of this chapter was to show that F49A-FTP could be used to treat allo-HSCTs, I confirmed that naturally latently infected monocytes were also targeted by F49A-FTP by demonstrating a reduction in the number of reactivation events after F49A-FTP treatment and monocyte differentiated and maturation.

Based on these observations, it is possible that such a fusion-toxin protein treatment, targeting US28, could act as the basis for a novel treatment to reduce the clinical threat of HCMV reactivation after allogenic HSCTs. Additionally beneficial, as infectious virus is not produced during latency, resistance to F49A-FTP due to selection of replicating, drug-resistant mutants is much less likely to occur during treatment of latently infected cells. I did notice, however, that there were occasional reactivation events, from F49A-FTP treated cultures, even at higher concentrations (see Figures 5.4, 5.6 and 5.7). In chapter 7, I

address this issue and show that there is a sub-population of virus, in our laboratory stocks of SV40-GFP-TB40E, that carries a US28 mutation, making it resistant to F49A-FTP treatment.

I also noted that F49A-FTP treatment of naturally latently infected cells did not completely ablate all reactivation events and that 72 hours of F49A-FTP treatment were required to clear most reactivation events from latently infected cells. This period may be inconveniently long in the context of an allo-HSCTs and that modification of F49A-FTP would likely be required to further optimise F49A-FTP efficacy.

I chose a concentration of $5x10^{-8}$ M F49A-FTP as drug titrations in experimentally infected monocytes indicated that this was the minimum dose required to clear latent HCMV, while minimising killing of uninfected monocytes (Figure 5.4). However, it is possible that higher concentrations of F49A-FTP could be used, especially in CD34+ cells, as there where no signs of cell death at anti-virally active concentrations of F49A-FTP (Figure 5.13). This is particularly important to note as allo-HSCTs are often CD34+ cells, depleted of other cell types [249], and so a higher concentration of F49A-FTP could be tolerable and more effective at killing latently infected cells. I, however, did not titrate F49A-FTP on CD34+ cells comprehensively, as these cells were in short supply.

Similarly, I have shown that the selectivity of F49A-FTP for US28, compared to CX3CL1-FTP, confers a stronger killing profile on F49A-FTP. If this specificity could be improved further, perhaps with more point mutations in the amino acid sequence of F49A-FTP, this may confer even better killing against latently infected cells. The recent solution of the US28 crystal structure in complex with CX3CL1 [50], may provide a basis for such further refinements to F49A-FTP, which could increase the selectivity of F49A-FTP for US28 and, therefore, the efficacy of F49A-FTP against naturally latently infected cells, which may be beneficial in the context of allo-HSCTs.

In summary, I have demonstrated that latently infected early myeloid cells express US28 on the cell surface, and that this can be targeted by the anti-viral fusion-toxin protein, F49A-FTP. This results in killing of both experimentally and naturally latently infected myeloid cells and the subsequent reduction of HCMV reactivation events. These findings are proof of principle that F49A-FTP can reduce the latent load of HCMV in early myeloid lineage cells which could form the basis for a novel approach to greatly reduce the clinical threat of HCMV positive grafts in haematopoetic stem cell transplants.

Chapter 6

The role of CTCF in HCMV latency

6.1 Introduction

HCMV establishes latency in early myeloid lineage cells, a state which is characterised by limited viral gene expression and, especially, no viral immediate early protein expression. This restricted IE gene expression is due to repression of the MIEP during latent infection of early myeloid lineage cells. The MIEP is an incredibly strong promotor; it drives expression of IE genes and the subsequent temporal cascade of lytic gene expression which leads to virus production and, so, is repressed by many factors during latency [391]. This includes both viral [136, 190] and cellular factors [18, 228, 507] inducing cellular transcriptional repression, all of which likely result in the establishment of repressive chromatin around the MIEP [387].

In Chapter 4, I showed that US28 is necessary for HCMV latency. To determine how US28 maintains latency, I collaborated with Dr James Williamson in Paul Lehner's Laboratory (Cambridge) to perform a mass spectrometry screen to measure changes in the abundance of different cellular proteins between THP-1 cells expressing US28 and THP-1 cells transduced with an empty vector (to control for possible changes induced by stochastic insertion of lentiviral vectors into the genome). This screen suffered from poor signal to noise ratio, but highlighted CCCTC-binding factor (CTCF) as a protein which increased in abundance in the presence of US28.

CTCF piqued my interest because it has been shown to repress the MIEP during lytic HCMV infection, by binding a DNA motif in the first intron of the MIE genes [244] and has also been implicated in the maintenance of latency by repression of lytic gene expression in HSV-1 [77], EBV [441] and KSHV [182, 418]. This cellular factor is a highly conserved

11-zinc-finger protein that regulates gene expression through the recruitment of transcription factors and subsequent rearrangement of higher-order chromatin structure [312].

I, therefore, hypothesised that CTCF may interact with, and repress, the MIEP during latent infection in order to repress IE gene expression and maintain latency and that US28 may induce CTCF expression in latently infected cells to achieve this aim. In this chapter, I will show that CTCF does indeed interact with the MIEP to repress activity, and that this repression serves to reduce IE gene expression. Finally, I discuss potential ways to continue this analysis as well as improvements which could be made to our mass spectrometry screen in order to investigate potential changes in THP-1 cell protein expression during ectopic US28 expression. Together, the findings in this chapter may lead to a better understanding of how US28 maintains HCMV latency in the myeloid lineage.

6.2 Results

In Chapter 4 I showed that US28 is necessary for HCMV latency. Consequently, I wanted to investigate the mechanisms by which US28 maintained latency in early myeloid lineage cells. To achieve this aim, in collaboration with Dr James Williamson, I performed a mass spectrometry screen, to measure changes in protein abundance between THP-1 cells expressing HA-US28-WT and cells transduced with an empty vector control. This screen measured changes in peptide abundance, from trypsin-digested proteins, using unlabelled protein samples, analysing changes in peptide abundance by measuring changes in the peak generated by mass spectrometry analysis of these peptides. The screen detected at least three peptides from both samples for 2723 proteins, of which 471 changed greater than 2-fold between samples, and 181 greater than 4-fold (Figure 6.1).

This is a very large number of changes, and I believe that it would be difficult to attempt to verify such a large number of potential leads. I believe that these many changes are due to cell stress induced by ectopic US28 protein expression. Supporting this assertion, most of the proteins which were increased in abundance by the greatest amounts were associated with apoptosis, the unfolded protein response and cellular trafficking (for a full dataset of this screen, see the Appendix). Nevertheless, six peptides originating from the transcriptional repressor protein CTCF were detected from THP-1 cell samples expressing US28, none of which were detected in THP-1 cells transduced with the empty vector control. As there were already publications linking CTCF to herpesvirus latency [182, 418, 441] and HCMV MIEP repression [244], I decided to investigate if CTCF played a role in HCMV latency.

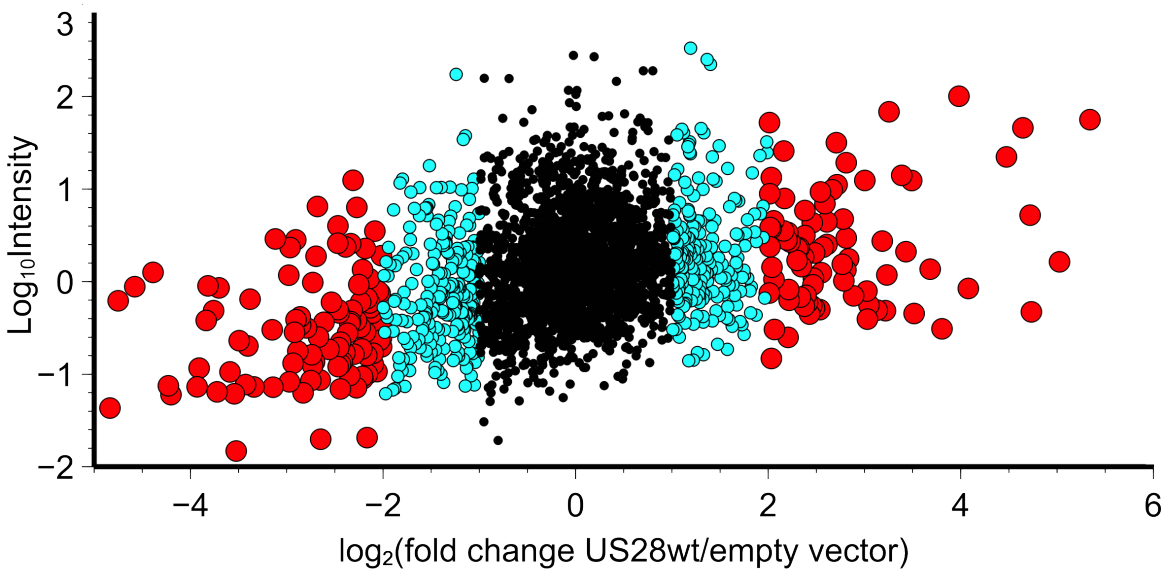

Figure 6.1 Expression of US28 protein in THP-1 cells leads to many changes in overall protein abundance, compared to cells tranduced with an empty vector control. Scatterplot of proteins identified by mass spectrometry screening, and quantified by >3 unique peptides. The summed ion intensity (y-axis) is shown as log10 while the fold difference between ion intensities is displayed as log2 (x-axis). Proteins which showed less than 2-fold change are displayed in black, those which showed between 2 and 4 fold change are in cyan and those which showed greater than 4-fold change are in red. Mass spectrometry analysis and data collection was performed by Dr James Williamson.

6.2.1 CTCF protein can be detected more easily in the presence of US28, but CTCF mRNA does not change.

I wanted to validate that CTCF protein increased in abundance in THP-1 cells expressing US28 in isolation, compared to THP-1 cells transduced with an empty vector control, as suggested by mass spectrometry analysis. To do this, I immunoblotted for CTCF protein in THP-1 cells expressing HA-US28-WT against those transduced with an empty vector; additionally, as I knew that the US28 mutant HA-US28-R129A is unable to maintain latency, but HA-US28-Y16F is able to maintain latency, I also included these mutant THP-1 cell lines in my analysis as well, to control for the problems associated with ectopic protein overexpression. Immunoblotting analysis appeared to detect more CTCF protein in THP-1 cells expressing HA-US28-WT and HA-US28-Y16F compared to either HA-US28-R129A or the empty vector controls (Figure 6.2A). However, RT-qPCR analysis showed no differences in CTCF mRNA levels (Figure 6.2B), suggesting that US28 expression alone does not induce increases in steady state CTCF RNA transcription, but may change the abundance of CTCF protein or the detectability of CTCF protein by immunoblot. To confirm this finding, I stripped the blot that had been used in Figure 6.2A, and reblotted using a different clonal antibody against CTCF (ab128873). As seen in Figure 6.2C, this antibody also showed a difference in CTCF protein abundance between THP-1 cells expressing HA-US28-WT and HA-US28-Y16F compared to either HA-US28-R129A or the empty vector controls, but also showed changes in a protein band at 70 kDa, in contrast to the change in the band at 150 kDa, as seen previously.

Finally, I infected monocytes with either Titan-WT, Titan-ΔUS28 or UV inactivated virus and analysed CTCF expression by RT-qPCR. In this analysis, latent infection with Titan-WT virus did appear to induce CTCF expression, while lytic infection, resulting from infection with Titan-ΔUS28, did not (Figure 6.2D).

6.2.2 CTCF interacts with the MIEP and mediates MIEP repression

As US28 signalling is necessary for the establishment of latency in monocytes, and appears to induce changes in CTCF in monocytic THP-1 cells, I reasoned that these changes in CTCF could act to repress the MIEP. Consequently, I reasoned that reducing CTCF expression in THP-1 cells should reduce repression of MIEP activity and overexpression of CTCF should increase MIEP repression. To analyse this, I transduced THP-1 cells with CTCF in phRSIN lentiviral vector, or with empty vector control, and also nucleofected THP-1 cells with a commercial anti-CTCF shRNA or used an empty vector control. I was able to

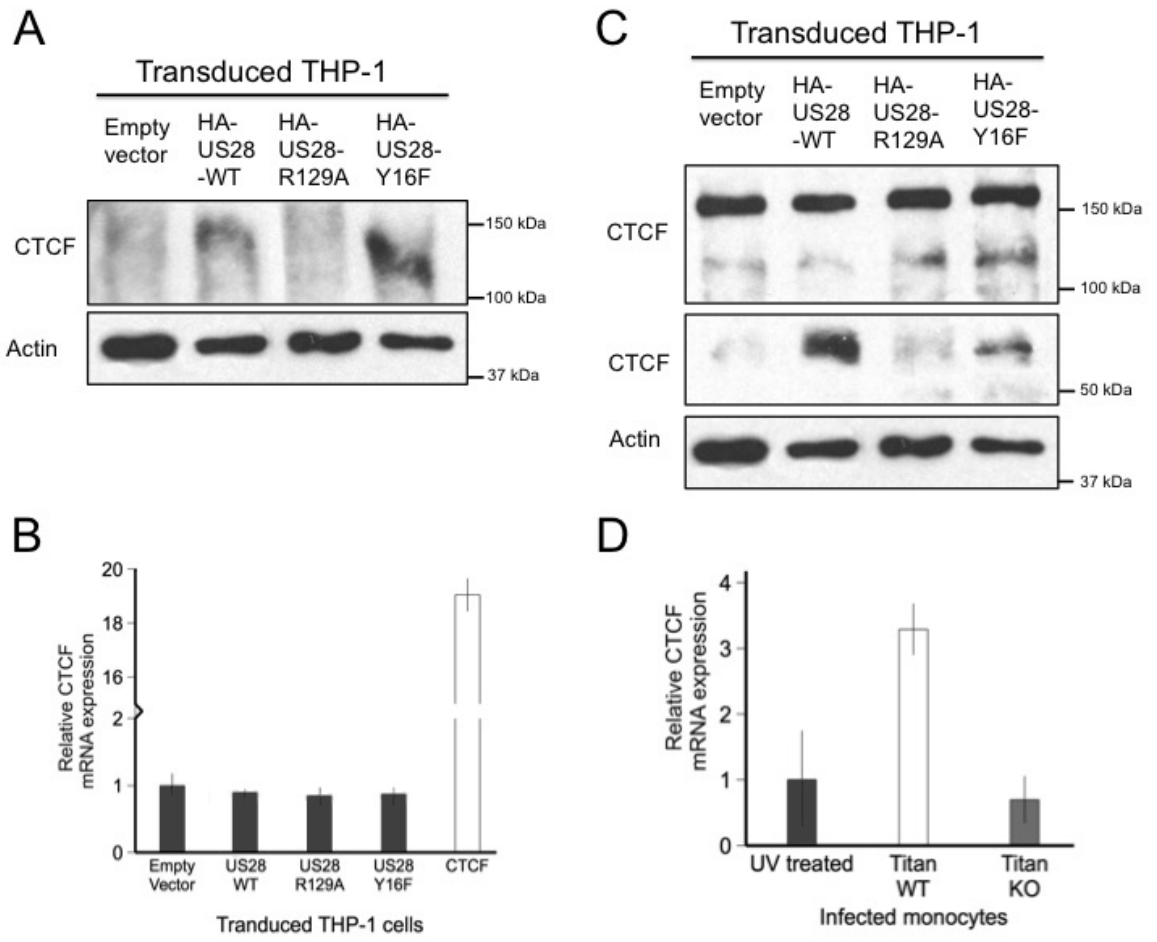

Figure 6.2 CTCF protein expression changes due to US28 expression, and CTCF mRNA expression increases during latent infection, but not due to US28 expression. THP-1 cells stably expressing HA-US28-WT, HA-US28-R129A or HA-US28-Y16F were generated by lentiviral transduction and puromycin selection, including an empty vector transduced control. A) Immunoblot analysis using a polyclonal antibody against CTCF (ab70303) was carried out on these four cell lines. B) RT-qPCR analysis on the change in CTCF mRNA levels between the THP-1 cells expressing these four US28 constructs, and THP-1 cells transduced with the same vector containing CTCF, as a positive control. RT-qPCR data was normalised to GAPDH controls, bars represent means from three values, and error bars show standard deviations. C) Repeated immunoblot analysis using a different polyclonal antibody against the CTCF (ab128873) was carried out on these four cell lines, this blot has been cropped to show bands that additionally appeared around 70 kDa as well as bands around 150 kDa. D) Finally, peripheral blood CD14+ monocytes were infected with either Titan-WT, Titan-ΔUS28 or UV inactivated virus, and five days post infection, RNA was harvested. This RNA was analysed by RT-qPCR for changes in CTCF expression, displayed relative to UV-inactivated virus. RT-qPCR data was normalised to GAPDH controls, bars represent means from three values, and error bars show standard deviations. Panel D was performed by Elizabeth Elder under my supervision.

reduce CTCF protein expression with this shRNA construct and also overexpress CTCF using the CTCF-phRSIN vector (Figure 6.3A). I then nucleofected these three THP-1 cell lines with an MIEP-luciferase construct [309] and assessed the ability of CTCF to regulate the MIEP. CTCF overexpression did indeed repress MIEP activity. In contrast, reducing CTCF expression led to an increase in MIEP activity (Figure 6.3B).

Additionally, I wanted to show that CTCF interacts with the MIEP in monocytes. To show this, I isolated CD14+ monocytes from blood donations and infected monocytes with Titan-WT, Titan-ΔUS28 or UV inactivated virus. Five days after infection I performed immunoprecipitation assays on the lysates from these cultures, immunoprecipitating with either a CTCF-specific antibody or an isotype control antibody. After this, I used qPCR analysis to quantify the abundance of MIEP in samples, and compared the fold enrichment of MIEP between CTCF precipitation and the isotype control (Figure 6.4). I found that in both Titan-WT and Titan-ΔUS28 infected monocytes, I saw some enrichment for the MIEP-CTCF immunoprecipitations, and that this effect was stronger in Titan-WT infected cells which establishes latent infection. This indicates that during HCMV infection, CTCF interacts with the MIEP and that it likely acts more strongly during latent infection, likely in order to repress the MIEP.

6.2.3 Changes in CTCF expression affects HCMV latency in THP-1 cells

To corroborate my findings that CTCF expression represses activity from the MIEP in THP-1 cells, I also infected THP-1 cells which were overexpressing CTCF or knocked down for CTCF, with either Titan-WT or Titan-ΔUS28, to see whether CTCF affected immediate early gene expression (which should be repressed during latency). As expected, infection of THP-1 cells, transduced with shRNA against CTCF (which reduced CTCF protein expression) with Titan-WT virus led to increased IE expression, as measured by RT-qPCR (Figure 6.5). Similarly, infection of THP-1 cells, transduced with a CTCF vector (which increased CTCF protein expression) with Titan-ΔUS28, led to reduced IE expression in these cells (Figure 6.5).

6.3 Discussion

Having established that US28 is necessary for HCMV latency in Chapter 4, I collaborated with Dr James Williamson to perform a mass spectrometry screen in order to identify cellular

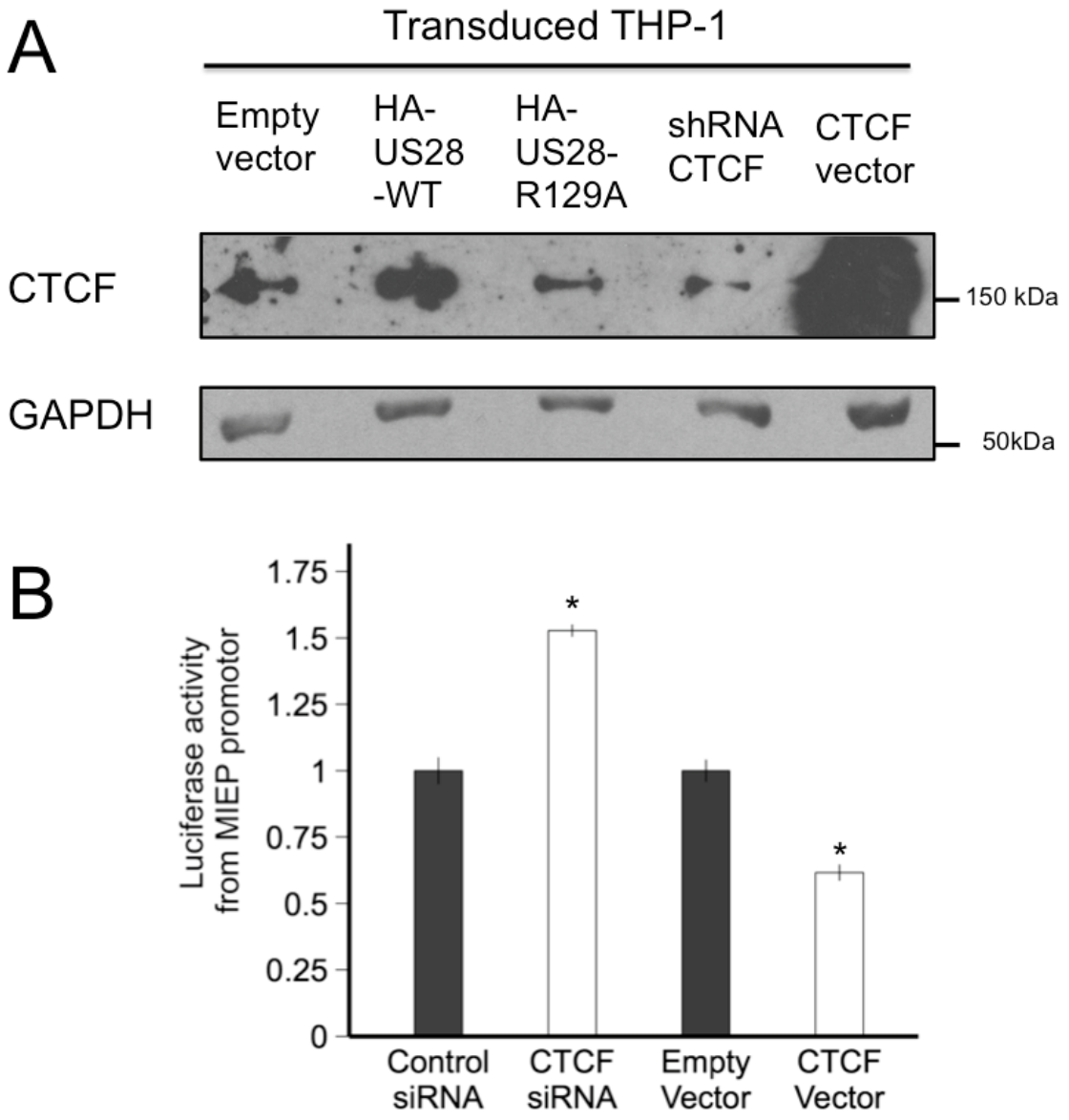

Figure 6.3 CTCF expression represses the MIEP, while knock down of CTCF expression reduces this repression. A) THP-1 cells were transduced by lentivirus with different constructs, from left to right: empty vector pHRSIN control, HA-US28-WT, HA-US28-R129A, shRNA against CTCF and phRSIN containing CTCF cDNA. After transduction, cells were lysed, proteins harvested and immunoblot analysis performed against CTCF using the polyclonal CTCF antibody ab70303. B) THP-1 cells were nucleofected with an MIEP-luciferase construct and luciferase assays were performed two days after nucleofection. Data was normalised to renilla-luciferase, all data points show means from two independent experiments, and error bars show standard deviations. P-values * (P=0.05) were considered significant. These two experiments were performed by Elizabeth Elder under my supervision.

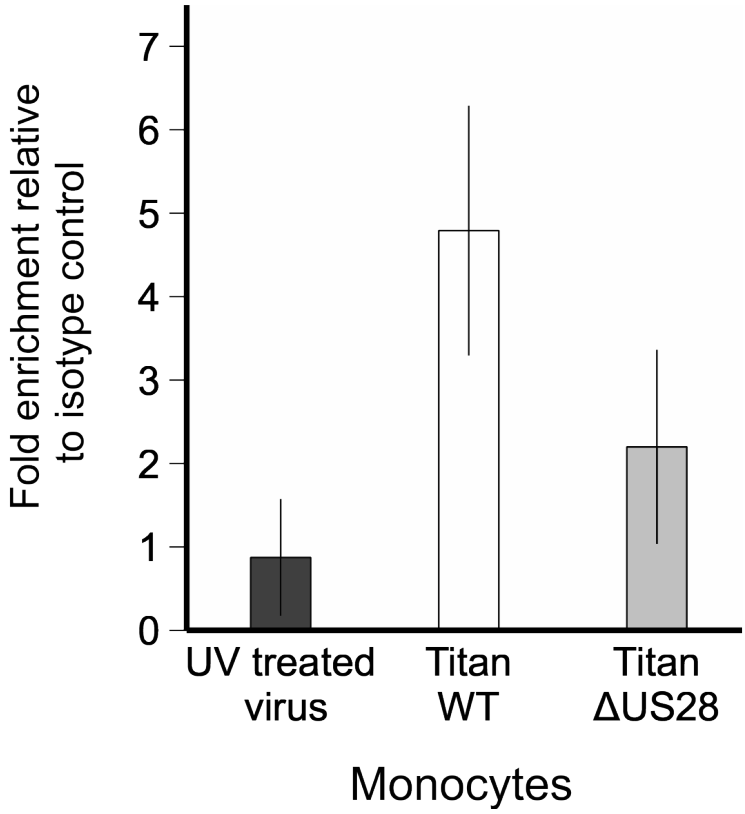

Figure 6.4 The MIEP can be immunoprecipitated with CTCF. Peripheral blood CD14+ monocytes were infected with UV inactivated virus, Titan-WT, or Titan-ΔUS28 respectively. Five days after infection, CTCF was immunoprecipitated or samples were mock treated with a rabbit isotype control. After this, MIEP was quantified by qPCR and changes in MIEP abundance between CTCF immunoprecipitation and isotype control were measured. qPCR data represents means from three values, and error bars show standard deviations. This experiment was performed by Elizabeth Elder under my supervision.

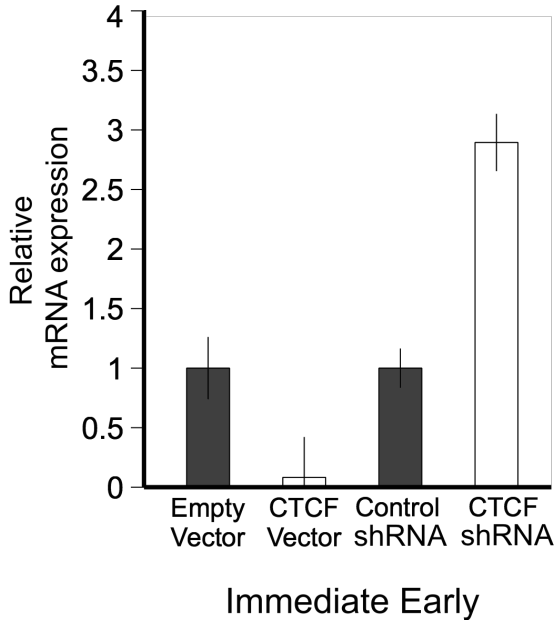

Figure 6.5 CTCF expression represses IE mRNA expression, while knock down of CTCF expression increases IE expression. THP-1 cells transduced to express shRNA against CTCF (or scrambled shRNA as a control), CTCF (or the empty vector control), were infected with Titan-WT (right two bars), or Titan-ΔUS28 (left two bars) respectively. Five days after infection, RNA was harvested. This RNA was analysed by RT-qPCR for changes in IE expression, displayed relative to controls. RT-qPCR data was normalised to GAPDH controls, bars represent means from three technical replicates, and error bars show standard deviations. This experiment was performed by Elizabeth Elder under my supervision.

proteins which change in abundance during ectopic HA-US28-WT expression in THP-1 cells. To help elucidate mechanisms by which US28 helps maintain latency, I started by comparing the abundance of proteins in THP-1 cells either expressing HA-US28-WT or transduced with an empty vector control, using unlabelled protein samples from these two cell lines. Despite the poor signal-to-noise ratio, I was able to identify CCCTC-binding factor (CTCF) as a potentially interesting hit, which was detected in the HA-US28-WT expressing THP-1 cells but not the empty vector transduced control cells. Although CTCF has known transcriptional activatory functions [296], in general, CTCF is a major transcriptional repressor which represses gene expression by modulating chromatin structure [296, 312]. I, therefore, hypothesised that CTCF might repress the HCMV MIEP, helping to maintain latency and that US28 increases CTCF expression in order to achieve this. This model is not without precedence, as CTCF has already been shown to repress the MIEP during primary, lytic HCMV infection [244] as well as maintain latency by repression of lytic gene expression from EBV [441], HSV-1 [77], and KSHV [182, 418].

To start, I wanted to confirm our mass spectrometry data, which indicated that CTCF protein abundance is increased in THP-1 cells expressing HA-US28-WT. Using immunoblotting, I was able to detect more CTCF protein in THP-1 cells expressing HA-US28-WT compared to empty vector transduced control cells, as well as cells expressing HA-US28-R129A. This confirmed the mass spectrometry data, and was consistent with the knowledge that HA-US28-R129A cannot maintain latency. However, RT-qPCR data of mRNA expression between these three cell lines showed no difference in mRNA levels. To confirm that this change in CTCF detectability was not antibody specific, I repeated my immunoblot with a different commercial antibody, which also showed changes in a CTCF protein band at 70 kDa.

Taking all these data into account, I believe that CTCF is likely to be post-translationally modified by US28, which improves its detection by mass spectrometry and immunoblotting, but does not change its mRNA levels. Supporting this assertion, both commercial antibodies used to detect CTCF by immunoblot (ab70303 and ab128873 from AbCam) were raised against a CTCF peptide corresponding to the region between amino acids 650-700, which also contains a site for SUMOylation [240]. CTCF is known to be SUMOylated, and this SUMOylation is known to activate transcription in at least one model [195]. In contrast, CTCF is desumoylated during hypoxic stress [479]. I, therefore, hypothesise that US28 in THP-1 cells mediates the deSUMOylation of CTCF, which makes it more detectable by immunoblot using the ab70303 antibody, and this deSUMOylation of CTCF mediates a repressive function of CTCF which acts to repress the MIEP. Regarding the second immunoblot, detection of

CTCF by ab128873 appears not to be masked by SUMOylation (at 150 kDa). However, there appears to be a new CTCF band at 70 kDa. This 70 kDa band for CTCF has been described previously [197, 245] as a possible N-terminally truncated CTCF protein generated by alternative translation of CTCF mRNA which is known to enhance CTCF transactivation of a p90TK-luciferase construct in COS6 cells [199]. Whether this 70 kDa version of CTCF plays a role in HCMV latency, is not clear.

In order to fully confirm that CTCF is deSUMOylated by HA-US28-WT, it would be necessary to find an antibody which binds to CTCF regardless of SUMOylation status. A survey of commercial antibodies against CTCF indicates that most companies raised antibodies against the same region (amino acids 650-700) or were raised against the N-terminal domain of CTCF which also contains a SUMOylation site [240]. One possibility could be to purchase a large number of antibodies against CTCF and screen them for sensitivity to SUMOylation. Another possibility would be to generate a tagged CTCF construct and nucleofect this construct into THP-1 cells expressing HA-US28-WT, HA-US28-R129A or the empty vector transduced control cells. I could then immunoprecipitate CTCF and immunoblot for SUMO protein. To these ends, I have generated a triple-FLAG tagged CTCF construct. As stated before, CTCF appears to interact and recruit histone deacetylases (HDACs) [158, 237] which are also associated with repression of the MIEP during HCMV latency [19, 141, 496], especially HDAC4 [207]. I will, therefore, also see if CTCF immunoprecipitates with HDAC4, as this could demonstrate one mechanism for CTCF-mediated repression of the MIEP. Additionally, it may be possible to immunoprecipitate CTCF using the FLAG-tag and analyse, by mass spectrometry, changes in CTCF post-translational modifications, such as phosphorylation [111, 198] and poly-(ADPribose)-ylation [503]. Finally, as I have already shown that CTCF expression represses the MIEP, it would be possible to generate CTCF mutants, with mutations in SUMOylation sites, to investigate whether expression of these mutants still represses the MIEP. This would help to demonstrate that CTCF SUMOylation plays a role in HCMV latency.

A number of other analyses were also consistent with CTCF playing a role in HCMV latency. In immunoprecipitation assays, CTCF binds the MIEP in Titan-WT infected monocytes and CTCF also represses the MIEP when overexpressed in THP-1 cells. This demonstrates that there must be CTCF binding sites in the MIEP, as well as in exon 1 of the MIE (since our nucleofected construct only contained the MIE promotor). Additionally, CTCF over-expression reduces immediate early gene expression in THP-1 cells lytically infected with Titan-ΔUS28, while knock down of CTCF expression leads to increased IE mRNA expression in THP-1 cells latently infected with Titan-WT. I believe that these data indicate that CTCF

plays a role in repressing IE mRNA expression during latency. I recognise that many of these differences are fairly small compared to the major changes seen in Titan-ΔUS28 infected monocytes, however, I believe that CTCF is likely to be only one part of a concerted set of mechanisms by which US28 helps to maintain HCMV latency. One such possibility is that CTCF protein binds to the HCMV genome in order to designate boundaries between regions of repressive and expressive chromatin structure, similar to its function in HSV-1 latency [77, 113]. In this model, CTCF likely binds to other regions of the HCMV genome, but not regions containing genes that are expressed during latent infection. It may, therefore, be possible to immunoprecipitate CTCF and use CHIP-Seq analysis to investigate where CTCF binds to the HCMV genome. This may help to identify latency-associated gene expression and potentially help our understanding of HCMV genome repression during latency.

Finally, a second proteomic screen was run, comparing THP-1 cells expressing HA-US28-WT to HA-US28-R129A, which overcame problems of cell stress due to protein overexpression (data can be found in the Appendix). This screen identified a number of other potentially interesting proteins modulated by US28; the levels of 48 proteins change by a factor of 2 or more when comparing wild type US28 and signalling mutant US28-expressing cells. I aim to select some of these proteins for further analysis, validate these in the context of latent infection and investigate their biological relevance. It will be essential to prioritise proteins based on their full validation and clear functional read outs for their latency-associated roles.

Chapter 7

Characterising viral isolates which are resistant to F49A-FTP

7.1 Introduction

HCMV disease results from productive infection of many cell types in multiple organs, which is uncontrolled by the immune system. The current favoured drug treatment for CMV disease in the immunocompromised is intravenous treatment with the nucleoside analogue ganciclovir [243]. Ganciclovir preferentially inhibits HCMV DNA polymerase [126] and is only active after its phosphorylation by the HCMV-enoded kinase UL97 [429]. Unfortunately, ganciclovir has associated cytotoxicity which can cause several severe side effects [117, 420] and viral resistance to ganciclovir is common, after long-term therapy, normally due to escape mutations in UL97 and also in the HCMV-encoded UL54 DNA polymerase (upon which ganciclovir acts) [225]. Although there are alternatives to ganciclovir, such as foscarnet, new antivirals against HCMV disease are needed.

For this reason, F49A-FTP, a fusion toxin protein which binds US28, was developed. F49A-FTP has potent antiviral activity against HCMV lytic infection *in vitro* and in mouse models of disease [412]. In Chapter 5 I demonstrated that F49A-FTP, could be used to target and kill latently infected cells as well. Spiess et. al, 2016 had already recognised that treatment with F49A-FTP raised the risk of viral resistance and, to address this, proposed to use F49A-FTP in conjunction with ganciclovir. These investigators also developed an CX3CL1-FTP-resistant viral isolate, by culturing infected fibroblasts with low concentrations of CX3CL1-FTP. This mutant, which has a premature stop codon at amino acid 258 (the viral isolate is now named US28 1-257) was trafficked to the cell surface, but could not bind chemokines [412]. It was proposed that this mutant may be unable to scavenge chemokines

and, therefore, be unable to evade the immune system. However there was no data to support this [412].

During work detailed in Chapter 5, I noticed that, occasionally, I observed viral reactivation events in F49A-FTP treated cultures, even at anti-virally activate concentrations (see Chapter 5). This was a concern, as it suggested that the FTP failed to kill some latently infected cells, which could then reactivate. Another possibility was that these reactivation events came from viral isolates which were already resistant to F49A-FTP. Either possibility could prove problematic in the context of treatment of allo-HSCTs, for example, where these reactivation events could lead to CMV disease. For this reason, I decided to characterise these reactivation events, that I observed in differentiated F49A-FTP treated monocytes, to see whether they were F49A-FTP resistant and, if so, how they developed this resistance.

In this chapter, I will show that I characterised twenty F49A-FTP resistant mutants and that a subpopulation of our SV40-GFP-TB40E viral stocks have a premature stop codon in the *US28* gene (called US28 1-219), which confers resistance to F49A-FTP. I will also show how F49A-FTP treatment generated another F49A-FTP-resistant mutant, US28 T11R, which has three point mutations in the N terminus of the US28 protein. Despite my concern that the US28 1-219 subpopulation might be unable to establish latency and could, therefore, be problematic for our experimental models of latent infection, I will show that US28 1-219 and US28 T11R are capable of establishing latent infection in CD14+ monocytes.

7.2 Results

7.2.1 Viral isolates, which reactivate after F49A-FTP treatment, are F49A-FTP resistant

To start, I wanted to determine whether virus which reactivated from F49A-FTP-treated monocyte cultures were F49A-FTP resistant mutants, or had survived a single treatment of F49A-FTP by chance. To test this, I experimentally infected CD14+ monocytes with SV40-GFP tagged HCMV and treated cultures for 72 hours with $5x10^{-8}$M F49A-FTP. I then differentiated and matured these monocytes to mDCs with cytokines and LPS, triggering HCMV reactivation. After this, I co-cultured these CD14+ monocyte-derived mDCs with HFFs and, because the virus has an SV40-GFP tag, analysed reactivation events by fluorescence microscopy as foci of GFP expression. I chose cultures where I saw only one reactivation event in the entire well after two weeks of infection, as these reactivation events would have come from a single viral isolate, allowing me to sequence the US28 from this

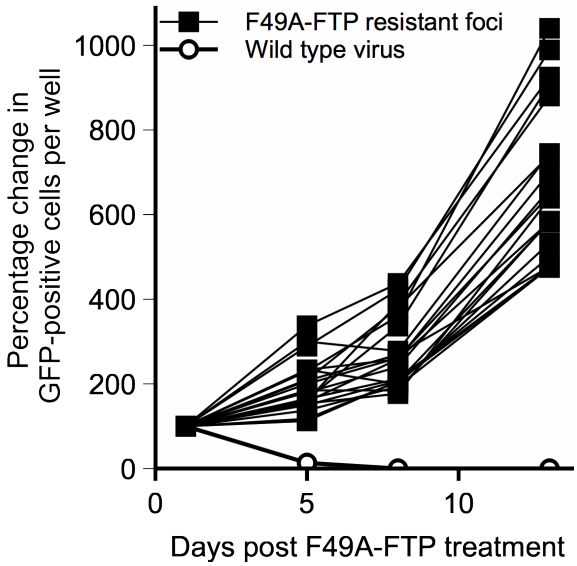

Figure 7.1 All twenty viral isolates, which reactivated after F49A-FTP treatment during latency, were F49A-FTP resistant. CD14+ monocytes from peripheral blood donations were isolated and experimentally infected with SV40-GFP-TB40E at an MOI of 5. After 24 hours, F49A-FTP was added to monocyte cultures at $5x10^{-8}$M. After 72 hours, media was changed and cytokines were added to induce monocyte differentiation and reactivation. These CD14+ monocyte-derived mDCs were co-cultured with HFFs and foci of viral reactivation observed by fluorescence microscopy against the GFP-tag expressed by the virus. Twenty cultures, in which only a single reactivation event was observed, were treated for a second time with F49A-FTP. As a positive control for F49A-FTP killing, HFFs were infected at an MOI of 0.1 with SV40-GFP-TB40E and treated with $5x10^{-8}$M F49A-FTP 48 hours post-infection. The number of GFP-positive cells were counted at the indicated days post-treatment. Values show a single result from a single experiment and so there are no error bars.

isolate by Sanger sequencing. This analysis identified twenty such cultures which I then retreated with F49A-FTP and quantified decreases in the number of GFP-positive cells, as a measure of how effective F49A-FTP was at killing cells lytically infected with these viral isolates. In all twenty cultures observed, F49A-FTP had no effect on lytically infected HFFs (Figure 7.1), compared to control HFFs, which were infected at an MOI of 0.1. This result demonstrated that these reactivation events were due to viral isolates that were resistant to F49A-FTP and not due to a failure of F49A-FTP to kill a few monocytes latently infected with wild type virus.

7.2.2 All resistant mutants isolated had the same premature stop codon in US28

It was important to know what sort of mutations could lead to resistance against F49A-FTP, as this could provide useful data on how US28 and F49A-FTP interact. In order to identify what mutations these F49A-FTP resistant mutants had, I harvested DNA from HFFs infected with all twenty isolates and amplified the *US28* gene, using primers against the *US28* region, and sequenced the *US28* genes from these twenty isolates. Surprisingly, all twenty isolates had the same point mutation, C655G, which mutates a glutamine to a premature stop codon (Figure 7.2). This truncated mutant of US28 (US28 1-219) has five of the seven transmembrane helices, but lacked two C terminal transmembrane helices, as well as the final extracellular loop and the C terminal intracellular domain. This mutant is also remarkably like the US28 1-257 mutation that was found by Spiess et. al, which cannot bind CX3CL1 [412].

7.2.3 The F49A-FTP resistant mutant, US28 1-219, is also present in laboratory stocks of SV40-GFP-TB40E

Given that there is, in our hands, no DNA replication during latent infection of CD14+ monocytes, mutants which confer antiviral-resistance are very unlikely to be produced during latent infection. Therefore, it seemed likely that US28 1-219 was already present in the virus stock that I used to latently infect the CD14+ monocytes. To test this, I infected HFFs at low MOI with the same stock of SV40-GFP-TB40E, and treated cultures with a low concentration ($1x10^{-9}$) of F49A-FTP for four weeks. After this, I treated the culture with $5x10^{-8}$M F49A-FTP, but saw no change in GFP-positive cells, indicating that the remaining virus was F49A-FTP resistant. I then plaque purified this virus, isolated ten plaques and sequenced the US28 genes from these plaques, as before.

Sequencing revealed that nine of these ten plaques had the same US28 1-219 mutation as discussed above. This strongly indicates that the US28 1-219 genotype is present as a minor subpopulation in our stock of SV40-GFP-TB40E, that is coincidentally resistant to F49A-FTP. As US28 protein expression is not necessary for HCMV lytic infection [252], this deletion may not confer a serious selective disadvantage.

The final mutant that I sequenced had a series of three point mutations in the N terminal region: T11R, D17N, and T25P, which I named "US28-T11R" (Figure 7.3).

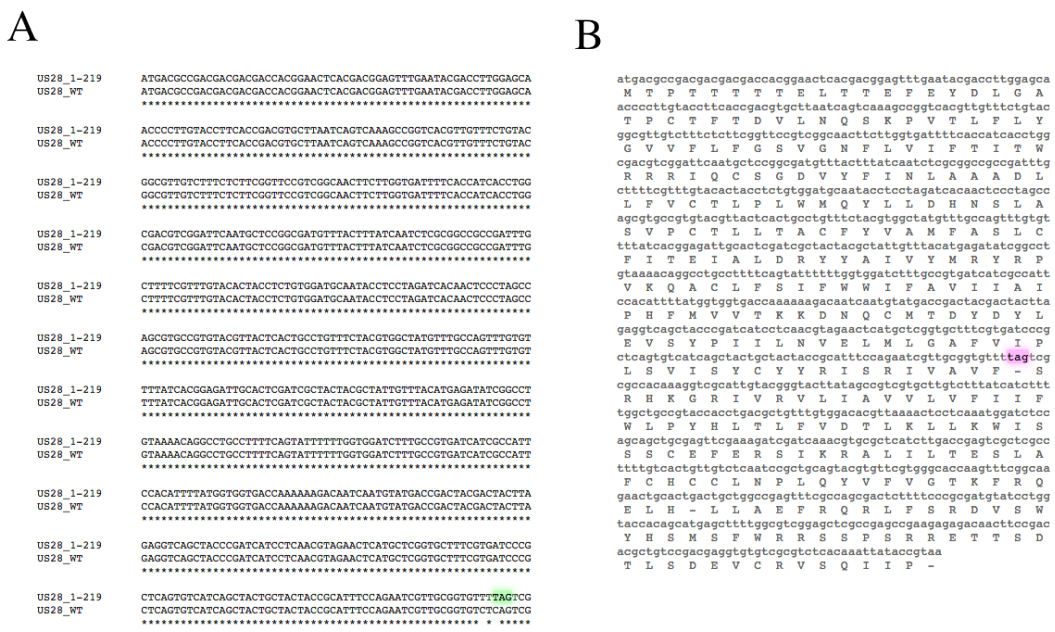

Figure 7.2 Sanger sequencing of the *US28* gene region for F49A-FTP resistant viral isolates revealed that all twenty isolates had a premature stop codon, conferring a US28 1-219 protein. F49A-FTP resistant clones where grown in HFFs and DNA was harvested. The US28 gene region was amplified and sequenced using Sanger sequencing by Source Bioscience (Cambridge, UK). A) Comparison of the twenty resistant clones (all identical) against the US28 gene from TB40E, using online Clustal Omega software, indicated that these clones have a C655G mutation (highlighted in green). B) Translation of the US28 1-219 sequence using online ExPASy software reveals that the C655G mutation confers a premature stop codon at amino acid 219 (highlighted in purple).

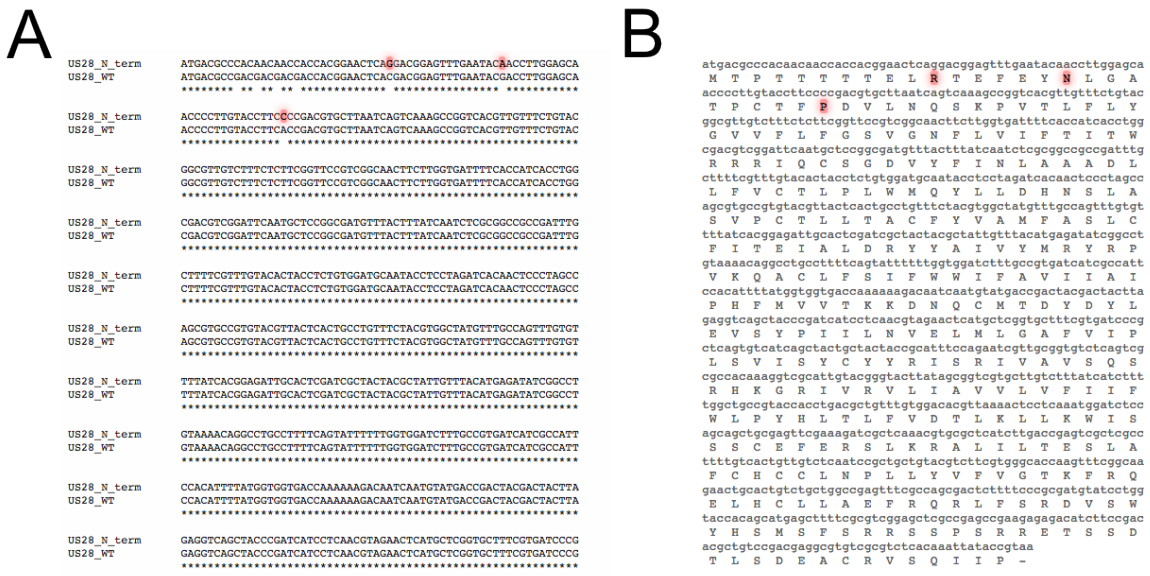

Figure 7.3 US28 T11R has three point mutations in the N-terminal region SV40-GFP-TB40E infected HFFs were grown in the presence of F49A-FTP to generate resistant clones. These clones were plaque purified and the *US28* gene region was amplified and sequenced using Sanger sequencing by Source Bioscience (Cambridge, UK). A) One mutant, which did not have the US28 1-219 mutation, was compared to the US28 gene from TB40E, using online Clustal Omega software, which indicated that this clone has six single nucleotide mutations, where the non-sense mutations are highlighted in red. B) Translation of the US28 T11R sequence using online ExPASy software reveals that these mutations confer three amino acid changes (highlighted in red)

7.2.4 US28 1-219 and US28-T11R establish latency

It has been previously observed that in a culture of experimentally latently infected monocytes, there are occasional cells (approximately 3 in 10^6 cells) which appear to be lytically infected, as detected by expression of viral immediately early 2 protein (personal observations). Additionally, by RT-qPCR analysis, I do see low levels of IE mRNA which could either be due to incomplete repression of the MIEP resulting in low levels of IE gene expression or due to a small number of *bona fide*, lytically infected cells which failed to establish latency (see Chapter 3). I reasoned that these observations could be due to cells being infected with US28 1-219, which is present in our stocks of SV40-GFP-TB40E, and may not be capable of signalling sufficiently to maintain latency (see Chapter 4). Consequently, in addition to this, I tested whether the US28 1-219 viral isolate could establish latency. Firstly, I infected CD14+ blood monocytes with US28 1-219 as well as wild type SV40-GFP-TB40E and, 5 days post infection, performed RT-qPCR analysis of viral gene expression in these monocytes (Figure 7.4A). I also infected monocytes with US28-T11R, as proof of principle that N-terminal mutations in US28 do not affect latent infection, (as seen in Chapter 4). RT-qPCR analysis showed no differences in viral gene expression between monocytes infected with these three isolates, indicating that US28 1-219 can, indeed, establish latent infection. Additionally, co-culture with HFFs indicated that these monocyte cultures do not release infectious virions, and so are not undergoing full, lytic infection (Figure 7.4B).

Finally, I was surprised to notice that US28 1-219, which is missing a large portion of its amino acid sequence, can still be expressed as a viable protein. As proof of principle that US28 1-219 is a viable protein, that can be expressed, I infected HFFs with US28 1-219, harvested the DNA, amplified the US28 gene region, adding an N-terminal HA tag onto the US28 gene, and cloned US28 1-219 into an expression vector. I then transfected HEK 293T cells with this vector, harvested protein after 3 days and immunoblotted for US28 using the HA tag (Figure 7.4C). This showed that I was able to detect US28 1-219, demonstrating that this protein can, indeed, be expressed.

7.3 Discussion

HCMV disease is a serious threat to the immunocompromised and is routinely treated by intravenous ganciclovir, oral valganciclovir or foscarnet. These antivirals all target the HCMV-encoded DNA polymerase but escape mutations in the viral DNA polymerase or the UL97 kinase are common [225]. Recently, Spiess et. al, proposed a novel antiviral

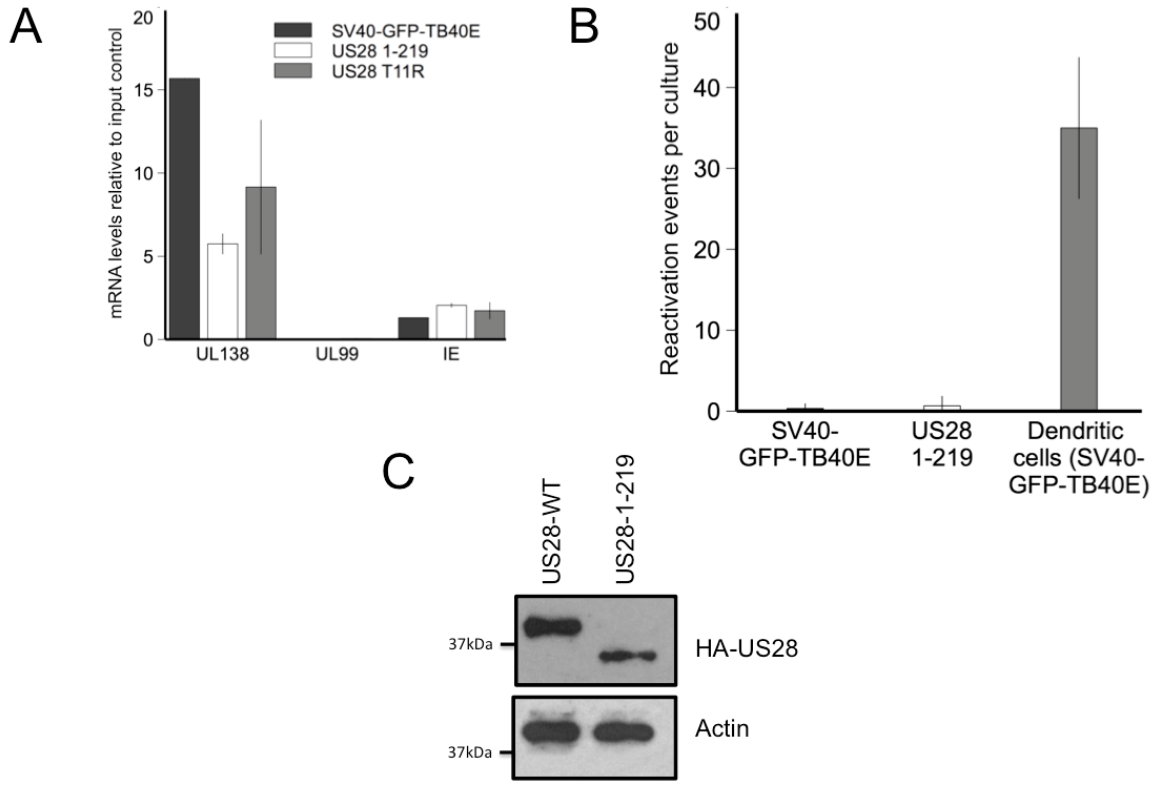

Figure 7.4 US28 1-219 and US28 T11R appear to establish latency. CD14+ monocytes from peripheral blood donations were isolated and experimentally infected with SV40-GFP-TB40E, US28 1-219 or US28 T11R at an MOI of 5. A) Five days post infection, RNA was harvested from samples and analysed for the latency-associated gene UL138 and the lytic genes IE, and UL99. Data were normalised to GAPDH RNA. B) Five days post infection, these monocyte cultures were then co-cultured with HFFs and the number of infected HFFs was measured, by staining for cells expressing HCMV IE protein, 7 days post-co-culture. I included mature dendritic cells, derived from monocytes latently infected with SV40-GFP-TB40E as a positive control. C) HEK 293T cells were transfected with pHRSIN vector containing either HA-US28WT (an N terminally tagged US28 construct) or US28 1-219, with an N terminal HA tag. Three days post transfection, cells were lysed and proteins analysed by immunoblotting against the HA-tag for US28 expression. Actin was used as a loading control.

approach to treating HCMV disease, using F49A-FTP which is a fusion toxin protein which binds US28 with high affinity [412]. They characterised one escape mutant, US28 1-257, which has a premature stop codon, resulting in a truncated protein which loses its affinity for fractalkine. In Chapter 5, I demonstrated that F49A-FTP also kills latently infected, early myeloid lineage cells but I noticed that there were occasional reactivation events from monocyte-derived dendritic cells that had been latently infected with SV40-GFP-TB40E and then treated with F49A-FTP, prior to differentiation. Based on the number of reactivation events from untreated cultures, I could estimate that these resistant isolates constituted around 0.2% of total infectious virus. I wanted to know whether F49A-FTP simply failed to kill a small proportion of *bona fide* latently infected cells, or whether these reactivation events were from viral isolates that had an initial resistance to F49A-FTP. I therefore chose to characterise the viral isolates.

Sequencing of the *US28* gene from twenty F49A-FTP-resistant isolates indicated that all twenty isolates had the same premature stop codon in their *US28* gene. This stop codon generated a truncated US28 mutant, which I named US28 1-219, and which was resistant to F49A-FTP-mediated killing. This mutant is remarkably similar to US28 1-257, identified by Spiess et. al and very likely has similar properties: expression and trafficking to the cell surface but greatly reduced binding to CX3CL1, and F49A-FTP (which is derived from CX3CL1) [412]. Although there has been little work to characterise these mutants, the crystal structure of US28 in complex with fractalkine suggests that fractalkine penetrates deep into the barrel structure of US28 [50], which would likely suggest that the loss of two transmembrane helices could greatly abrogate the binding efficiency of chemokines.

Given that there is likely no DNA replication during latent infection of CD14+ monocytes, it seems very likely that US28 1-219 represents a small subpopulation genotype which is present in our laboratory stocks of virus which was selected for by F49A-FTP. To test this, I grew SV40-GFP-TB40E in HFFs in the presence of low concentrations of F49A-FTP in order to generate and select for resistance mutants. As expected, almost all F49A-FTP-resistant isolates had the same US28 1-219 mutation as before, which strongly suggests that this mutation is found in a subpopulation of our viral stocks of SV40-GFP-TB40E. The one resistant mutant which did not have the US28 1-219 mutation had three N-terminal amino acid substitutions: T11R, D17N, and T25P, which I named "US28-T11R". These point mutations are consistent with previous studies of US28 binding to fractalkine which show that the N-terminal region is necessary for fractalkine binding. As a previously analysed T11A mutant, described by Casarosa et. al, [61] did not show any reduction in binding affinity to fractalkine, it seems likely that one of the other mutations, particularly D17N,

(which is next to the key Y16 residue), may be essential for F49A-FTP binding. I did not pursue this question as it was beyond the scope of this project.

Finally, I was interested in whether the US28 1-219 mutant could establish latency. This question is important because, if US28 1-219 cannot establish latent infection in monocytes, it would result in a small population of cells undergoing lytic infection into our model of latency. Indeed, I have previously seen occasional, (approximately 3 in 10^6 cells) cells from latently infected monocyte cultures which appear to be lytically infected due to their expression of immediately early protein (personal observations). Similarly, RT-qPCR analysis of latently infected monocytes does show low levels of IE mRNA (see Chapter 3), which may also be due to a small number of contaminating, lytically infected cells. I, therefore, infected monocytes with US28 1-219 and used RT-qPCR analysis for viral gene expression, as well as measuring virus release, to see whether the US28 1-219 viral isolate could establish latent infection. Surprisingly, I saw no indications that US28 1-219 was impaired in its ability to establish latent infection. I was, therefore, confident that US28 1-219 was not contaminating our viral stocks of SV40-GFP-TB40E with a virus genotype that could not establish latent infection.

Interestingly, the fact that US28 1-219 can establish latency indicates that this truncated US28 mutant can still signal in monocytes to maintain latent infection. In Chapter 4, I suggested that phosphorylation of the US28 C terminus may modulate US28 signalling, to confer a change in phenotype from signal activation (during lytic infection), to signal attenuation (during latency). However, my data indicates that the C terminal region is not necessary for US28 signalling, which is consistent with the literature [472], but also that phosphorylation of the C terminus of US28, which is known to regulate US28 signalling [263, 273, 383], is not necessary for US28 signalling to maintain latency. To further support these data, it would be good to use the US28 1-219 clone, which I demonstrated can be expressed in HEK 293T cells, and show that US28 1-219 expression in THP-1 cells attenuated cellular signalling, in the same manner as US28-WT, as well as activating cellular signalling after THP-1 cell differentiation. Finally, it should be noted that I did not show that the US28 1-219 isolate can reactivate from latently infected monocytes, after cellular differentiation. This experiment is necessary to confirm that phosphorylation of the US28 C-terminus plays no role in the change of US28 signalling properties during cellular differentiation.

Returning to the original aim of this Chapter, I was able to show that viral resistance to F49A-FTP is likely to be conferred by mutations in the *US28* gene, either in the N terminus or by C terminal truncations. As both mutants can establish latent infection, these F49A-FTP resistant mutants still undergo latency and, therefore, should have little effect

on viral persistence. It would be interesting to see whether F49A-FTP resistant mutants exist in the populations of virus which infect seropositive individuals, as this may indicate how successful F49A-FTP could be as an antiviral treatment against either CMV disease or HCMV latency.

Chapter 8

Isolating latently infected cells

8.1 Introduction

HCMV establishes latent infection in early myeloid lineage cells, which includes CD34+ progenitor cells and CD14+ monocytes [215, 258, 391, 439]. Latent infection generally occurs at very low frequency in these cells, which can be as low as 1 in every five thousand cells [398]. This low frequency of infection means that natural latency is very hard to study, as uninfected cells often dilute any effects displayed by infected cells. This means that latency-associated gene expression can only be analysed by extremely sensitive RT-qPCR and the viral proteins from this gene expression cannot be detected. Therefore, I reasoned that if I could isolate or enrich latently infected cells, this could have major benefits for the study of HCMV latency. In Chapter 3, I used the latency-associated expression of GFP by SV40-GFP-TB40E to sort a population of infected monocytes for the GFP-positive, latently infected cells. Although this is a powerful technique, this only works for *in vitro* infection with labelled viruses. If I wanted to sort, perhaps, naturally latently infected cells, I would need to exploit aspects of HCMV latent infection, such as latency-associated gene products.

HCMV expresses only a small subset of its genome in CD14+ monocytes, known as the latency-associated genes, which include *US28*. *US28* encodes a chemokine receptor homologue [75], which is expressed on the cell surface during latent infection (Chapter 5). In Chapter 5, I exploited this cell-surface expression of US28 to target latently infected cells with an antiviral fusion-toxin protein, F49A-FTP. In this chapter, I also ruled out the possibility that HCMV latent infection increases expression of CX3CL1 - the fractalkine receptor - which would increase affinity of F49A-FTP towards these cells. However, I did not exclude the possibility that HCMV infects CD14+ monocyte subsets which express more CX3CL1; this model would also be consistent with increased killing of latently infected cells

with F49A-FTP, however, not due to US28 expression. There is one well-defined subset of monocytes which express more CX3CL1 on their cell surface: these are CD14+/CD16+ monocytes, which are roughly 10% of the monocyte population.

Interestingly, in the mouse, the CD14+/CD16+ subset of monocytes are a patrolling phenotype, immune-privileged after differentiation and are also likely to be important for MCMV dissemination [94]. It has, therefore, been suggested that HCMV may preferentially infect CD14+/CD16+ monocytes [130, 131]. If this were the case, it would provide an alternative mechanism for why F49A-FTP selectively kills latently infected monocytes, in that it binds to CX3CR1-high cells. Additionally, if true, this would explain why, when I infect monocytes at an MOI of 5, only roughly 10% of cells become latently infected [208, 217, 325]. Finally, it would also provide a mechanism to enrich for naturally latently infected cells, by isolating only CD14+/CD16+ monocytes from seropositive donors, we may be able to study natural latency in new ways.

In this chapter, I isolated CD14+/CD16+ monocytes and experimentally infected these cells, in order to see whether they could be infected more easily with SV40-GFP-TB40E. I was able to isolate CD14+/CD16+ monocytes, however I saw only a small, statistically insignificant, increase in infectivity in these cells.

8.2 Results

I wanted to isolate CD14+/CD16+ monocytes using the magnetic activated cell sorting (MACS) protocol, that I had routinely used to isolate CD14+ monocytes throughout this project. However, to isolate CD14+ monocytes, I routinely selected for these monocytes using anti-CD14 magnetic beads. Unfortunately, this positive selection for CD14+ monocytes would make it impossible to then sort for CD14+/CD16+ monocytes by positive selection, as all CD14+ monocytes would already be labelled. Consequently, to overcome this, I used negative selection of CD14+ monocytes, using the human monocyte isolation kit II from Miltenyi, followed by positive selection for the CD16+ subpopulation in these negatively selected CD14+ monocytes. To start, I wanted to confirm that I did indeed isolate the correct cell types, using flow cytometry. After negative selection of CD14+ cells, I stained a sample of my cells for cell surface CD14 and found, by flow cytometry, that I had isolated only CD14+ cells (Figure 8.1A). After this, I performed positive selection, on these CD14+ cells for CD16, and then analysed for cell surface CD16 by flow cytometry (Figure 8.1B). This indicated that the cells I positively selected for were CD16+ enriched, and that the cells

which were negative selected were depleted for CD16+ cells. This indicated that my isolation protocol did, indeed, enrich for CD14+/CD16+ cells (Figure 8.1).

I next wanted to determine whether CD14+/CD16+ monocytes were more susceptible to infection by HCMV, as this would determine if HCMV selectively infects this monocyte subtype. Consequently, I infected CD14+ (total) monocytes, CD14+/CD16- and CD14+/CD16+ monocytes with the same amount of SV40-GFP-TB40E, and counted the number of GFP positive cells 3 days post-infection, by fluorescence microscopy. Figure 8.2A shows that I saw only a marginal increase in the number of GFP positive monocytes, from the CD14+/CD16+ monocyte subset. Additionally, I was concerned that different monocyte subsets may express the GFP cassette differently, and so I harvested DNA from these cells and quantified the number of HCMV-positive genomes by qPCR. In agreement with my observations by fluorescence microscopy, I saw little difference in the number of HCMV genomes between these cell types (Figure 8.2B).

8.3 Discussion

HCMV establishes latent infection in early myeloid lineage cells, which includes CD34+ progenitor cells and CD14+ monocytes [215, 258, 391, 439]. Naturally latently infected myeloid cells are generally infected at very low frequency [398], which means that natural latency is very hard to study, as uninfected cells often dilute any effects displayed by infected cells. Indeed, even in experimental models of infection, using an MOI of 5, infection is usually around 10% of total monocytes [208, 217]. I hypothesised that this phenomenon could be due to HCMV preferentially infecting certain monocyte subtypes, such as CD14+/CD16+ monocytes, which constitute roughly 10% of the total monocyte population. This model would also provide an alternative explanation for why F49A-FTP kills latently infected monocytes specifically: CD14+/CD16+ monocytes express higher cell surface levels of CX3CR1, which binds F49A-FTP at low levels [130, 131, 208], (see Chapter 5). If this were the case, it would provide a new method for isolating naturally latently infected monocyte, as I could isolate CD14+/CD16+ monocytes, where there would be a high portion of latently infected cells. The possibility that CD14+/CD16+ monocyte constitute a major source of HCMV latency has been a keen research topic in the field [94, 130].

To test this, I isolated CD14+/CD16+ monocytes, using negative selection for CD14+ monocytes and positive selection, within this population, for CD14+/CD16+ monocytes. I confirmed that I did, indeed, isolate these cells, using flow cytometry. I then infected these three monocyte populations: total CD14+, CD14+/CD16- and CD14+/CD16+ with

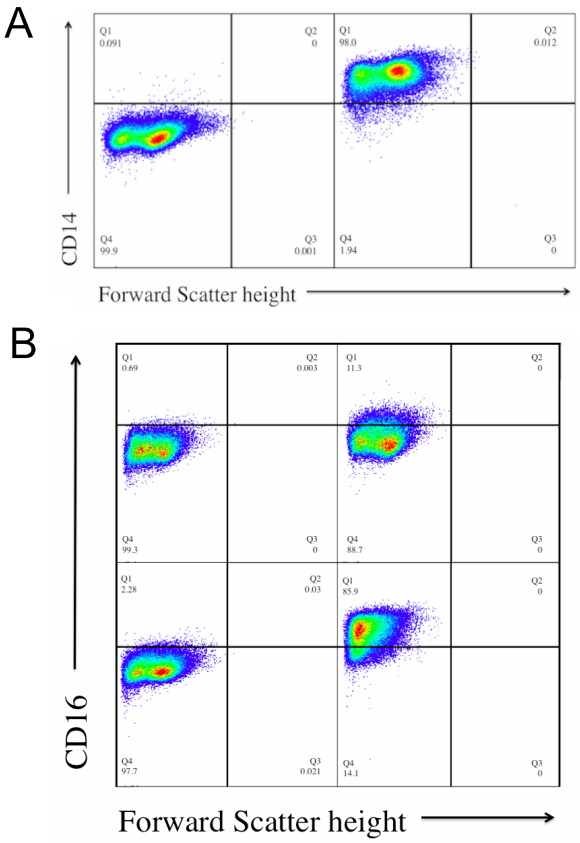

Figure 8.1 CD14+/CD16+ monocytes isolated and confirmed by flow cytometry. A) CD14+ monocytes from peripheral blood donations were isolated by negative selection, and effective isolation was confirmed by labelling with an anti-CD14 antibody followed by flow cytometry. Compared to an isotype control (left), almost all cells appeared positive for CD14 (right). B) Following selection for CD14+ monocytes, cell surface expression of CD16 was analysed by anti-CD16 antibody labelling and flow cytometry, compared to an isotype control (top left), around 10% of CD14+ monocytes express high levels of cell surface CD16 (top right). After this, CD16+ monocytes were isolated by positive selection, and effective selection was confirmed by labelling cells using an anti-CD16 antibody. Compared to an isotype control (bottom left), most selected cells express cell surface CD16 (bottom right).

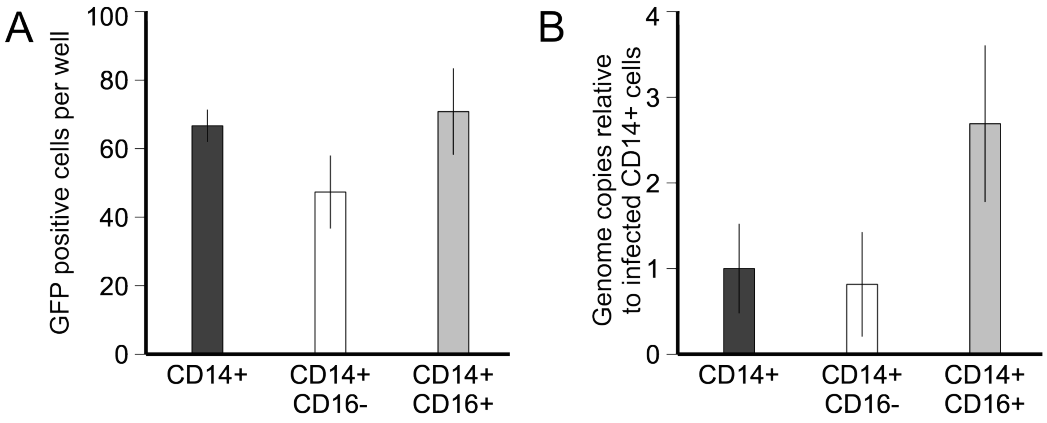

Figure 8.2 CD14+/CD16+ monocytes were only marginally more susceptible to infection by HCMV. CD14+, CD14+/CD16- and CD14+/CD16+ monocytes were isolated and infected with SV40-GFP-TB40E at an MOI of 1, from the same vial of viral stock. A) Three days post infection, I counted the number of GFP positive cells per well, with 10,000 cells in each well. B) Five days post-infection, I harvested DNA and performed qPCR analysis on the MIEP region to quantify the number of viral genomes in the different, latently infected cell types. No data points were statistically significant using Student's T-tests.

SV40-GFP-TB40E, and counted the number of GFP positive cells, three days after infection. Additionally, I used qPCR quantification of viral genomes to measure viral infectivity. Using both measures, I saw only a slight increase in infectivity of CD14+/CD16+ monocytes by SV40-GFP-TB40E, indicating no significant preference, by HCMV to latently infect this monocyte subtype.

Although there had been suggestions that HCMV may preferentially infect CD14+/CD16+ monocytes, I found no evidence to suggest this to be the case. Indeed, after these data were generated, it was found, in healthy people over 70 years of age, that HCMV latent load was identical in CD14+/CD16+ monocytes, as in total CD14+ cells [301].

Chapter 9

Discussion

9.1 Discussion

The work in this thesis provides the first insights into the role of the HCMV-encoded gene *US28* during HCMV latency, one of only a small number of genes expressed during HCMV latent infection of early myeloid lineage cells.

During this project, I and others observed that the deletion of the *US28* gene resulted in an HCMV virus that cannot establish latent infection. Expanding on the work that was published by Humby and O'Connor [161], I showed that US28 expression in THP-1 cells attenuates MIEP activation, potentially by attenuating cell signalling pathways that are normally activated by viral infection of monocytes, which would otherwise activate the MIEP and trigger lytic infection of monocytes. Of particular clinical relevance, inhibition of US28 by the small molecular inhibitor VUF2274 triggered lytic gene expression, which could be detected by the immune system of a healthy, seropositive blood donor. US28 inhibition could therefore be a new immuno-therapeutic strategy to target and kill latently infected cells. We are planning to establish collaborations with Dr Katja Spiess and Professor Mette Rosenkilde (Technical University of Denmark), to develop and test small molecule inhibitors against US28, as well as larger peptide-analogue inhibitors of US28 in a collaboration with Professor Martine Smit (University of Amsterdam). These collaborations could potentially lead to an immunotherapeutic approach to inhibiting US28 to remove latent HCMV infection.

This work also demonstrated that US28 protein appears to have very different signalling properties in myeloid lineage cells, which change during cellular differentiation. There is already evidence that US28 has different signalling properties in different cell types [264, 469, 470], however why US28 signalling properties change is unknown. I believe that our THP-1 cell model is a powerful tool for investigating this phenomenon, and that

BioID tagging US28 may help us understand how US28 changes its protein interactions, and subsequent signalling properties, in a cell-type dependent manner.

Intriguingly, cytomegaloviruses from other species also encode CCR homologues, suggesting that these CCR homologues may also signal to enforce latency in a manner similar to US28. However, US27 and US28 are only conserved among primate CMVs [57]. UL33 and UL78 are more highly conserved in other cytomegaloviruses and, in particular, MCMV M33 induces smooth muscle cell migration similar to US28 [226, 426], and has also been linked to latency in mice [57]. At first, it would be interesting to see if either M33 or M78, or indeed any of the five US28 homologues encoded by rhesus CMV [306], could substitute for US28 in maintaining HCMV latency, as this would indicate a common mechanism of latency. It seems likely that human CMV and rhesus CMV could share a common mechanism of latency, given the relatively recent speciation between their respective hosts. However, murine CMV and rat CMV may not follow this same pattern and, indeed, the sites of CMV latency in these organisms appear to be salivary glands rather than the early myeloid lineage [57].

This observation leads to another key question regarding CMV persistence. I have demonstrated that the immune system can detect and kill monocytes that are infected with Titan-ΔUS28. It is believed that CMV latency underpins CMV persistence [134], because the immune system does not kill latently infected cells, which act as a viral reservoir. It would therefore be quite informative to see whether a US28 deletion virus can persist in a human host, as this would answer the question of whether latency underpins viral persistence or whether viral immune evasion is sufficient to maintain persistent infection. Of course, this experiment would have to be performed in an animal model of HCMV infection, and so understanding the mechanisms of CMV latency in rhesus and murine models would allow us to perform the experiments necessary to answering this question.

Finally, if a US28 deletion virus is unable to maintain a persistent infection in a human host, it may be an ideal vaccine candidate in healthy individuals. This is because the virus would be able to infect all cell types, thereby triggering a strong MHC-I response due to endogenous antigen expression and processing, which ought to be strongly immunogenic [262, 428]. Unlike current successful vaccines, which expose people to only a small number of immunodominant antigens [139, 268, 302, 303, 357, 358], a ΔUS28 virus would express all but one HCMV antigen, and so may trigger a more broad immune response.

During these investigations, I also noticed that the secretome of latently infected monocytes appears to repress lytic infection of monocytes by Titan-ΔUS28. This observation suggests that secreted cytokines may be acting in a paracrine manner to suppress aberrant

lytic gene expression in neighbouring, infected monocytes. It will be interesting to see whether this effect is due to TGF-β, which is known to signal to repress the MIEP via upregulation of HDAC4 [207], but also whether this effect is cell-type specific (does the latent secretome repress lytic gene expression in HFFs, for example?).

As we have clear knowledge that US28 is expressed during latent infection, in a fruitful collaboration with Dr Katja Spiess, Dr Thomas Kledal, and Professor Mette Rosenkilde, I showed that a fusion-toxin protein, F49A-FTP, which binds US28, could target and kill latently infected cells. To show this, I used a FACS sorting protocol to isolate cells that were latently infected with a GFP-tagged virus, SV40-GFP-TB40E, and showed that these cells expressed US28 protein on the cell surface with radiolabelling assays. After this, I developed a protocol to target and kill latently infected cells using F49A-FTP, which was also effective at killing CD34+ progenitor cells infected with SV40-GFP-TB40E as well as monocytes that were naturally latently infected, donated from HCMV-seropositive individuals.

This project is now being moved forward in collaborations with Dr Victor Ferreira (Toronto), where F49A-FTP will be introduced into patients receiving multi-organ transplants, as well as a separate project with Professor Luka Cicin-Sain (Hannover) to test the efficacy of F49A-FTP in clearing HCMV from cadaverous tissue. Hopefully, these projects will lead to a method to reduce the latent load of HCMV, as well as killing HCMV infected cells from donated tissue, which will hopefully reduce the risk of HCMV disease and improve the outcome for transplant recipients.

Finally, I have begun to further investigate how US28 protein helps to maintain HCMV latency, by performing a mass spectrometry screen, in collaboration with Dr James Williamson from Paul Lehner's laboratory, which identified CTCF, a repressive transcription factor, as a potential mechanism that US28 may be manipulating to maintain HCMV latency. Additionally, a second mass spectrometry screen, not discussed in this thesis, identified PYHIN proteins, which are involved in DNA sensing and the inflammatory response, as being modulated by US28 signalling. These investigations have now been taken over by Elizabeth Elder, a PhD student in John Sinclair's laboratory, who will hopefully build upon the observations in our mass spectrometry screen.

There is an intriguing possibility that CTCF post-translational modifications change due to US28 expression during HCMV latency. One way to investigate this possibility would be to generate a FLAG-tagged mutant of CTCF, which could be transfected by nucleofection into THP-1 cells either expressing HA-US28-WT or HA-US28-R129A and then immunoprecipitated and immunoblotted for SUMO. Although this approach is likely to be fruitful, there is a risk that overexpressing CTCF could affect post-translational modifications. Another

approach would therefore be to modify the CTCF gene in the genome using CRISPR-Cas9 mutagenesis [352]. There are several options here, including generating a triple-FLAG tagged mutant of CTCF, so that I could immunoprecipitate endogenous CTCF and immunoblot for SUMO, or indeed I could selectively remove SUMOylation sites from CTCF and investigate how these mutations affect establishment of HCMV latency.

This body of work has built foundations upon which we can further investigate the role of US28 during HCMV latency. It has reinforced the observation that US28 has very different functions in different cell types and that myeloid differentiation status greatly effects how US28 expression acts upon the infected cell. This work also shows that US28 could be a key therapeutic target against HCMV latent infection, which could play a major role in reducing latently infected cells in a transplant setting, to reduce the risk of HCMV disease in immunosuppressed patients. Further understanding the actions of US28 will be key to treating HCMV latency.

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Appendix A

Full data arrays and proteomic screens

Gene	log2 fold change	Gene	log2 fold change	Gene	log2 fold change
H1C	5.3416	PRCC	3.0305	MEPCE	2.5369
RER1	5.0257	AP3S1	3.0251	SP100	2.4824
RCN2	4.7332	ERP29	3.0035	COQ5	2.4814
CLASP2	4.7203	DARS2	2.887	NOP16	2.4699
RPS15A	4.6452	DUSP23	2.8353	HSPA14	2.4652
RPL38	4.4745	CPSF2	2.8108	EBP	2.4579
AUP1	4.0785	VDAC3	2.8104	RAB3GAP1	2.4524
TRAP1	3.9812	AGPAT2	2.8072	ZAK	2.4295
SHTN1	3.8052	MT-ND5	2.7859	CIAO1	2.409
GPX4	3.6823	H1FX	2.7793	CCDC58	2.393
CEP170	3.5157	SF3B4	2.7698	ACACA	2.3812
PTGES3	3.4903	RPL32	2.7154	RAB10	2.3811
RPA3	3.4309	DDX17	2.7081	PCYOX1L	2.3763
RPL19	3.3867	EEF1B2	2.6656	LARP1B	2.3688
HMGB1	3.2550	NUCKS1	2.6107	GIT1	2.3455
NOP14	3.2324	TAF15	2.6098	TRIM24	2.3353
PNO1	3.2179	ZNF207	2.5894	DNAJB11	2.3222
RAB8B	3.1848	SNX12	2.5714	LMAN2	2.3057
KATNA1	3.1326	DHX8	2.5525	RANBP3	2.3004
RNF14	3.0467	ATP5D	2.543	PUS1	2.2266
VAT1L	2.2176	IGF2BP2	-2.014	RPS7	-2.1835

Gene	log2 fold change	Gene	log2 fold change	Gene	log2 fold change
SLC9A3R1	2.2137	TSNAX	-2.0156	BDH1	-2.2094
TBC1D23	2.207	PSMD12	-2.0163	MNDA	-2.2122
PSPH	2.2042	ENOPH1	-2.0179	COMT	-2.2124
GSS	2.1953	COPG2	-2.0299	CCDC86	-2.2196
H2BK	2.1700	IDI1	-2.035	CDC16	-2.232
SLC25A3	2.1617	PSMB9	-2.0472	VPS25	-2.2358
VAPB	2.1610	UBE2O	-2.0626	MLEC	-2.251
SRD5A3	2.0652	RPS18	-2.0846	RPL11	-2.2716
ABHD16A	2.0603	ABCB7	-2.1005	MRPL47	-2.2745
CBX1	2.0572	GMPPA	-2.1013	PELO	-2.275
DHX16	2.0393	CRKL	-2.1102	CARHSP1	-2.302
INTS4	2.0334	SCAMP3	-2.1328	H1B	-2.3107
ARPC3	2.0328	TFG	-2.1394	TMED9	-2.3203
RAD23B	2.032	TPD52	-2.1426	GSTP1	-2.335
GNB4	2.0201	CRTAP	-2.1621	ANAPC7	-2.3366
FARSA	2.0190	GSTK1	-2.1635	MRPS27	-2.3447
RPS27A	2.0162	KIAA0319L	-2.1651	C1orf50	-2.345
SUB1	2.0136	C19orf52	-2.1681	SDF2L1	-2.368
DCTD	2.0120	MPP1	-2.1813	TPM4	-2.3871
TBL2	-2.3950	ACP1	-2.7293	UGDH	-3.3806
CLP1	-2.4226	HIRIP3	-2.7308	SCAF8	-3.4033
GOLT1B	-2.4400	RAP2C	-2.7401	ATOX1	-3.427
SMS	-2.4410	TRABD	-2.7492	CNBP	-3.4935
AKAP8	-2.4426	SCAF4	-2.7933	TRIP4	-3.5245

Table A.1 Full table of changes in protein abundance in THP-1 cells expressing US28. Expression of US28 protein in isolation in THP-1 cells leads to many changes in overall protein abundance, as quantified by mass spectrometry analysis on the whole cell proteome. THP-1 cells expressing US28 were compared with THP-1 cells transduced with an empty vector control. In this table these changes are listed by Gene ID and log2 of the fold change in abundance in US28 expressing THP-1 cells.

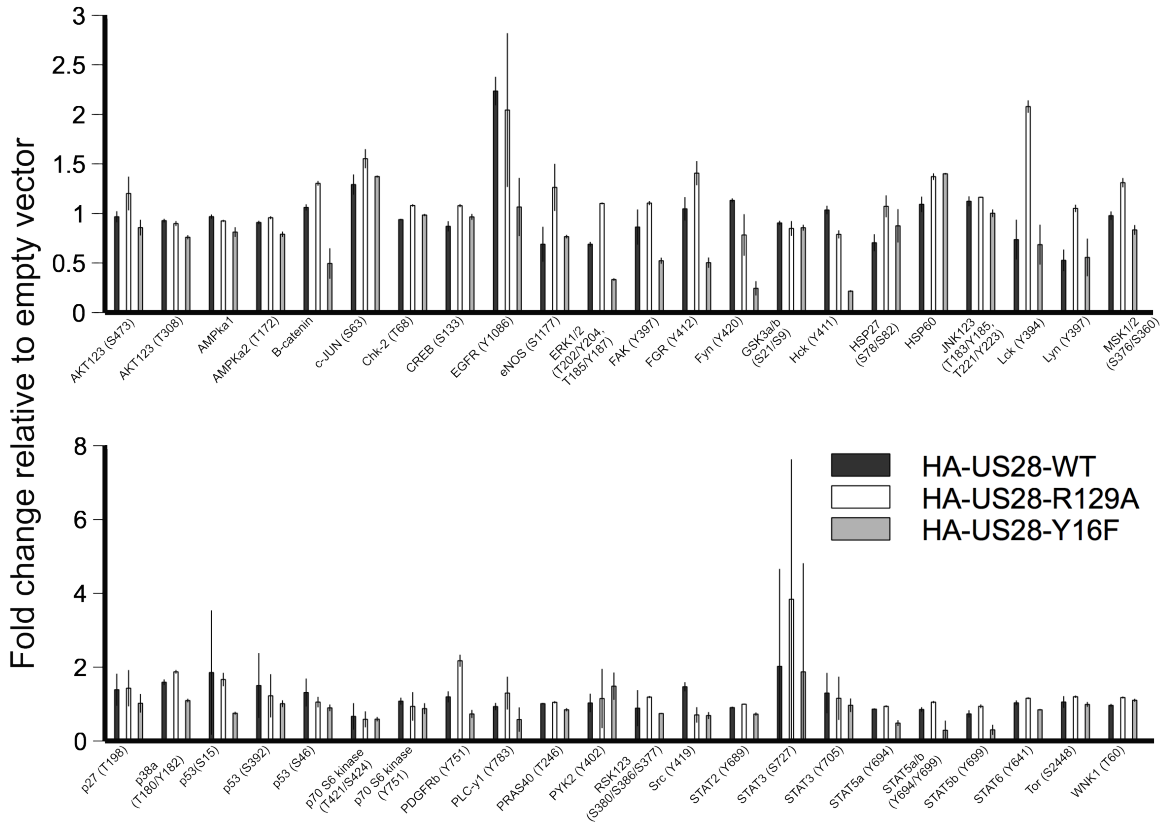

Figure A.1 THP-1 cells which had been induced to express either HA-US28-WT, HA-US28-R129A (which cannot maintain latency) or HA-US28-Y16F (which can maintain latency) were lysed and analysed for changes in cellular kinase phopshorylation levels by antibody array. Data represent fold change in intensity relative to empty vector transduced THP-1 cells.

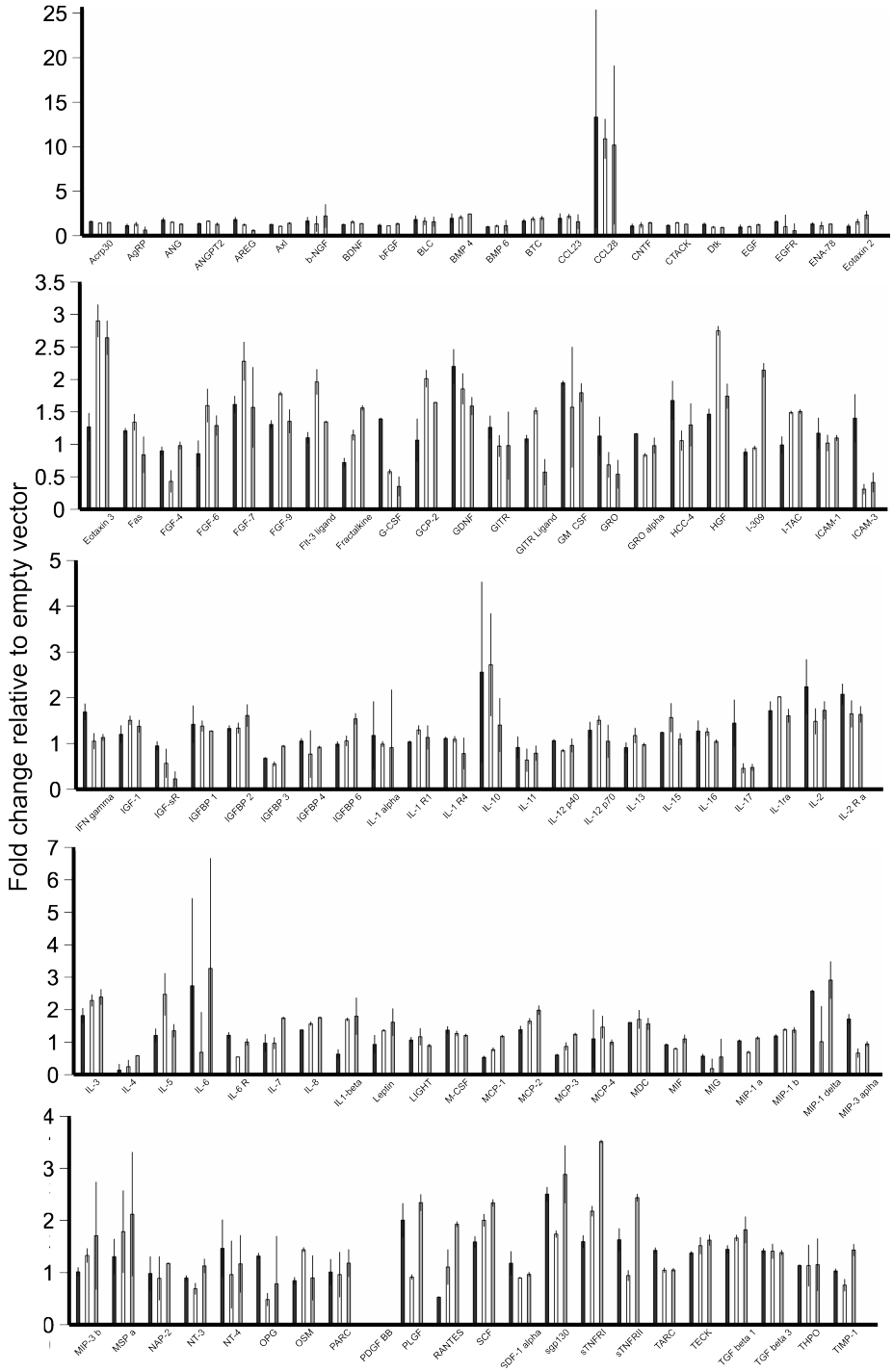

Figure A.2 Ectopic US28 expression induced changes in THP-1 cytokine secretion. THP-1 cells which had been induced to express either HA-US28-WT, HA-US28-R129A (which cannot maintain latency) or HA-US28-Y16F (which can maintain latency) were washed in PBS then cultured in fresh media for 1 day, and the media was then harvested, clarified by centrifugation, and then analysed for secreted cytokines using an antibody array against human cytokines. Data represent fold change in intensity relative to empty vector transduced THP-1 cells.

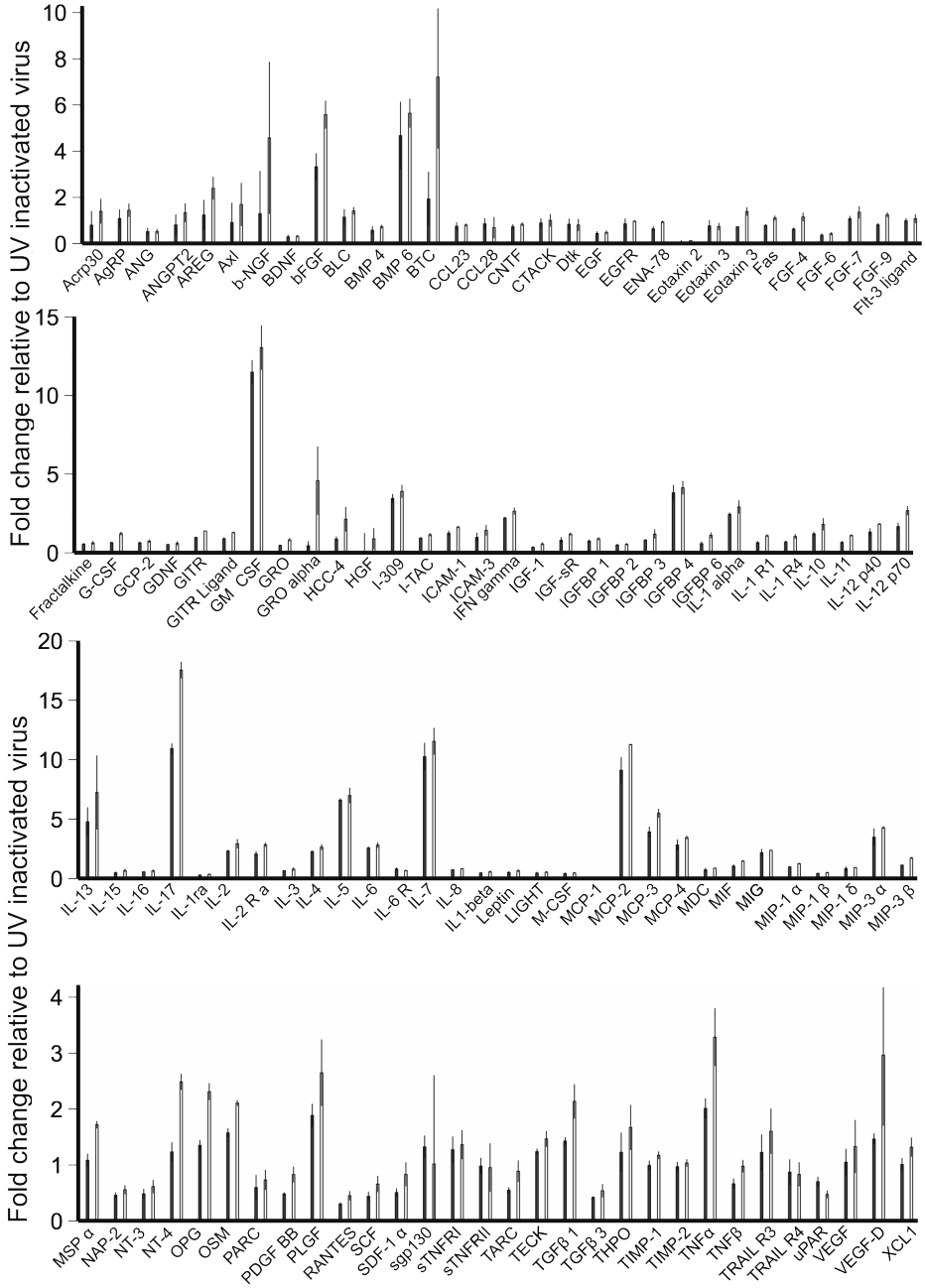

Figure A.3 Although US28 induced changes in THP-1 cytokine secretion, there was no significant change in cytokine secretion between Titan-WT and Titan-ΔUS28 infected monocytes. CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-WT or Titan-ΔUS28. Six days post infection, these cultures were washed in PBS and then the following day, the media from these cells was harvested, clarified by centrifugation, and then analysed for secreted cytokines using an antibody array against human cytokines. Data represent fold change in intensity relative to monocytes treated with UV inactivated virus.

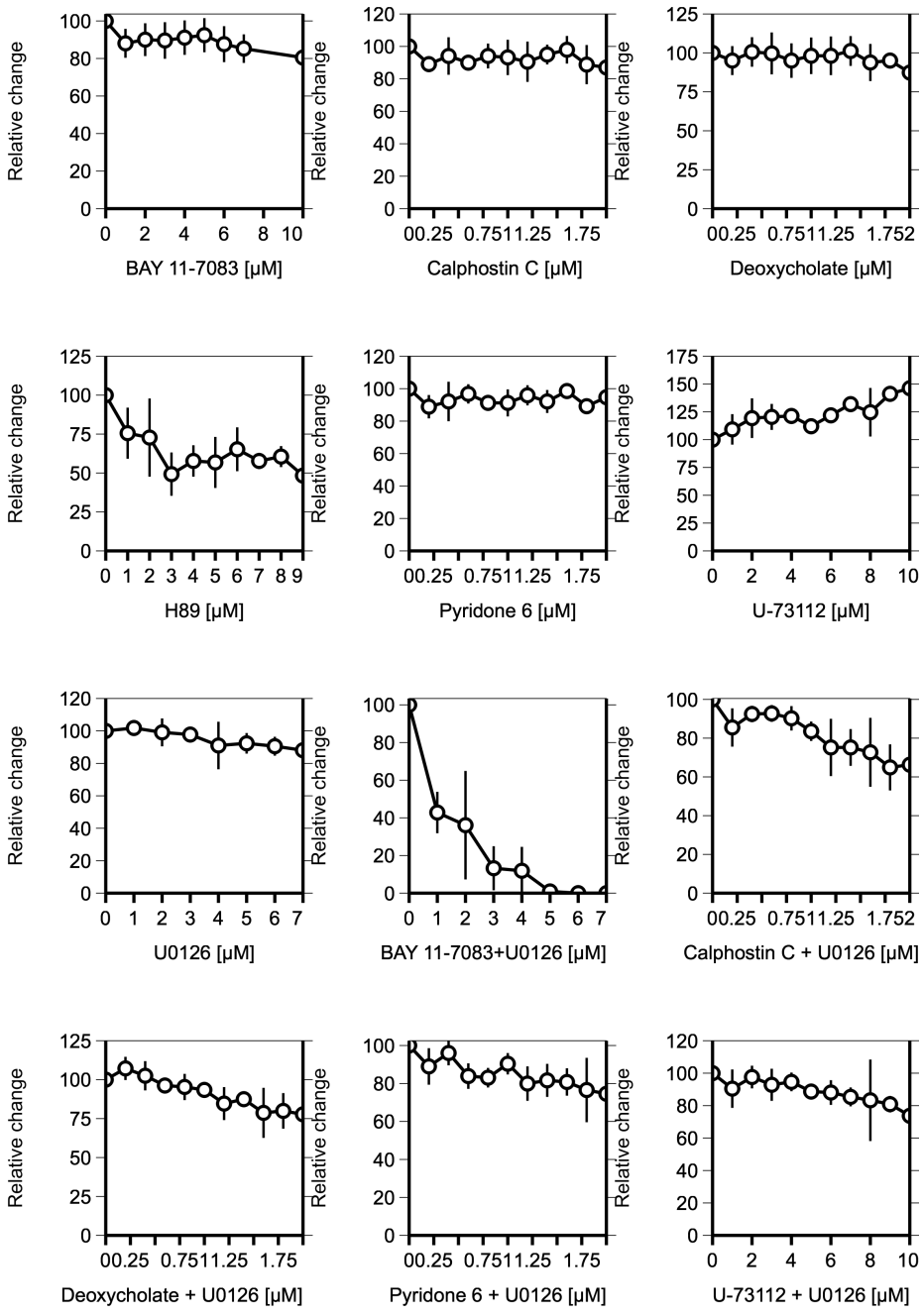

Figure A.4 Although US28 induced changes in THP-1 cytokine secretion, there was no significant change in cytokine secretion between Titan-WT and Titan-ΔUS28 infected monocytes. CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-ΔUS28 in the presence of an increasing concentration of the small molecule inhibitors above. These inhibitor are: BAY 11-7083 (NFκB), Calphostin C (PKC), Deoxycholate (Wnt activator), H89 (MSK-1 inhibitor), Pyridone 6 (JAK-STAT), U-73112 (PLC), U0126 (MAP kinase). Each inhibitor, except H89, was also combined with U0126. Subsequently, GFP-positive cells were counted three days post-infection as an indicator of lytic infection.

RPL23	QPRT	EED	MAD1L1	PDXDC1
SNRPD2	MRI1	EPS15	CD33	GSK3B
KRT1	RPP30	PRKCB	TIMELESS	PCF11
EIF5A2	GNPDA1	SEPHS1	ELOVL1	HOOK3
PPP2CB	COIL	LSM14A	LRCH3	PKN1
ANXA2	MAPRE2	RACGAP1	NMD3	METTL1
CDK2	THOC1	ARHGAP17	NBN	CTCF
ATP1B3	MCU	GGH	POLR1B	CHAMP1
RAB11B	PIN4	DNAJC3	GATM	NOM1
TM9SF3	HMOX2	MTX1	PPP1R10	TNKS1BP1
ME2	TATDN1	CD3EAP	PNPLA6	RBM7
CAPNS1	COPS6	TELO2	ABCF3	ARHGAP18
PPIE	EEFSEC	RBBP6	APLP2	ST14
RPS6KA3	ISOC2	PPP1R2	HMMR	TAP1
CD63	HRAS	SLFN11	URB2	UBE3C
EEF1E1	TM9SF2	MOB3A	CHMP7	NCAPG2
CBWD3	MRPS35	ZCCHC8	FOXP4	TBC1D10B
TSTA3	YTHDC1	USP8	PRKAR1A	ZC3H4
DSTN	ILVBL	ATPAF1	KNTC1	STAG1
WDR5	NOL7	RBM19	XRN1	CENPF
CIT				

Table A.2 A table of proteins that were only detected in THP-1 cells expressing US28. Expression of US28 protein in isolation in THP-1 cells leads to many changes in overall protein abundance, as quantified by mass spectrometry analysis on the whole cell proteome. This table shows proteins which were detected in THP-1 cells expressing US28, but not in control THP-1 cells. Data are listed as Gene IDs.

KRT4	RAB11A	RPS6KA4	MROH1
RB1	RPS15	CPD	DDX55
KRT2	PPP2CA	NDUFB8	GNPDA2
ANXA2P2	PSME4	ARIH2	RQCD1
KDM4A	AAK1	PARN	EXOSC8
KRT16	AGK	TTC4	RSF1
KRT5	LAMTOR1	BAG2	MYCBP
RABL6	GPALPP1	BCKDHB	LYST
WDR82	TBC1D5	SRI	NIPSNAP1
EIF5A	TUBGCP3	NCAPD3	ELAC2
MRPS5	TRMT6	RPL27A	DHRS4
PRKAA1	YTHDF2	PRIM1	MECR
SDSL	OAS3	ORC2	RTFDC1
TRNT1	APAF1	PIN1	MRPL9
GOLPH3	DNPH1	DRAP1	VIPAS39
RCOR1	PDE6D	PPP6R3	GPHN
KBTBD11	CNOT3	NOL9	PXMP2
SEC24D	NDUFS7	RNF20	IMP3
NDUFV2	NCOR1	INTS8	DIP2B
ITGAL	MTX2	RSRC2	VPS28

Table A.3 A table of proteins that were only detected in THP-1 cells expressing US28. Expression of US28 protein in isolation in THP-1 cells leads to many changes in overall protein abundance, as quantified by mass spectrometry analysis on the whole cell proteome. This table shows proteins which were not detected in THP-1 cells expressing US28, but were detected in control THP-1 cells. Data are listed as Gene IDs.

Gene ID	R129A /WT	q-Value R129A /WT	Gene ID	R129A /WT	q-Value R129A /WT
CSPG4	3.558		AIM1	1.1833	0.023488159
CAV1	3.3112	1.85E-09	HLA-DRB1	1.1725	0.000284102
RPS4Y1	3.0252	1.07E-07	FAM213A	1.1686	3.42E-05
NCAM1	2.6889	6.91E-07	CPS1	1.1506	2.43E-05
MNDA	2.332	1.61E-07	PLD4	1.1197	5.68E-05
PADI2	2.1077	2.76E-05	RPL22L1	1.0963	1.45E-05
KYNU	1.9237	6.53E-06	HLA-DRA	1.0949	0.000531429
ALOX5AP	1.8074	3.65E-05	CD53	1.0888	0.007741026
HSD17B8	1.7845	4.62E-05	CKB	1.0731	3.30E-07
AKT3	1.6978	0.000640302	SYNPO2	1.0607	1.81E-06
S100P	1.6149	0.000106363	IFITM3	1.0391	0.000410095
DDX3Y	1.6083	1.55E-07	TGM2	1.0321	0.007301067
CTH	1.5734	5.04E-06	MYO1G	1.0201	0.000103962
CAND2	1.5474	1.85E-09	CCNA1	-1.0439	0.004861721
NCAM2	1.5306	1.18E-05	CD109	-1.077	2.03E-07
TRIM72	1.5008	3.30E-07	PDLIM1	-1.1047	3.30E-07
RAB6B	1.4657	4.75E-05	ST3GAL4	-1.1078	7.20E-05
SULT1A1	1.2869	3.62E-06	ITGAL	-1.1552	1.07E-07
IFI16	1.2425	8.55E-06	ANPEP	-1.3659	1.55E-07
NMNAT3	1.2394	4.04E-05	IPCEF1	-1.454	0.00014358
OAS2	1.2234	2.69E-06	MYO1E	-1.5312	1.94E-07
PLXND1	1.2179	0.000103249	MICAL2	-1.7809	0.000469089
ALDH2	1.1871	0.000100523	ANXA8	-2.0233	3.30E-07

Table A.4 Changes in protein abundance, as detected by TMT-tagged peptide mass spectrometry. This table lists all proteins which changed more than 2 fold in abundance between THP-1 cells expressing HA-US28-WT and those expressing HA-US28-R129A. The table displays Gene IDs, log2 fold change between R129A against US28-WT and the q-values for these changes, based on 3 experimental repeats.