

The role of human cytomegalovirus encoded viral G protein-coupled receptors in onco-modulatory signalling

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List of Abbreviations

7TM:	7-trans-membrane protein
ΔUS28:	delta US28
AEC:	3-amino-9-ethyl-carbazole
AIDS:	acquired immune deficiency syndrome
AP-1:	activator protein 1
AP-2:	activator protein 2
APS:	ammonium persulphate
AU:	arbitrary units
BAC:	bacterial artificial chromosome
BSA:	bovine serum albumin
cAMP:	cyclic adenosine monophosphate
CCL:	chemokine ligand
CCR:	chemokine receptor
CNTF:	ciliary neurotrophic factor
CPE:	cytopathic effect
CT:	cardiotrophin
DAPI:	4', 6-diamidino-2-phenylindole
DMEM:	Dulbecco's Modified Eagle's Medium
DMF:	dimethylformamide
DMSO:	dimethylsulfoxide
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
E:	early
EBV:	Epstein Barr Virus
EDTA:	ethylene diamine tetraacetic acid
ELISA:	enzyme-linked immunosorbent assay
EMEM:	Eagle's Minimum Essential Medium
FBS:	fetal bovine serum
GPCR:	G-protein-coupled receptor
vGPCR:	viral G-protein-coupled receptor
GEF:	guanine nucleotide exchange factor
GDP:	guanosine diphosphate



GRK:	GPCR kinase
h.p.i:	hours post infection
HCl:	hydrochloric acid
HCMV:	human cytomegalovirus
HFF:	human foreskin fibroblast
HHV-5:	human herpesvirus 5
HIV:	human immunodeficiency virus
HSV:	herpes simplex virus
IE:	immediate-early proteins
ICC:	immunocytochemistry
IFA:	immunofluorescence assay
Ig:	immunoglobulin
IL:	interleukin
JAK:	Janus activated kinase
Kb:	kilo basepairs
KCl:	potassium chloride
K ₂ HPO ₄ :	di-potassium-hydrogen phosphate
KH ₂ PO ₄ :	potassium dihydrogen phosphate
KSHV:	Kaposi's sarcoma-associated herpesvirus
L:	late
LIF:	leukemia inhibitory factor
m.o.i:	multiplicity of infection
MCP:	Monocyte Chemo-attractant Protein
MEM:	Minimal Essential Medium
MG:	malignant glioblastoma
MIP:	Macrophage Inflammatory Protein
NaCl:	sodium chloride
NaF:	sodium fluoride
Na ₂ PO ₄ :	sodium phosphate
Na ₃ VO ₄ :	sodium orthovanadate
NEAA:	non essential amino acids
NFAT:	Nuclear Factor of Activated T-lymphocytes
NFκB:	Nuclear Factor kappa B
NK:	natural killer cells

NP-40:	nonyl phenoxypolyethoxyethanol
ORF:	open reading frame
OSM:	oncostatin-M
p.f.u.:	plaque forming units
p.i.:	post infection
PAA:	polyacrylamide
PBS:	phosphate buffered saline
Pen/Strep:	Penicillin/Streptomycin
PFA:	paraformaldehyde
PI3Ks:	phosphoinositide 3-kinases
PKA:	protein kinase A
PKC:	protein kinase C
PLA ₂ :	phospholipase A ₂
PLC:	phospholipase C
PMSF:	phenylmethanesulfonylfluoride
PVDF:	polyvinylidene fluoride
RANTES:	Regulated upon Activation, Normal T-cell Expressed and Secreted
RIPA:	radioimmunoprecipitation assay
RNA:	ribonucleic acid
RT:	room temperature
SDS:	sodium dodecyl sulphate
SMC:	smooth muscle cells
STAT:	Signal Transducer and Activator of Transcription
TAD:	transcriptional activation domain
TBST:	tris buffered saline with Tween
TEM:	transmission electron microscopy
TEMED:	N,N,N,N'- tetramethylethylenediamine
TNF:	Tumour Necrosis Factor
Tris:	Tris (hydroxymethyl) amine
UL:	unique long
US:	unique short
VEGF:	Vascular Endothelial Growth Factor
vGPCR:	viral GPCR
WT:	wild type

Abstract

Human cytomegalovirus (HCMV) is a ubiquitous virus of the herpes type that infects a high percentage of some populations. One of the most researched genes expressed by HCMV with close homology to human chemokine receptors is the US28 G protein-coupled receptor.

Study design: This study was initiated to elucidate the intracellular signalling pathways of an inflammatory factor (IL-6) and an angiogenic factor (STAT3) triggered by the viral US28 oncogene and the presence of US28 in the HCMV viral particle. These pathways were observed by introducing the US28 gene into two human cell lines by infection with a HCMV strain that expresses the US28 gene (wild type), and two HCMV strains where the US28 gene was deleted (Δ US28 and Δ US28/UL33). Special attention was directed at the expression of IL-6 after promotion of the US28 gene and subsequent phosphorylation of STAT3. A new US28 antibody was validated and a method developed in an attempt to determine US28 on the viral particle.

The following techniques were applied: **Cell culture work**, two mammalian cell lines were used, HFF's and U373 MG. **Virus stock titre determination** to determine the multiplicity of infection. **Protein quantitation** to determine very small quantities of protein for Western blot analysis. **ELISA** for the quantitative determination of IL-6. **Western blotting** for phospho-STAT3 determination and validation of the US28 antibody. **Immunocytochemistry** was used for back titrations of virally infected cells. **Immunofluorescence assay and use of confocal microscopic techniques** was used for the location of the US28 gene in the virion and for tSTAT3 translocation to the nucleus.

Conclusion: A clear increase in IL-6 secretion ($495\% \pm 1\%$) was seen, and this was after only an hour in HCMV WT infected cells. From the increase in IL-6 secretion a subsequent increase in STAT3 phosphorylation was detected in the same samples. A clear link has been established between IL-6 and STAT3. A method to determine whether US28 was present in the HCMV viral particle was designed and preliminary results obtained. The results were inclusive.

Keywords: Human cytomegalovirus (HCMV), G protein-coupled receptors (GPCR), US28, Oncogene, Tumour, Antibody, Ligand, Cytokine, Interleukin 6 (IL-6), Signal Transducer and Activator of Transcription (STAT3).

Chapter 1: Introduction

Human cytomegalovirus (HCMV) is a ubiquitous virus of the herpes type that infects a high percentage of some populations. Under immunocompetent individuals this infection poses no health threats, but in the event of immunocompromised individuals it can lead to pathologies that include hepatic and respiratory diseases, and it has also been implicated in several chronic diseases. In tumours HCMV infection has been shown to up-regulate several growth factors and cytokines which enhance cell proliferation, cell survival and has also been reported to stimulate angiogenesis. This would imply that HCMV could promote malignant behaviour in tumour cells in the event of infection with this virus.

Many of the cell growth enhancing effects are due to cytokine mimicking or cytokine stimulated receptors of host or viral origin with death and apoptotic evading intracellular signalling pathways being activated. One of the most researched genes expressed by HCMV with close homology to human chemokine receptors is the US28 G protein-coupled receptor (which is also regarded as an oncogene). The gene for this viral GPCR homolog is believed to have been acquired from the host genome and is maintained within the viral genome throughout its co-evolution with the host. This strategy aids in its evasion of the host immune system and plays an important role in various signalling pathways. The human immune response presents a virus with many challenges as well as opportunities. Viruses have developed various strategies to evade the innate and acquired immune responses that could eliminate them from their infected hosts. The relationship between viruses and chemokines has become an area of intense scientific investigation since the observation that HIV makes use of several chemokine receptors as co-receptors for binding prior to entry into cells, thus certain chemokines can block HIV target cell entry.

This study was initiated in an attempt to elucidate the intracellular signalling pathways of certain inflammatory and angiogenic factors (IL-6 and STAT3 respectively) triggered by the viral US28 oncogene. These pathways were observed by introducing the US28 oncogene into two human cell lines by infection with a HCMV strain that expresses the US28 gene (wild type), and two HCMV strains where the US28 gene was deleted (Δ US28 and Δ US28/UL33).

Also a newly available US28 antibody was validated and used in an attempt to determine US28 on the viral particle.

Experiments done during this study used two different clinical strains of HCMV – the Titan strain and TB40-BAC4 strain. These two strains have genomes cloned as bacterial artificial chromosomes (BACs). These virus strains were generated by delivery of the BAC plasmid into mammalian cells that support viral replication. BAC mutagenesis has become a successful tool to manipulate the HCMV genome and to investigate the function of vGPCRs in the context of viral infection in biologically relevant cells. The Titan strain was a newly isolated clinical HCMV strain that was prepared for research (Maussang et al., 2006).

TB40-BAC4 is an HCMV strain which demonstrates efficient replication and has a broad cell tropism (Sinzger et al., 2008).

Due to the large number of ligands that can elicit an intracellular response from expressed US28 receptors, this receptor has become a target for further research to determine the mechanism by which it affects the immune evading responses. Special attention was directed at the expression of IL-6 after promotion of the US28 gene and subsequent phosphorylation of STAT3.

Chapter 2: General literature review

2.1 G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) have been receiving a great deal of attention in the world of research science. Over the years a more comprehensive look has been taken into the structure, mechanism of activation, receptor regulation and drug development targeted at these membrane proteins. GPCRs make up the largest family of trans-membrane proteins in the human genome with over 800 unique GPCRs found to date (Kobilka, 2007). Their primary function is to transduce extracellular stimuli into intracellular signals (Kroeze et al., 2003). GPCRs mediate many important cellular signal transduction events which are related to differentiation, proliferation, angiogenesis, cancer development, and cell survival (Lundstrom, 2005). It is because of this that these membrane proteins have been profitable targets for pharmaceutical companies regarding drug development efforts.

All GPCRs have a similar general structure with seven hydrophobic transmembrane α -helices, an extracellular N-terminus region and an intracellular C-terminus region. These proteins arrange themselves into an ordered tertiary structure. The seven-transmembrane helices form the core of the GPCR, and because of this they are also known as seven-transmembrane domain receptors. This core consists of the seven helices connected by three extracellular loops and three intracellular loops. A fourth intracellular plasmic loop, believed to be orientated to form a ligand binding pocket is formed when the C-terminal segment is post-translationally palmitoylated at the two cysteine residues in the cytoplasmic tail (Figure1) (Baldwin, 1993). Glycosylation and ligand binding of peptides, hormones and proteins occur at the elongated extracellular N-terminal. Small organic agonists bind between the α -helices within the extracellular parts of the transmembrane regions and part-way across the membrane, while phosphorylation and posttranslational palmitoylation occur intracellularly at the C-terminal domain for desensitisation and internalisation. Closely associated GPCRs can physically interact with each other, thereby modifying intracellular signalling and cellular functions.

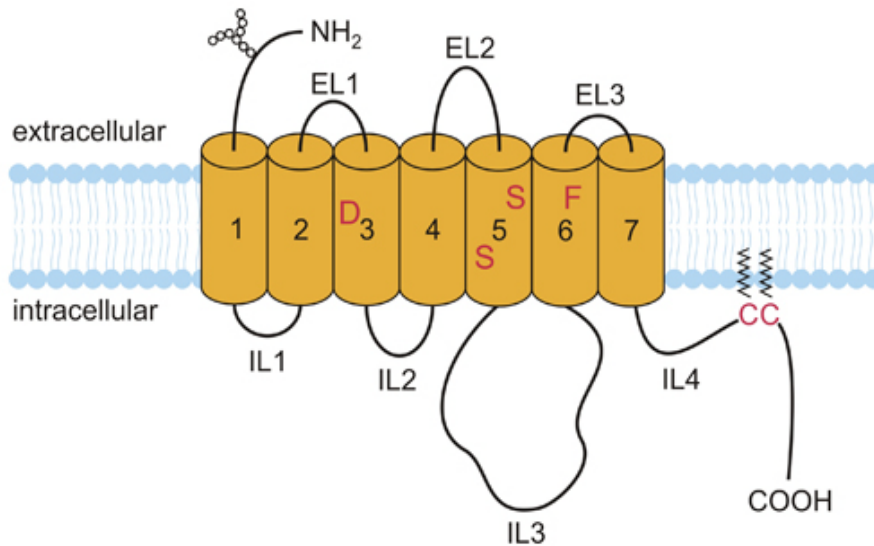


Figure 1: The general structure of a GPCR. The transmembrane core (TM 1-7) is represented as cylinders. The N-terminus (NH₂) is located extracellularly. The C-terminus (COOH) is located intracellularly. The trans-membrane regions are linked by three extracellular loops (EL) which alternate with three intracellular loops (IL). Residues which participate in ligand binding on the N terminal are indicated in red: an aspartic acid residue (D) in TM3, serine residues (S) in TM5, and a phenylalanine residue (F) in TM6. A fourth intracellular loop (IL4) is formed when the protein is posttranslationally palmitoylated at the cysteine residues (C) in the cytoplasmic tail. Retrieved from the Institute of structural biology and biophysics, (Gensch, 2009).

G protein-coupled receptors are highly homologous, and are activated by external stimuli in the form of a specific ligand. GPCRs mediate the very rapid intracellular effects of neurotransmitters, hormones, ions, odorants, light, biogenic amines, lipids, proteins, amino acids, nucleotides and chemokines (Calebiro et al., 2010). It has been estimated that 80% of hormones and neurotransmitters activate intracellular signal transduction mechanisms by triggering GPCRs (Kristiansen, 2004). Ligand binding results in a conformational change in the receptor, which causes the activation of a specific G protein complex closely associated with the intracellular region of the receptor. Most of the intracellular actions in a cell are brought about by pathways which are activated by G proteins (Kristiansen, 2004). The larger G protein complexes are called heterotrimeric molecules and are made up of alpha (α), beta (β) and gamma (γ) subunits. The binding of a ligand to a GPCR triggers the interaction between the receptor and G protein inside the cell membrane. This interaction occurs at the intracellular loops of the GPCR – primarily intracellular loops 2 and 3 (Wess, 1997). This interaction catalyses guanine nucleotide exchange factor (GEF) that allosterically exchanges guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G α subunit of the G protein complex. The G α subunit dissociates from the G $\beta\gamma$ dimer on binding to the GTP (Figure 2).

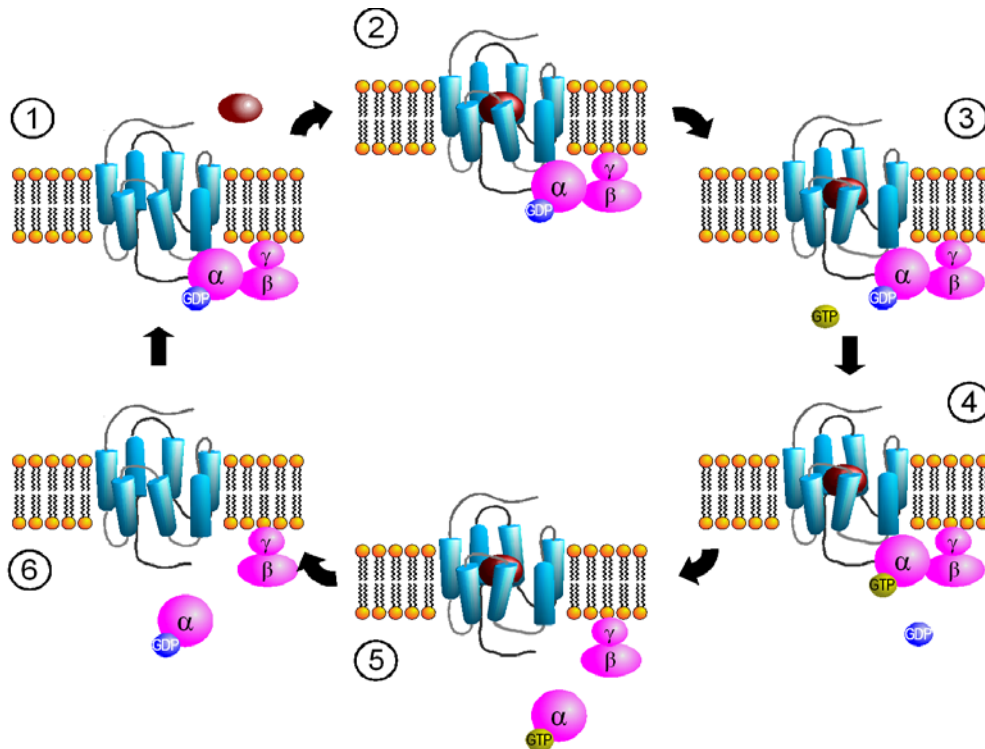


Figure 2: Activation cycle of G-proteins by G-protein-coupled receptors. 1) G protein-coupled receptors are activated by external stimuli in the form of a ligand. 2) This causes a conformational change in the receptor, which results in the activation of a closely coupled G protein. 3) G proteins are made up of alpha (α), beta (β) and gamma (γ) subunits. The binding of a ligand to a GPCR initiates the interaction between the receptor and G protein inside the cell. 4) This interaction catalyses guanine nucleotide exchange factor (GEF) to exchange a bound guanosine diphosphate (GDP) for a guanosine triphosphate (GTP) on the $G\alpha$ subunit of the G protein. 5) The $G\alpha$ subunit dissociates from the $G\beta\gamma$ dimer and after binding to the GTP. This brings about the activation of select intracellular signalling pathways and effector proteins. 6) $G\alpha$ has a slow GTP \rightarrow GDP hydrolysis capability; regenerating the inactive form of the α -subunit ($G\alpha$ -GDP). This allows the re-association to the $G\beta\gamma$ dimer and the inactive G-protein complex which can again bind to a GPCR and be reactivated. Taken from (Jähnichen, S. (2006)).

This brings about the activation of different signalling pathways and effector proteins such as enzymes and ion channels (Ferguson, 2001). The $G\alpha$ and $G\beta\gamma$ subunits stimulate several effector molecules, which include adenylyl and guanylyl cyclises, phosphodiesterases, phospholipase A₂ (PLA₂), phospholipase C (PLC) and phosphoinositide 3-kinases (PI3Ks), thereby activating or inhibiting the production of various second messengers (Figure 3) (Marinissen and Gutkind, 2001).

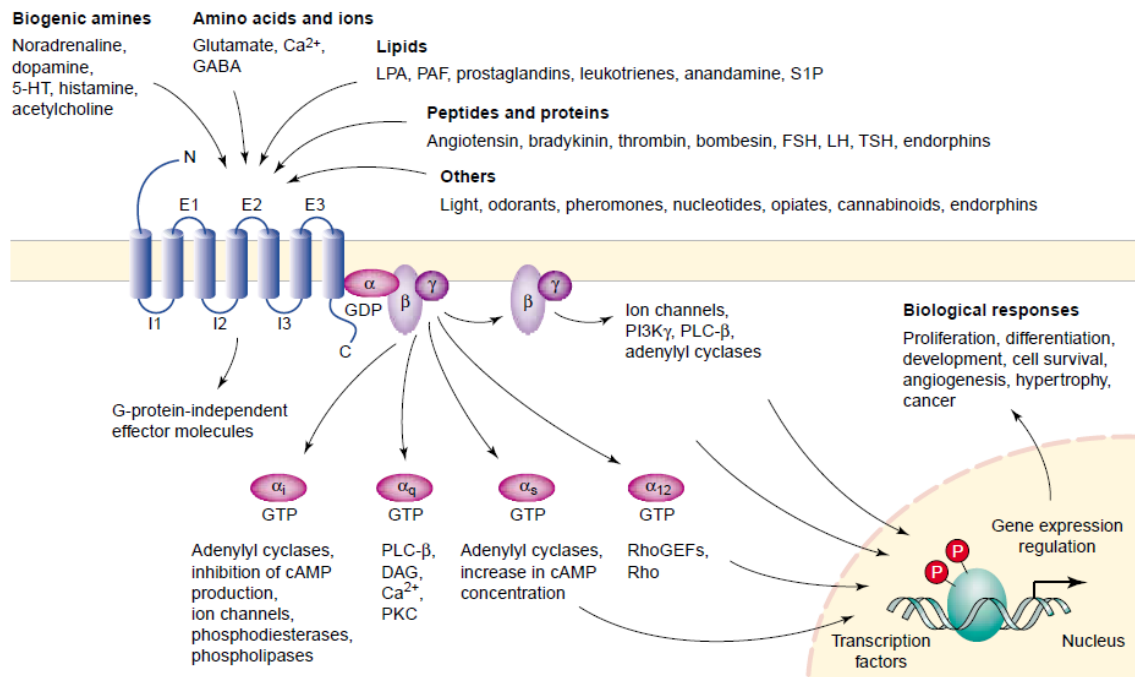


Figure 3: Diversity of GPCRs mediated signalling. A wide variety of ligands, including biogenic amines, amino acids, ions, lipids, peptides and proteins, use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G-protein-dependent and independent pathways. Such signalling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis.

Abbreviations: DAG, diacylglycerol; FSH, follicle-stimulating hormone; GEF, guanine nucleotide exchange factor; LH, luteinising hormone; LPA, lysophosphatidic acid; PAF, platelet activating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine-1-phosphate; TSH, thyroid-stimulating hormone. Taken from (Marinissen and Gutkind, 2001).

After a GPCR has been activated it cannot remain in this state forever. A regulatory mechanism is in place to control the extent and time of activation. This involves desensitisation and internalisation of ligand activated GPCRs. The sequence of receptor activation and desensitisation is an important process that must be recognised when developing a drug targeting the GPCR.

Early desensitisation is seen as a protective mechanism towards excessive stimulation. It brings about a decrease in cellular responsiveness to further stimulation. It occurs within seconds to minutes following receptor activation by agonist exposure and involves phosphorylation of serine residues located in the third cytoplasmic loop of the C-terminal domain of the GPCR. Phosphorylation is brought about by protein kinase A (PKA), which is cAMP dependent and by G protein-coupled receptor kinases (GRK). This results in uncoupling of the receptors from their respective heterotrimeric G proteins and loss of downstream-signalling events without a detectable change in the total number of receptors

present in cells or tissues (Ulloa-Aguirre et al., 1999, Freedman NJ, 1996, Krupnick and Benovic, 1998, Clark et al., 1989, Sterne-Marr and Benovic, 1995).

There are two recognised forms of desensitisation namely homologous desensitisation and heterologous desensitisation. Homologous desensitisation is a very rapid two-step process. It is agonist-dependent (specific) and results in a cell's response decreasing after high receptor occupancy by a specific ligand. The ligand-occupied receptors are phosphorylated by a GRK which prepares the receptor by increasing its affinity for binding with soluble intracellular inhibitory proteins called arrestins. These inhibitory proteins prevent signal transfer to the G protein by uncoupling the receptor. It further targets the receptor for internalisation, which redirects signalling to alternative G protein-independent pathways (Lefkowitz, 1998). Receptor phosphorylation can occur in the absence of a ligand. An example of this can be seen with heterologous desensitisation, where the activation of one GPCR can result in the inhibition of another heterologous GPCR. It involves second messenger-dependent kinases (PKA and PKC) and is non-ligand specific. Heterologous desensitisation is observed after several minutes of low amounts of ligand exposure. It occurs when a cell's response to various ligands acting through different receptors decreases after receptor activation by a specific ligand.

Homologous desensitisation may be mediated through receptor phosphorylation which can be brought about by both second messenger-activated protein kinases and GRKs (Ulloa-Aguirre et al., 1999). The combined action of GRKs and arrestins leads to the attenuation of G protein signalling and internalization of the stimulated receptor (Sherrill and Miller, 2008). The endocytosis of G protein-coupled receptors is called internalisation. Very simply, this process is thought to promote dephosphorylation by bringing receptors to endosome-associated phosphatases. Dephosphorylated receptors can then be recycled back to the plasma membrane. This reversal of the desensitised state is known as resensitisation. Recycling receptors enables the cell to return to an inactivated state and by this mechanism enables the quick recovery of signalling potential after ligand withdrawal.

One pathway of internalisation is that of ligand-activated G protein-coupled receptors being mediated by non-visual arrestins - β -arrestins 1 and 2. Arrestins are thought to act as adaptors in the endocytosis process. They bind directly, stoichiometrically, and with high affinity to clathrin (Gaidarov, 1999 , Lefkowitz, 1998). The function of clathrin is to facilitate the formation of small vesicles in the cytoplasm. Clathrin-coated vesicles selectively store and transport substances for various membrane trafficking pathways. Clathrin-mediated endocytosis is the most efficient and fastest pathway for receptor endocytosis. Formation of endocytic vesicles are an important part of membrane recycling. Specialised regions of the intracellular plasma membrane are coated by a lattice of clathrin forming a structure called a coated pit. The internalised receptors are trafficked via endosomes; following the removal of the clathrin coating of the vesicle and fusion with endosomal membranes, this process requires dynamin (Calebiro et al., 2010). A molecular chaperone in protein-assisted refolding (cytosolic Hsc70) stimulates the removal of clathrin from the coated vesicles. The clathrin triskelions can be re-used in the formation of new coated pits and vesicles after internalisation, while ligand-receptor complexes traffic through endosomal compartments. Sorting mechanisms determine the fate of the internalised receptors. Either they return to the plasma membrane, remain in the endosomes for a longer period of time or are transported to lysosomes or other organelles for degradation or dismantling (Figure 4).

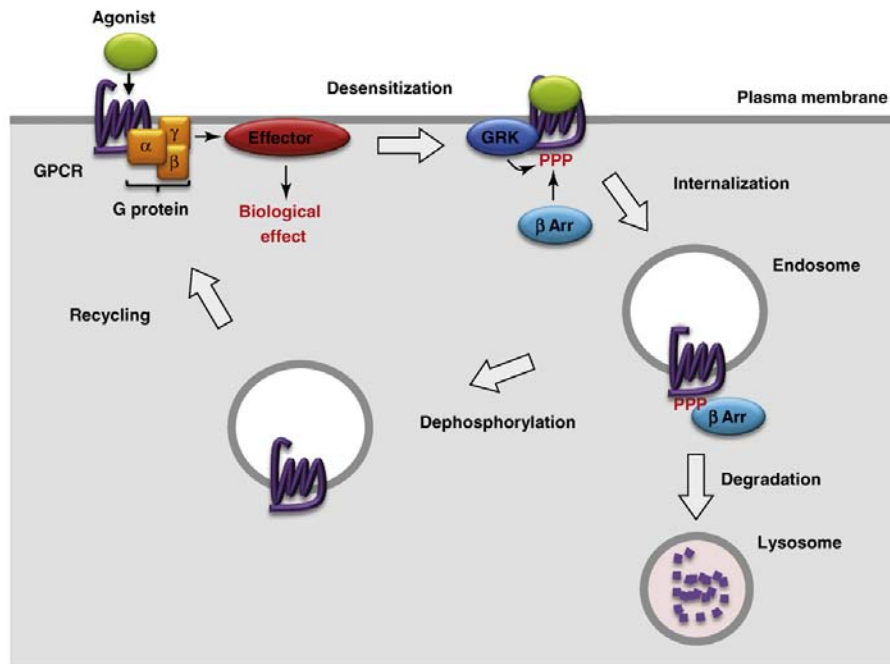


Figure 4: Depicting desensitisation and internalisation in a GPCR. Desensitisation brings about a decrease in cellular responsiveness to further stimulation. Phosphorylation occurs by protein kinase A (PKA) and by G protein-coupled receptor kinases (GRK). This results in uncoupling of the receptors from their respective heterotrimeric G proteins with a loss of downstream-signalling events. Upon GRK phosphorylation, the GPCRs affinity for β -arrestin increases. Once β -arrestin binds to a GPCR it undergoes a conformational change and recruits clathrin for internalisation and the process of opsonisation begins. Individual vesicles fuse to form early endosomes. The receptor will be trafficked either back to the plasma membrane or delivered to lysosomes for degradation. Taken from (Calebiro et al., 2010).

Not all GPCRs are internalised via clathrin-coated pits. Several are known to undergo clathrin-independent internalisation (van Koppen and Jakobs, 2004).

This knowledge was important in understanding why GPCRs became so essential in drug discovery and development, and especially in oncogenesis. The involvement and onco-modulatory functions of GPCRs will be discussed further under Section 1.3.

2.2 Human cytomegalovirus, viral G protein-coupled receptors and unique short 28

The human cytomegalovirus (HCMV) also known as human herpesvirus 5 (HHV-5) is a member of the herpesviridae family and β human herpesvirus subfamily. It is the largest known human herpesvirus with its 230 kbp genome encoding approximately 200 genes, of which more than 70 are for viral proteins (Varnum et al., 2004). The structure of the human cytomegalovirus is as follows : it has a double stranded linear DNA genome which is enveloped by a proteinaceous matrix referred to as the tegument. The tegument together with

the genome is encased in an icosahedral lattice called the nucleocapsid. The capsid is 130nm in diameter and is surrounded by a lipid bilayer that contains viral glycoproteins such as gB and gH. The complete particle is known as the virion (Chen et al., 1999) (Figure 5). The envelope of the HCMV virion is derived from the host cell at both nuclear and cytoplasmic intracellular membranes. There are at least 25 viral proteins packaged within the tegument. Most of the tegument proteins are encoded by conserved open reading frames (ORF's) and are both phosphorylated and immunogenic (Varnum et al., 2004).

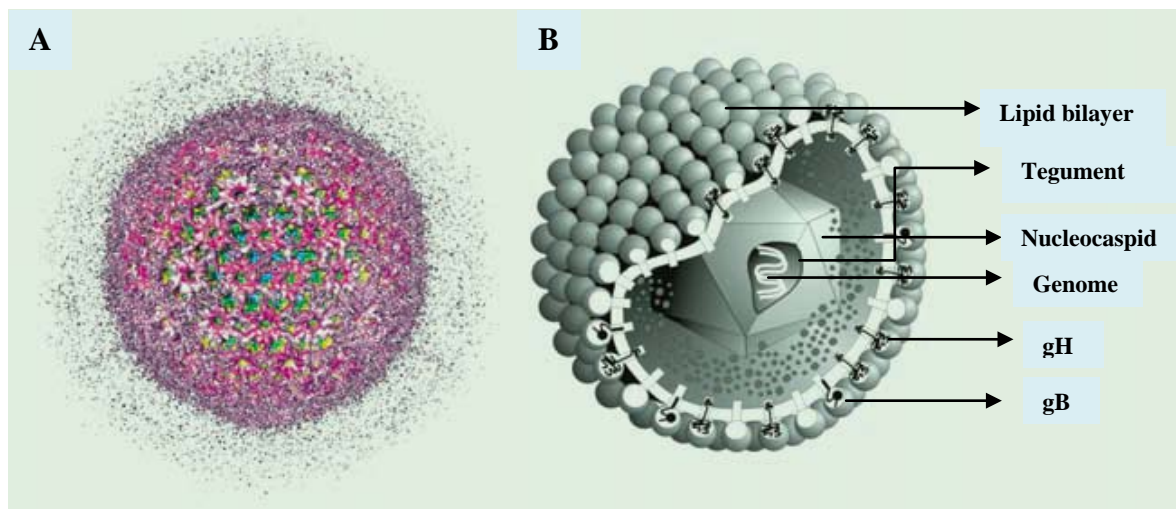


Figure 5: Structure of the Human cytomegalovirus (HCMV). (A) A shaded surface representation of a 3D reconstruction of the icosahedrally ordered portion of an intact human cytomegalovirus particle as viewed along a three-fold symmetry axis (B) Virtual 3D model, showing various components of the HCMV virion. Adapted from (Gandhi and Khanna, 2004).

HCMV is widely distributed throughout the world's population with up to 90% of the individuals in some populations harbouring a latent viral infection (Garcia et al., 2006). HCMV infection varies with geographic location and socioeconomic status. It is more widespread in developing countries, homosexual men, and in communities with lower socioeconomic status. It is important to note that it represents the most significant viral cause of birth defects in the Western world and that total HCMV clearance is hardly ever achieved. The viral genome remains at selected intracellular sites when the virus is in a latent state (Pass et al., 2009). After a primary infection the virus can establish lifelong latency and persistence, as the immune system cannot clear the infection. HCMV has adapted to survive in a host with normal immune function this reflects tremendous evolutionary pressure on the virus to develop strategies to avoid recognition and elimination by the immune system (Söderberg-Nauclér, 2008).

HCMV infections are frequently associated with the salivary glands, due to persistent and recurrent virus shedding through saliva. Besides being transmitted by saliva it can also be transmitted via sexual contact, placental transfer, breastfeeding and blood transfusions. The majority of immunocompetent hosts with HCMV infections are asymptomatic; however HCMV infections are important to certain high-risk population groups. These infections can be life threatening for pre-natal (causing encephalitis) or post-natal patients, as well as individuals who are immuno-compromised. These include organ transplant recipients, persons with leukemia, or those infected with human immunodeficiency virus (HIV). In HIV infected individuals a co-infection with HCMV is considered an ‘AIDS-defining infection’. The reason for this is because the HCMV infection reduces the T-cell count even faster and thereby accelerates the progression to AIDS. HCMV can also lead to other severe pathologies such as pneumonitis, hepatitis, and retinitis (Deayton et al., 2004, Gandhi and Khanna, 2004). HCMV infections are associated with high mortality rates in recipients of bone marrow transplants and with increase morbidity in recipients of solid organ transplants due to chronic rejection. Conventional vaccination with attenuated HCMV or HCMV proteins have failed to prime protective immune responses, most probably due to the antigens failing to be presented effectively *in vivo* (Bennekov, 2004).

According to an evolutionary theory, over the ages viruses have ‘pirated’ cellular genes from their hosts that are involved in cell-cycle control, immune modulation, apoptosis, signal transduction of growth factors, differentiation and cell trafficking to support their life-cycles (Couty and Gershengorn, 2005). HCMV appears to be one of the viruses having ‘pirated’ genes encoding key regulatory cellular proteins, many of which display a high homology to human cellular GPCRs and chemokines. The virus incorporates these genes into its own genome thereby enabling these HCMV-encoded GPCRs to elude the immune system, redirect cellular signalling and contribute to pathologies (Vischer et al., 2006). This is due to the fact that GPCR proteins can physically interact with each other, thereby modifying intracellular signalling and cellular functions. This makes GPCR’s very beneficial and worthwhile for incorporation of viruses into a host genome. This ability enables the virus to alter its host cell’s homeostasis in the absence of a receptor ligand and to use the intracellular signalling pathways for its own benefits. The functional properties of these viral receptors are different from their host homologs (Couty and Gershengorn, 2005). Constitutive signalling by GPCRs contributes to virus survival and host invasion.

Since GPCRs have proven to be such successful drug targets and their role in viral infections is becoming clearer, viral GPCRs are also emerging targets for drug development research (Smit et al., 2000, Maussang D, 2009). A high frequency of HCMV DNA, mRNA and antigens have been well documented in past years in tumour samples from patients with different malignancies (Cinatl et al., 2004, Söderberg-Nauclér, 2008) - ranging from colon cancer (Harkins et al., 2002), malignant glioblastomas (Cobbs et al., 2002), EBV-negative Hodgkin's lymphoma (Huang G, 2002), prostatic carcinoma (Minu et al., 2003) and breast cancer (Cobbs C, 2007). Together with epidemiological data, this suggests a role for HCMV in cancer (Slinger et al., 2010a). However, the exact role of the virus has not yet been established. Unlike its counterparts, the gamma herpes viruses, which include Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV), HCMV is not regarded as an oncogenic tumour virus. Instead HCMV may have an onco-modulatory role, catalysing an oncogenic process that has already been initiated (Slinger et al., 2010a). Tumour cells with disrupted regulatory or signalling pathways enable HCMV to modulate these properties including stimulation of cell proliferation, survival, invasion, production of angiogenic factors, and immunogenic properties (Cinatl et al., 2004).

In fully permissive cells, expression of the HCMV genome is controlled by a series of transcriptional events that leads to the synthesis of three categories of viral proteins: immediate-early (IE), early (E), and late (L). The term onco-modulation has been used to describe the ability of HCMV to modify tumour cell biology (Cinatl et al., 2004, Cinatl et al., 2005). Onco-modulation means that HCMV may infect tumour cells and modulate their malignant properties in a manner not involving direct transformation. It is believed that tumour cells provide an altered genetic environment, characterised by disturbances in intracellular signalling pathways. Transcription factors and tumour suppressor proteins in the transformed cells enable HCMV to exert its onco-modulatory potential and because of this HCMV has been suggested to be a therapeutic target in some cancer patients (Martin Michaelis, 2009). This is partly supported by the fact that HCMV also produces a number of anti-apoptotic proteins that may interfere with the action of chemotherapy, rendering virus-infected tumour cells resistant to standard anticancer treatment (Söderberg-Nauclér, 2008).

Beside HCMV infection being linked to the development of proliferative pathologies, it is also linked to cardiovascular pathologies (Maussang et al., 2009). This study however focuses on the proliferative pathologies surrounding HCMV. The HCMV genome can be divided into two parts, a unique long part - known as UL and a unique short part - US (Slinger et al., 2010a) (Figure 6).

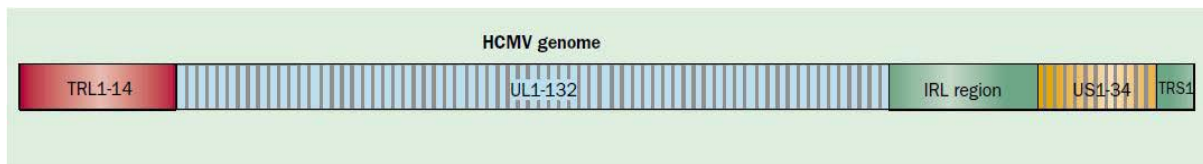


Figure 6: A schematic representation of the HCMV genome. Expressing the long and short regions-UL and US respectively. Adapted from (Gandhi and Khanna, 2004).

HCMV encodes four GPCRs, which contain four chemokines receptors which are encoded in the open reading frames (ORFs) US27, US28, UL33 and UL78 (Chee et al., 1990). These GPCR homologs are believed to be involved in HCMV-induced pathogenesis. One of the HCMV-encoded proteins, known to induce a proliferative and angiogenic phenotype *in vitro* and *in vivo*, is the viral chemokine receptor US28 (Maussang et al., 2006). The US28 gene is located in the unique short region of the HCMV genome and is transcribed during infection of permissive cells. Transcription has also been detected in semi-permissive and non-permissive cells (Zipeto et al., 1999). It belongs to the GPCR -chemokine receptor branch of the rhodopsin family and has homologs to UL33 and US27 which are transcribed as an early gene of HCMV. This receptor signals constitutively and interacts with a broad range of chemokines which are essential to the pathophysiological significance and immunoregulatory aspects of this receptor (Moser et al., 2008). The US28 sequence has features that are shared by all human and viral chemokine receptors. These include a highly acidic amino-terminal segment, a Pro-Cys motif in the amino-terminal segment, a conserved cysteine in the third extracellular loop, and a 16-residue basic third intracellular loop. It does however lack a consensus sequence for N-linked glycosylation (Gao and Murphy, 1994). To date US28 is the best characterised of all HCMV vGPCRs.

US28 is related (33 % amino acid identity) to the receptor for the human Macrophage Inflammatory Proteins (MIP-1 α and MIP-1 β), Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES) and Monocyte Chemoattractant Protein-1(MCP-1). These CC chemokines receptors have been suggested to be the human homolog of US28. It was found that the US28 receptor binds these ligands with a rank order of RANTES > MCP-1 > MIP-1 α = MIP-1 β (Kuhn et al., 1995). US28, US27 and UL33, all have homology to the CC chemokine receptors, yet only US28 can function as a receptor for the CC chemokines (McElroy et al. 2000). Other definitive characteristics of US28 include that it is an integral plasma membrane protein, it binds four distinct human β chemokines; it is capable of transducing specific chemokine signals to the cytoplasm by inducing transient elevations of [Ca²⁺] (this can best be seen in response to RANTES). US28 mRNA is expressed *in vitro* in the late phase of lytic infection of fibroblasts (Welch et al., 1991). US28 activates monocyte motility for all its ligands (Baggiolini M, 1994) and *in vivo* MIP-1 α can regulate the proliferation of hematopoietic progenitor cells and is a stem cell inhibitory factor (Gao and Murphy, 1994).

Beside the CC chemokines that have two cysteines adjacent to each other, US28 binds with the strongest affinity to the CX3C chemokine: fractalkine that have three intervening amino acids between the two cysteine residues. The binding affinity for fractalkine is such that this interaction is thought to mediate adhesion of virus-infected cells to membrane-bound fractalkine. US28 does not however exhibit any affinity for the CXC chemokines that have one intervening amino acid between the cysteine residues (Randolph-Habecker et al., 2002).

It is believed that US28 promotes dissemination of HCMV-infected cells in response to chemokine gradients *in vivo* (Lalani et al., 2000). Given that this receptor signals in both a ligand-dependent and constitutive manner, US28 could modulate cell behaviour. As HCMV infection progresses, the cells expressing US28 on their surface are able to bind β chemokines like: CCL3/Macrophage Inflammatory Protein (MIP)-1 α and CCL5/RANTES from the extracellular environment and probably internalise them in a β -arrestin independent manner (Droese et al., 2004). This prevents the targeting of HCMV-infected cells by chemokine-activated immune cells. This implies that, by effective chemokine sequestration, HCMV-infected cells can avoid elimination by chemokine-activated lymphocytes and NK cells. The CC chemokine MCP-1 is also down regulated during HCMV infection (Fortunato et al., 2000). Viral derived antigens are found mainly in neutrophils and monocytes during acute infection and these chemokines may facilitate dissemination of HCMV from the initial

replication site by attracting neutrophils and monocytes (Landolfo et al., 2003). Leukocytes and vascular endothelial cells can aid the spread of HCMV in this way.

It has been suggested that the expression of US28 may function as a decoy receptor that binds CC chemokines and that this prevents triggering of the immune system against the infected host cells rather than functioning with a principal role of transmitting a signal. An alternative hypothesis by (Billstrom et al., 1998) proposed that during HCMV infection the virally encoded US28 is expressed on the surface of the infected cell as a functionally active receptor that mediates downstream effector functions in the host cell following chemokine stimulation (Billstrom et al., 1998). In a study evaluating HCMV infection of vascular smooth muscle cells, the infection showed a greater migration (chemokinesis) when infected with US28-expressing viruses compared with controls (Streblow et al., 1999). This led to a model suggesting that the migration of HCMV-infected cells toward sites of inflammation is a means of virus dissemination as well as a factor contributing to chronic vascular disease. Most activities attributed to US28 are pro-inflammatory rather than anti-inflammatory (Mocarski, 2002). US28 has been found to induce various oncogenic responses when expressed in NIH 3T3 fibroblasts, including increased production of cyclin D1, cyclooxygenase-2 (COX2), and vascular endothelial growth factor (VEGF) (Maussang et al., 2006, Maussang et al., 2009). Moreover, US28 contributes to HCMV-induced VEGF promoter activity and COX2 expression in HCMV-infected cells (Maussang et al., 2006, Maussang et al., 2009) and promotes tumour formation in a mouse xenograft model (Maussang et al., 2006, Maussang et al., 2009). With this in mind US28 may function either as a chemokine receptor, a phospholipase C activator, or a pro-apoptotic factor (Bego and St. Jeor, 2006). Just like other GPCRs US28 can also undergo rapid receptor endocytosis and recycling in a ligand-independent fashion. The US28 C-terminal domain is constitutively phosphorylated by GRK family of proteins. β -arrestin recruitment decreases or weakens constitutive signalling and allows constitutive receptor endocytosis and recycling via the clathrin mediated mechanism (Pleskoff et al., 2005).

In summary, US28 is a vGPCR that exerts anti-tumourigenic effects in two melanoma cell lines, Sbc12 and 451Lu (where it is expressed on the cell surface) (Moser et al., 2008). Contrary to this recent work on US28 provides a molecular link between HCMV and cancer. US28 stably transfected NIH-3T3 cells gave origin to tumours when introduced sub-cutaneously into nude mice (Maussang et al., 2006, Alcamì and Lira, 2010). Further *in vivo* data would be needed to confirm the role of US28 in oncogenesis.

This study reports on an investigation of the effects that US28 had on IL-6 and the phosphorylation of STAT3. These two markers were chosen based on results from previous work done in the Department of Medicinal chemistry on US28. In an experiment to identify factors secreted by US28-expressing NIH 3T3 cells, a mouse antibody array for angiogenic factors recognising different chemokines, growth factors, and cytokines showed that US28 increases the secretion of both IL-6 and VEGF. This study investigated IL-6 secretion in HCMV infected HFF and U373 cells *in vitro*. This was followed by investigating STAT3 phosphorylation since the STAT3 activation pathway being one of the downstream targets of IL-6. This study differed from previous work in making use of virally infected rather than transfected cells.

2.3 Oncogenesis, Interleukin 6, Signal Transducer and Activator of Transcription 3 and their cellular function

Oncogenesis is a complex process of cellular transformation that can lead to uncontrolled cell division either by altering genetic coding within one or more genes or through inhibition of suppressor genes that normally control the rate of cell division. This uncontrolled cellular proliferation can result in the formation of malignant tumours in the body. Oncogenesis is characterised by a progression of changes that occur at both cellular and genetic level. Cancer is the result of alterations in oncogenes, tumour-suppressor genes, and microRNA genes (Croce, 2008). An oncogene is a normally functioning gene that, when mutated or expressed at high levels, establishes conditions which are favourable for normal cells to undergo uncontrolled cell division, thus forming a malignant mass (a cancerous tumour). A number of oncogene products are analogs of the normal cellular components (Rhoads, 1991). Oncogenes encode proteins that are involved in the control of cell proliferation, apoptosis, or both (Croce, 2008). These genes can be introduced into a normal cell through viral infection,

or may be switched on by genetic damage through ionising radiation or chemical agents. Viral GPCRs appear to play a role in oncogenesis and tumour growth (Muller et al., 2001, Iwakiri et al., 2009).

Interleukins are a subset of a larger group of proteinaceous cellular messenger molecules called cytokines. They are a group of naturally occurring highly active proteins that are involved in cell-to-cell communication that are secreted by leukocytes, especially by macrophages and T lymphocytes which amongst others promote the development and differentiation of most hematopoietic cells as well as recruitment and activation of leukocytes. Interleukins are generally not stored within cells but are instead secreted rapidly, and briefly, in response to various stimuli.

IL-6 is an interleukin which is important in host defence and acts as both a pro-inflammatory and anti-inflammatory cytokine. Due to both its pro- and anti-inflammatory actions, IL-6 is a pleiotropic cytokine that regulates some immune responses. It plays a central role in both the innate and acquired immune response and is essential for antibody production, playing a role in the final differentiation of B-lymphocytes into Ig-secreting cells. It is the predominant inducer of fever and the acute-phase response in the liver, both of which are innate immune mechanisms which are triggered by infection and inflammation (Gealy et al., 2005). IL-6 is produced by many different cell types. The main sources *in vivo* are activated monocytes, fibroblasts, and endothelial cells. Macrophages, T-lymphocytes and B lymphocytes, granulocytes, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cells, and keratinocytes can also produce IL-6 after stimulation. IL-6 is involved in lymphocyte and monocyte induction of nerve cells differentiation. It can induce myeloma and plasmacytoma growth. IL-6 acts as a "myokine": a cytokine produced by muscle cells which is discharged into the bloodstream after muscle contraction and acts to increase the breakdown of fats and increase glucose uptake (Figure 7).

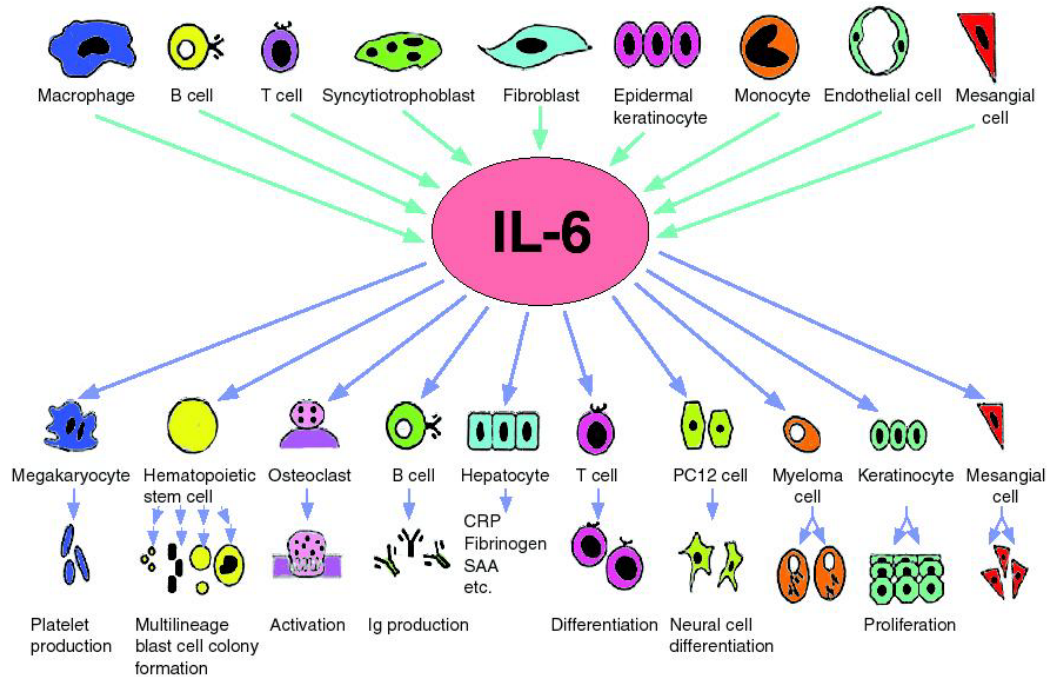


Figure 7: The biological activities of IL-6. This schematic shows cells known to produce interleukin-6 (IL-6) and the actions of IL-6 in the human body. Immunoglobulin (Ig), C-reactive protein (CRP), serum amyloid A-protein (SAA). Taken from (Naka T, et al.2002).

The IL-6 is a single-chain glycoprotein with a reported molecular mass ranging from 21kDa to 30kDa. This is due to the fact that monocytes express at least five different molecular forms of IL6. These forms mainly differ by post-translational alterations such as glycosylation and phosphorylation. IL-6 is a single-chain glycoprotein with its primary structure consisting of 184 amino acids (Rose-John et al., 1992). Just like all cytokines it contains four cysteine residues that are highly conserved which form disulphide bridges. It has a 4 α -helical bundle with an up-up-down-down topology (Hammacher, 1994). The IL-6 gene maps to the human chromosome 7p21-p14 between the markers D7S135 and D7S370. It is approximately 5 kb and contains four introns and five exons. The heterogeneous nature of IL-6 is due to extensive post-translational modification with most forms of IL-6 phosphorylated at multiple serine residues, although the extent of phosphorylation is very tissue specific (Simpson et al., 1997). The IL-6 gene promoter contains many different regulatory elements which allows for the induction of gene expression by numerous different stimuli, including glucocorticoids and cAMP. In non-lymphoid cells the NF- κ B binding site is responsible for the induction of the IL-6 gene expression after IL-1 or Tumour Necrosis Factor-alpha (TNF- α) exposure (Figure 8). Human IL-6 exerts a wide variety of biological functions including growth and anti-apoptotic effects via it's receptor (Osborne et al., 1999).

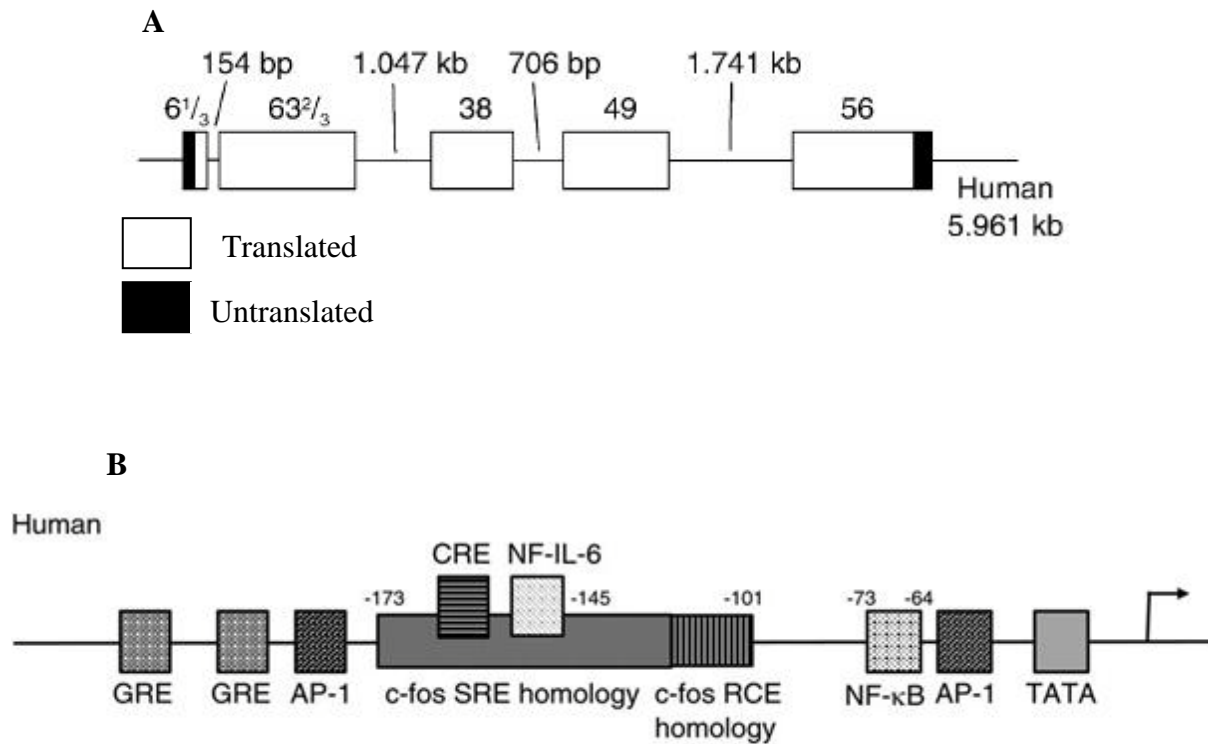


Figure 8: (A) Gene structures of human IL-6. Exons are shown as boxes, introns as lines joining the boxes. Numbers above the exons indicate the number of amino acids encoded by each exon. **(B)** The promoter of human IL-6, showing the different transcription factor binding sites present in each. GRE -glucocorticoid responsive element; CRE -cAMP responsive element; SRE -serum responsive element; RCE -retinoblastoma control element. The arrows indicate the transcriptional start points. The human IL-6 promoter details are taken from (Hirano T.1998). This figure was taken from (Kaiser et al., 2004)

The receptor complex through which IL-6 exerts its effects is expressed on T- lymphocytes, mitogen-activated B-lymphocytes, peripheral monocytes as well as by some macrophage and B-lymphocyte derived tumour cell types. It is not expressed on resting B- lymphocytes but is present on resting T-lymphocytes. The receptor consists of two heterologous membrane-bound glycoprotein subunits, the ligand binding IL-6R α (also known as CD126) and the signal transducing gp130 (also known as CD130) (Jones et al., 2001) (Figure 9).

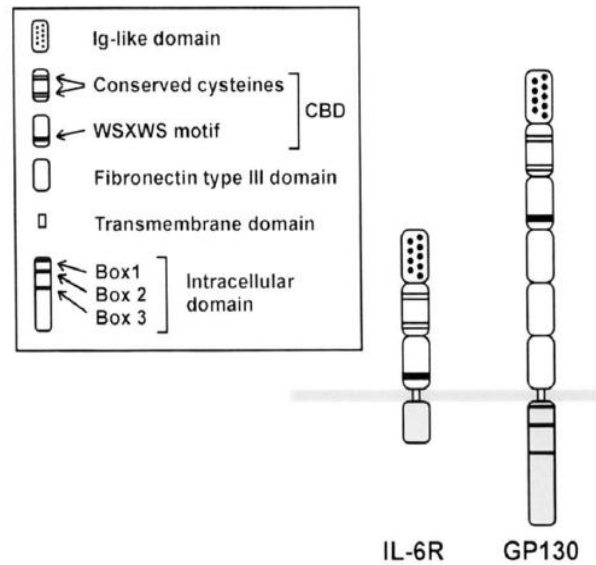


Figure 9: Structure of IL-6R and gp130. Schematic representation of the domains of the IL-6R and gp130 proteins. The shaded horizontal line represents the cell membrane. Taken from (Simpson et al., 1997).

The receptor contains an immunoglobulin-like sequence domain in the amino terminal region of the extracellular receptor domain. The intracellular domain of the IL-6 receptor does not show any homology to other proteins involved in intracellular signal transduction. To initiate signalling by the receptor complex, the IL-6 must first make a low affinity contact with the IL-6R α subunit which then recruits the signal transducing gp130 subunit. Binding of IL-6 to this receptor leads to disulfide-linked homodimerisation of gp130 and the associated activation of cytoplasmic JANUS activated kinases (tyrosine kinases - JAK1, JAK2, and TYK2). This is accepted as the first step of signal transduction and phosphorylation of the Signal Transducers and Activators of Transcription (STATs) STAT1 and STAT3. Activation of the JAK-STAT pathway can result from numerous cytokine receptor systems but known to be activated specifically by the IL-6 family of cytokines (Molden et al., 1997) (Figure 10).

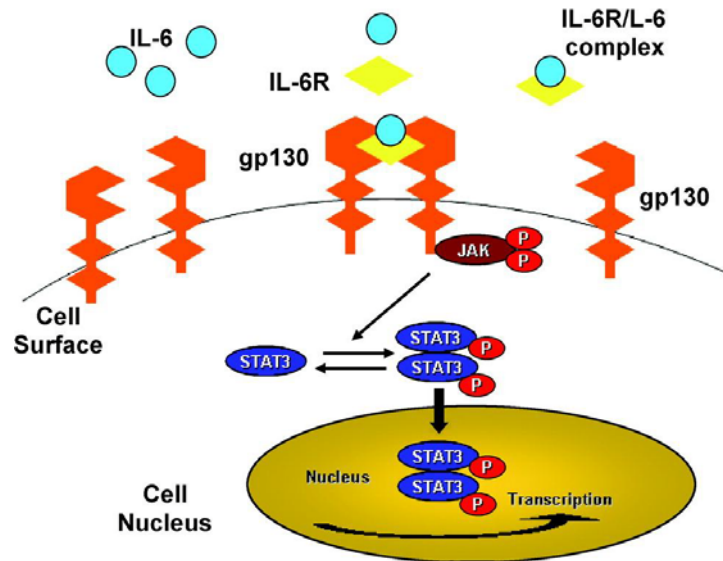


Figure 10: IL-6 mechanism of action, triggering of intracellular signalling pathways.

IL-6 functions by binding to either its membrane-bound receptor or its soluble receptor. The binding of IL-6/sIL-6R to the gp130 signalling subunit induces the homodimerization of gp130. This, in turn, triggers an intracellular signalling cascade through several pathways including Ras/Raf, mitogen-activated protein kinase, and Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT). Taken from (Ahmed et al., 2007)

Gp130 was initially identified as the signal-transducing component of the IL-6 receptor. New insight into gp130 has found that it also forms part of the receptor-complexes of leukemia inhibitory factor (LIF), oncostatin-M (OSM), ciliary neurotrophic factor (CNTF), interleukin 11 (IL-11) as well as cardiotrophin-1 (CT-1) and the novel neurotrophin-1/B cell stimulating factor-3 (Simpson et al., 1997). IL-6 has recently been implicated in human cervical cancer by influencing the pathogenesis of the disease, though the mechanism remains unclear. IL-6 is a central pro-inflammatory cytokine involved in female genital infection and cervical cancer which frequently develops in close association with chronic inflammation caused by infection with various sexually transmitted agents. This suggests a strong connection between IL-6 and cervical cancer. (Richter et al., 1999). Additionally, IL-6 was found to be highly expressed in invasive cervical carcinomas, but not expressed or only barely expressed in normal cervix tissue or in pre-neoplastic lesions (Tartour E, 1994) (Wei LH, 2001). IL-6 has been found to function as an autocrine or paracrine tumourigenic factor (Kuilman et al., 2008). Human IL-6 may also contribute to a variety of B cell neoplastic disorders (Molden et al., 1997).

The biological activities of IL-6 are important in regulating tumour cell growth, and can be produced by various types of cancer cells, including multiple myeloma, renal cell carcinoma, prostate carcinoma, ovarian carcinoma, and cervical carcinoma by autocrine or paracrine mechanisms. IL-6 can act as an apoptosis inducer or inhibitor depending upon the types of cells. Due to this, the determination of IL-6 serum levels may be useful to monitor the activity of myelomas and to calculate tumour cell masses. Interleukin concentrations have been suggested to serve as a clinical parameter of severity of HCMV infection, and due to this studies have been performed to investigate the possible role of IL-6 as prognostic parameter (Zedtwitz-Liebenstein et al., 2009). It was also noted that advanced/metastatic cancer patients have higher levels of IL-6 in their blood (Chiche et al., 2001). In addition, IL-6 may play a role in enhancing the growth and tumorigenicity of B lymphocytes immortalised by Epstein-Barr virus (EBV), another herpes virus known to cause cancer. At least 1 polymorphism in the IL-6 gene renders HIV-infected men susceptible to Kaposi sarcoma. This is most commonly seen in patients who suffer from AIDS. Genetic variations in IL-6 are associated with susceptibility to many other diseases: diabetes, atherosclerosis, depression, systemic lupus erythematosus, prostate cancer, and systemic juvenile rheumatoid arthritis being a few.

The genome of the human herpes virus 8 (HHV-8/KSHV) encodes a structural homolog of IL-6, referred to as viral IL-6 (vIL-6) (Neipel et al., 1997). Viral IL-6 shows 62 % amino acid similarity to human IL-6 and has been suggested to be involved in the pathogenesis of a variety of diseases. The detection of an IL-6 gene homologue in HHV-8 and its respective gene product led to the suggestion that vIL-6 may play a functional role in the pathogenesis of other lymphoproliferative diseases (Burger et al., 1998). Due to the activity of vIL-6 as an inducer of IL-6 production, it may be an important factor in the development of HCMV associated diseases. Viral IL-6 has also been shown to activate the signalling pathways of cytokines involving STAT proteins and Janus kinases via interactions with the gp130 signal transducing subunit (Molden et al., 1997).

Previous studies have shown that the production of TNF- α , IL-1, IL-6, and IL-8 increase in HCMV-infected cells *in vitro*. HCMV's ability to induce production of IL-6 has therefore been suggested to contribute to HCMV associated mucosal inflammation (Rahbar et al., 2003). Elevated levels of IL-6 have been reported to accompany HCMV replication in transplanted lungs and bone marrow during episodes of inflammation or rejection. Due to the ability of HCMV to survive long term in the infected host, it might be likely that the virus has evolved mechanisms to alter the expression or signalling of IL-6 as part of the viral arsenal of immune evasion strategies (Gealy et al., 2005). This is not fully understood and the effects of HCMV infection on IL-6 expression are not well documented and need to be further addressed.

Almeida et al. (1994) showed that HCMV can cause a disturbance of the cytokine cascades involved in the regulation of haematopoiesis, in particular the expression of IL-6 by endothelial cells (ECs). In the data presented it was shown that HCMV was able to induce a 100-fold increase in IL-6 production detectable as early as 2 hours post infection at the protein level and within 30 minutes at the mRNA level (Almeida et al., 1994). It was also shown by (Evers et al., 2004) that IL-6 was induced in HCMV-infected fibroblasts, reaching maximal concentrations approximately 20 hours post infection. Additionally, HCMV infection in fibroblasts has been found to induce the transcription of the IL-8 receptor. Thus, the virus could regulate the secretion and uptake of these ILs from different cell types in order to maximize the viral particle dissemination in the host (Fortunato et al., 2000). Increased IL-6 levels have been associated with increased activation of STAT3. This involved upstream activation of JAK1. The mechanisms of constitutive STAT3 activation include (among others) both autocrine and paracrine production of IL-6 which leads to cytosolic STAT3 phosphorylation.

Signal Transducers and Activators of Transcription (STAT) proteins are a family of 7 transcription factors (STATs 1, 2, 3, 4, 5a, 5b, and 6) that form part of the JAK-STAT signalling cascade. This cascade is the basis of the signal transduction mechanism for many cytokine receptors (Toshio Hirano, 2000). In response to cytokines, growth factors hormones and oncogenes, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators (Figure 11).

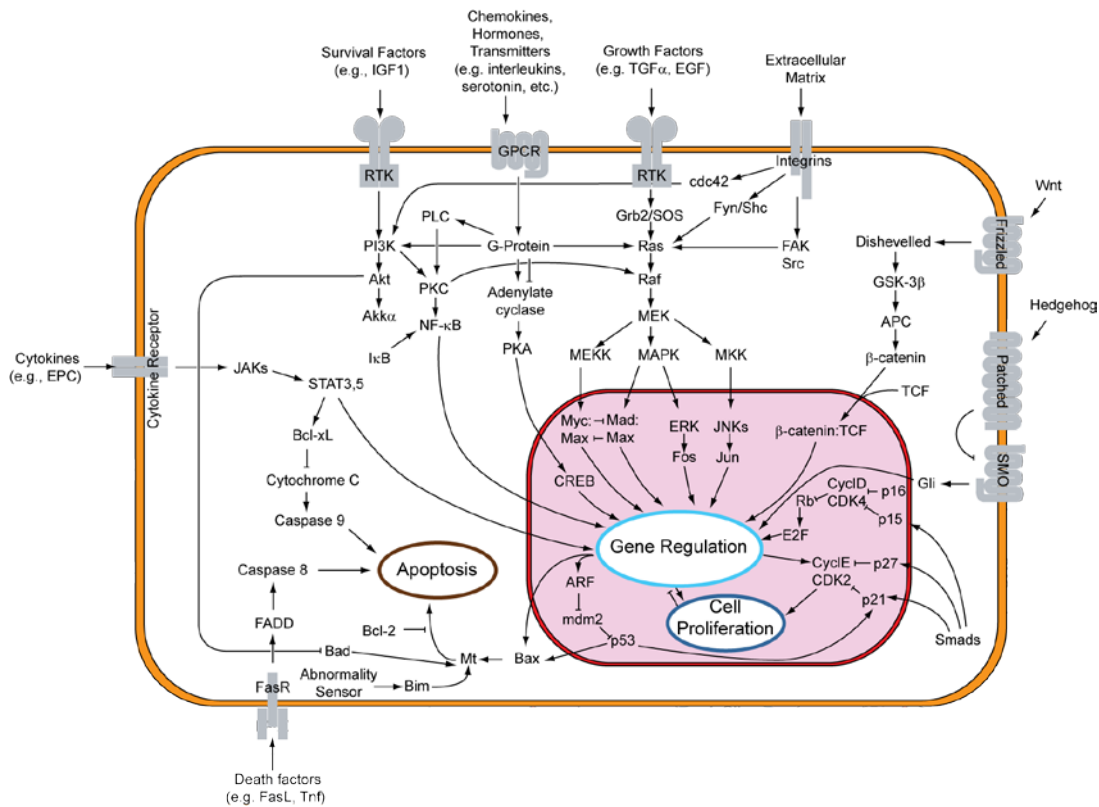


Figure 11: The Signal Transducers and Activator of Transcription proteins regulate many aspects of cell growth, survival and differentiation. The transcription factors of STATs are activated by the Janus Activated Kinase (JAK) and dysregulation of this pathway is frequently observed in primary tumours and leads to increased angiogenesis, enhanced survival of tumours and immunosuppression. These complexes bring together the intracellular regions of gp130 to initiate a signal transduction cascade through certain transcription factors. (Taken from (Lodish, Harvey, 2003))

Accumulation of STATs in the nucleus is both rapid and tightly controlled (Hsieh et al., 2005). Once in the nucleus the activated STAT dimers can bind to consensus DNA-recognition motifs, called gamma-activated sites (GAS), in the promoter regions of cytokine-inducible genes initiating transcription (Figure 12) (Mitchell and John, 2005).

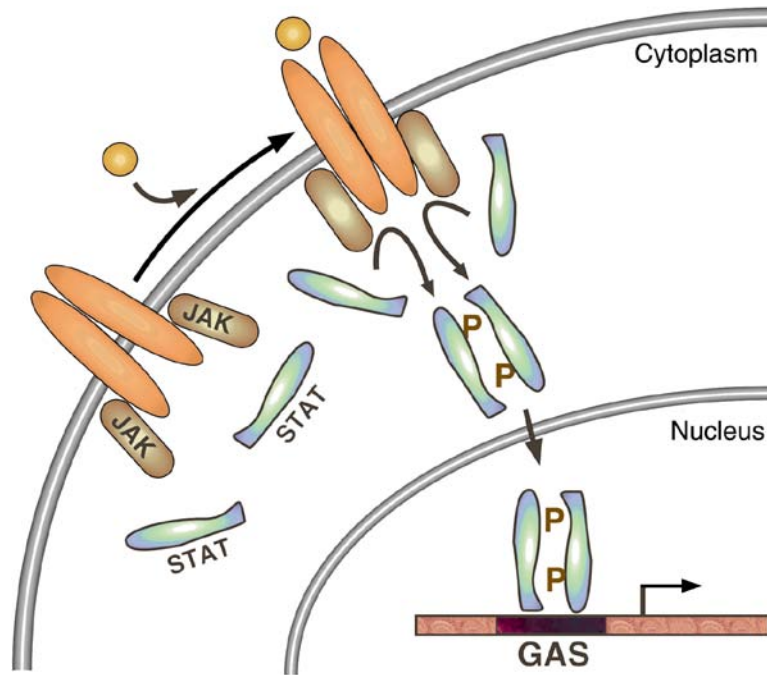


Figure 12: The JAK-STAT signalling pathway. Once a ligand binds to its appropriate receptor, it brings forth the activation of receptor-associated JAKs and mediates the phosphorylation of specific receptor tyrosine residues. This leads to the recruitment of specific STATs, which are then also phosphorylated at the tyrosine residues. Activated STATs are then released from the receptor, dimerise, translocate to the nucleus, and bind to members of the γ -activated site (GAS) family of enhancers. Taken from (Schindler, 2002).

STAT proteins range from 750-850 amino acids in size. They share structurally and functionally conserved domains. The amino terminal domain (NH₂) mediates the formation of STAT tetramers, and the coiled-coil domain (CC) mediates the interaction with other helical proteins. A linker domain connects the DNA-binding domain with the Src homology 2 (SH2) domain, which is required for receptor binding and the formation of STAT dimers. The carboxy terminal transcriptional activation domain (TAD) shows less sequence homology among the members of the STAT family and interacts with co-activators and co-repressors (Figure 13A) (Groner et al., 2008).

STAT3 activation was required for the gp130-mediated G1 to S phase cell-cycle transition, indicating its importance not only for cell survival but also for cell cycle progression. STAT3 activates the expression of target genes involved in cell cycle progression and the regulation of apoptosis. It also directs the secretion of factors from tumour cells which regulate angiogenesis and the immune response in surrounding cells. STAT3 plays a crucial role in haematopoiesis, in mediating immune responses and in the regulation of cell differentiation, but can also promote cellular transformation and abnormal cell proliferation (Groner et al., 2008).

Furthermore, STAT3 activation is essential for the up-regulation of the cyclins D2, D3, and A (Fukada, 1998). However, STAT3 has been known as an oncogenic transcription factor, due to it being constitutively activated in a wide variety of human malignancies. It has recently been proven to be critical for tumour initiation and growth *in vivo* in a model of skin cancer (Grivennikov et al., 2009). As a transcription factor, STAT3 principally mediates its effects by regulating gene expression (Bromberg and Wang, 2009).

There are four members of the mammalian JAKs receptor-associated tyrosine kinases: JAK1, JAK2, JAK3, and TYK2. JAKs have seven conserved JAK homology (JH) domains. The carboxy-terminal portion of these molecules includes a distinctive tyrosine kinase domain (JH1) and a pseudokinase domain (JH2). The amino-terminal JH domains, JH3–JH7, constitute a FERM (four-point-one, ezrin, radixin, moesin) domain that mediates association with receptors (Figure 13B) (Schindler, 2002).

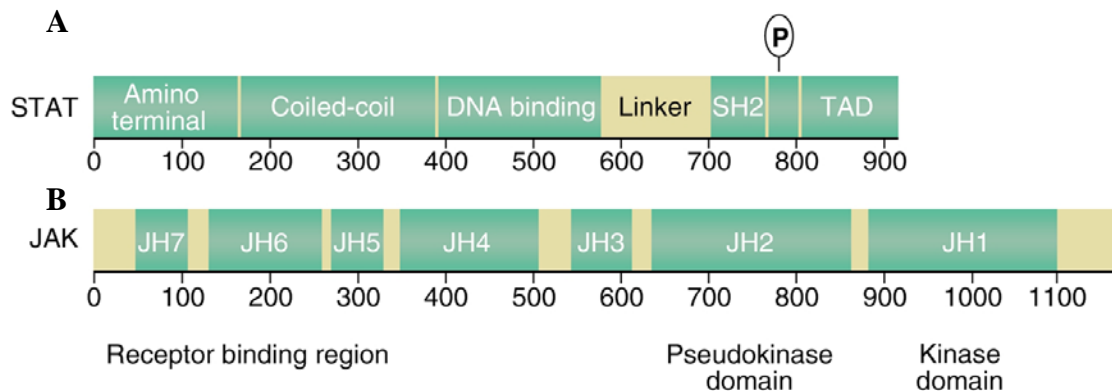


Figure 13: STAT and JAK structure. (A) The STATs share several conserved domains, including an amino-terminal domain (NH2), a coiled-coil domain, the DNA binding domain, a linker domain, an SH2 domain, and a tyrosine activation domain (P). The carboxy-terminal transcriptional activation domain is conserved in function, but not in sequence. (B) The JAKs share seven regions of high homology, JH1–JH7. JH1 has been shown to encode the kinase. JH2 represents a pseudokinase domain, which appears to regulate JH1 catalytic activity. JH3–JH7 have been implicated in receptor association. (Taken from (Schindler, 2002)).

Among all the STATs STAT3 is most significantly correlated to tumourigenesis, and is considered as an oncogene (summarised in Figure 14). Where STAT3 appears to function as a transcriptional activator the biological outcome can be proliferation, survival, or apoptosis, depending on the target tissue (Levy and Lee, 2002). STAT3 activity has been reported in nearly 70% of solid and haematological tumours, including multiple myeloma, several lymphomas and leukaemia's, breast carcinoma, head and neck cancer, prostate cancer, cervical cancer, ovarian carcinoma, melanoma, renal carcinoma, colorectal carcinoma and thymic epithelial tumours (Zhou et al., 2010). Several tumour viruses are also associated with STAT3 activation. For example, it is constitutively activated in human T-lymphotrophic virus I-transformed T-lymphocytes and Epstein–Barr virus (EBV)-related lymphoma cell lines (Muromoto et al., 2009).

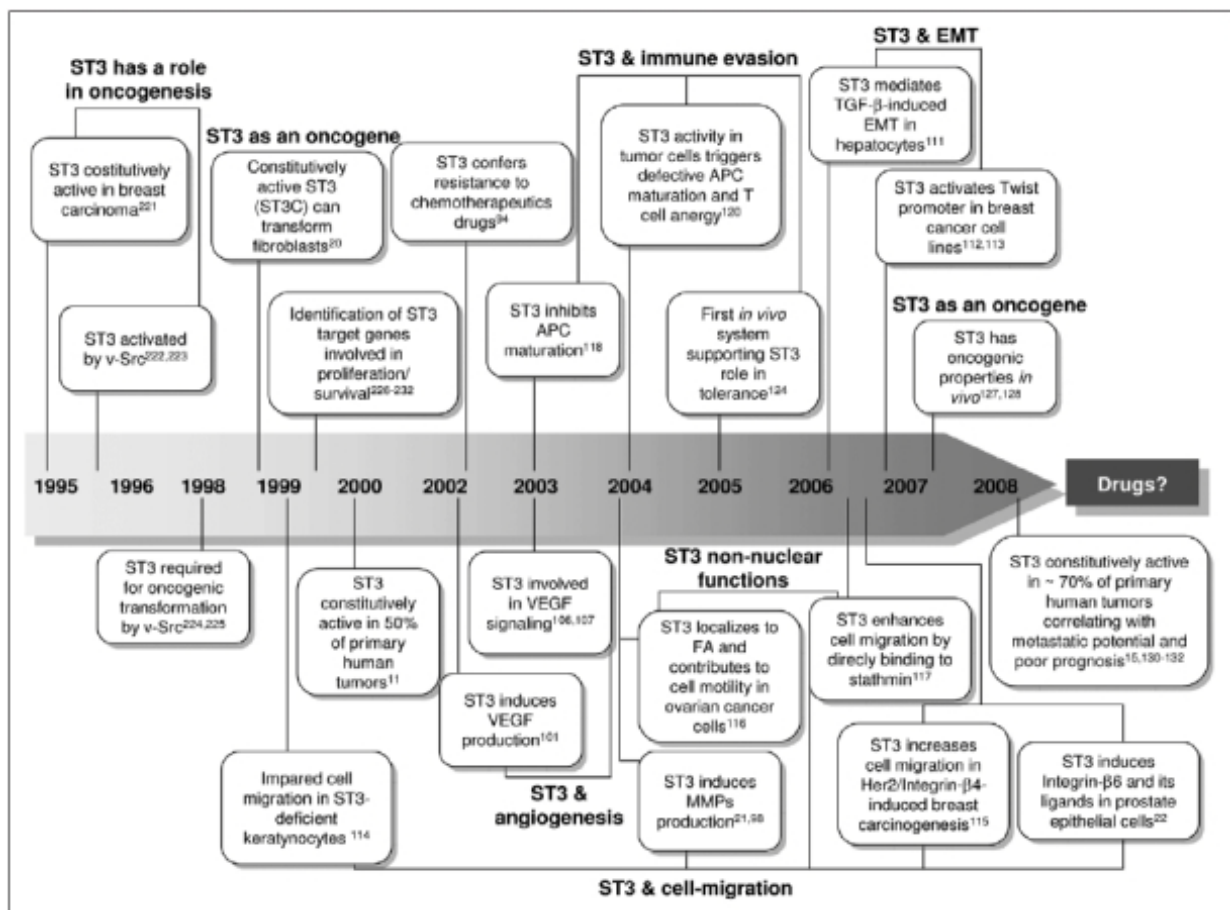


Figure 14: Uncovering the role of STAT3 in cancer over the past few years. The findings identifying the multiple functions of STAT3 in oncogenesis are illustrated along a timeline. ST3, STAT3; ST3C, STAT3C; FA-focal adhesions.

Taken from (STAT1 and STAT3 in Tumourigenesis: Two Sides of the Same Coin, 2000).

The successful co-existence of viruses with their host requires a variety of mechanisms of viral immune evasion. These falls into three different categories: viral escape from recognition by immune cells, resistance to apoptosis, and viral counter attack which can lead to generalised immunosuppression (Raftery et al., 2004). Most STAT3-deficient animals die early in embryogenesis, emphasising the vital importance of this gene. In addition, because these molecules play key roles in immune responses, defective STAT signalling can favour tumour development by compromising immune surveillance. STAT3 activation is typically dependent on dysregulated growth factor receptor tyrosine kinases or their associated JAK kinases (Bromberg, 2002).

Due to its involvement in tumour development/ cell regulation/ transcription/ pathways described above, a growing number of studies are focussing on STAT3 as a potential therapeutic target in cancer (Diaz et al., 2006, Fossey et al., 2009, Miranda et al., 2010)

Chapter 3: Outline of the aims of this study

In earlier studies it was revealed that expression of US28 induces a proangiogenic and transformed phenotype by up-regulating the expression of vascular endothelial growth factor and enhancing cell growth and cell cycle progression (Maussang et al., 2006). Analysis previously done on secreted growth factors, chemokines and cytokines using an Angiogenesis Antibody Array for expressed proteins (RayBiotech) confirmed increased VEGF and interleukin-6 (IL-6) secretion in supernatants of US28-expressing NIH 3T3 cells (Slinger et al., 2010b). Also in earlier work done in the Department of Medicinal Chemistry at Vrije Universiteit using gene reporter assays, and VEGF ELISA compared differences between WT and delta US28 HCMV. It indicated that US28 could be present in the HCMV viral particle as the complete protein. However in the past a high quality antibody against the HCMV-encoded US28 receptor was not available. The Medicinal Chemistry Department at Vrije Universiteit was the first research group to be able to obtain a US28 antibody in 2009. With the US28 antibody at their disposal, a method was developed and the location of the US28 protein in the viral particle was attempted.

3.1 Research question

Does the presence of viral US28 upregulate IL-6 expression and constitutive signalling via STAT3 in two different HCMV infected mammalian cell lines?

3.2 Primary aim

The primary aim of this study was to investigate whether there was a significant raise in the inflammatory and angiogenic factor IL-6 and STAT3 respectively in HCMV infected HFF and U373 cells.

3.3 Secondary aim

A secondary aim of this study was to determine whether US28 was present in the HCMV viral particle.

3.4 Objectives

- (i) Optimisation of the protocol to infect U373 cells with HCMV to obtain stably transformed cells
- (ii) Measure the extent of STAT3 phosphorylation and IL-6 expression after virus transformation
- (iii) Quantify the IL-6 and STAT3 extracted from cells infected with different strains of HCMV using a commercial Enzyme-Linked Immunosorbent Assay, Western blot techniques and Immunofluorescence Assays.
- (iv) Validation of the US28 antibody.
- (v) Method development for the detection of US28 in the viral particle.

3.5 Techniques applied during this study

1. Cell culture work
2. Virus stock production and titre determination
3. Protein quantitation
4. Enzyme-linked immunosorbent assay (ELISA)
5. Western blotting
6. Immunocytochemistry (ICC)
7. Immunofluorescence assay (IFA) and use of confocal microscopic techniques

Chapter 4: Materials and Methods

4.1 Definitions of important terms

Multiplicity of infection

The multiplicity of infection (m.o.i) is the ratio of infectious agents (e.g. phage or virus) to infection targets (e.g. cells). When referring to a group of cells inoculated with infectious virus particles, the m.o.i is the ratio defined by the number of infectious virus particles deposited in a well (ml added x PFU/ml) divided by the number of target cells present in that well (ml added x cells/ml).

Plaque-forming unit

A plaque-forming unit (pfu) is a measure of the number of particles capable of forming measurable plaques per unit volume (concentration), such as virus particles. It is a functional measurement rather than a measurement of the absolute quantity of particles. Viral particles that are defective or which fail to infect their target cell will not produce a plaque and thus will not be counted. Using pfu rules out possible multiple-hit phenomena and includes only the particles capable of infecting cells on their own. Thus, one pfu equates to one lytic event (or one infectious virus particle).

4.2 Viruses

The HCMV stock was the concentrated supernatant containing infectious particles.

The Titan strain is an isolated clinical HCMV strain. It was cloned in the Ulm labs in Germany using a bacterial artificial chromosome (BAC) construct. The TB40-BAC4 is an endothelial trophic viral strain. It was cloned in Ulm using a BAC construct. Delta US28 is a mutant strain of HCMV, in which the US28 ORF has been completely deleted through mutagenesis. All the virus isolates from which viral stocks were made were kindly provided to the Department of Medicinal Chemistry by M. Detlef (Abteilung Virologie, Universitätsklinikum Ulm, Germany).

4.3 Human Foreskin Fibroblast passaging

Human foreskin fibroblast (HFF) cells are derived from neonatal human foreskins. HFF's are versatile cells which proliferate rapidly. They are important in a variety of disciplines including use as stem cell feeder lines and in virological applications, or virus propagation and titer determination. Unfortunately these cells cannot be used clinically since numerous adventitious viruses and infectious agents exist that can theoretically infect humans. Normal human fibroblasts have two extreme modes of existence in culture: quiescent and proliferative.

a) Materials

- Primary human foreskin fibroblasts (HFF) cells passaged to between P10-P25 were used in this study.
Cells passaged between P1-P10 are used only for increasing available HFF cells and not for transformation experiments. Cells passaged between P25-P30 are only used for viral stock production.
- Polyacrylamide (PAA)
- Dulbecco's modified Eagle's medium (DMEM) (faster cell growth)
(Purchased from PAA Laboratories)
- Minimum Essential Medium (MEM) (slower cell growth)
- Glutamine solution
- Penicillin/Streptomycin solution
- Fetal bovine serum (FBS) (purchased from Integro)
- Non essential amino acids (NEAA)
- Trypsin
- Phosphate buffered saline (PBS)/ ethylenediaminetetraacetic acid (EDTA)

b) Preparation of Solutions

- Phosphate Buffered Saline (PBS):
 - 20 mM KCl
 - 137 mM NaCl
 - 1.5 mM KH_2PO_4
 - 8.1 mM Na_2HPO_4
- PBS/EDTA
 - (1/50) dilution was done from 50x stock solution of PBS
 - 1% EDTA was added to this stock solution
- DMEM/MEM supplemented with :
 - 10% FBS
 - 1% Glutamine
 - 1% NEAA
 - 1% Pen/Strep.

c) Method

Passaging the HFF fibroblasts

The culture dishes (150 mm outer diameter, x 21.9 mm) of HFF fibroblasts were observed with an inverted microscope to determine whether the cells were either confluent or dense enough to split. DMEM/MEM supplemented media, trypsin, and PBS/EDTA were placed into a 37°C water bath and allowed to warm up. The working surface in the hood was sprayed with 70% ethanol and wiped down. The DMEM/MEM supplemented media, trypsin, and PBS/EDTA were removed from the water bath and the outside dried and sterilised by spraying with 70% ethanol and placed into the flow cabinet. New sterile dishes were placed into the laminar flow hood and labelled medium from the dishes containing the cells was removed by aspiration and the cells were rapidly washed twice with 2,5ml PBS/EDTA solution. The PBS/EDTA was kept for two minutes on the cells and then aspirated; 1ml of trypsin was added to each dish (making sure the bottom was covered) and then incubated for 5-10 minutes at 37°C in a 5% CO_2 atmosphere. The dish was lightly tapped and when the cells were clearly detached, 15µl of freshly supplemented medium was added. The cells in

media were pipetted up and down ten times to make sure the maximum numbers of cells were collected. The cells were then split 1:3 into new dishes, resuspended in 5-7 ml of media and placed back into the incubator, this was done weekly.

4.4 Human cytomegalovirus stock production

For viral stock production, cells that generate a large number of HCMV particles in a short time are required. Human foreskin fibroblasts (HFFs) are cells that best fit this role.

a) Materials

- 10 T 175 cm² flasks with confluent HFF cells passaged between P10-P30.
- Supernatant from two fully infected HFF dishes or alternatively cells 3 days after infection.
- Sterilised SW28 buckets, this was done with the use of 70% ethanol and more than 30 minute UV radiation in laminar air flow bench.
- Clean ultra-centrifuge and SW28 rotor, both wiped with 70% ethanol before use.
- Sterile polyallomer SW28 tubes (Beckman Coulter # 326823) were autoclaved and sterilised for 30 minutes by UV radiation.
- Sterile cryo-tubes for virus storage.

b) Preparation of Solutions

- DMEM/MEM
- Sucrose buffer
 - 37,31g Sucrose
 - 0,609g K₂HPO₄
 - 0,260g KH₂PO₄
 - Dissolved in 500ml of 18MΦ water

This solution was filter sterilised by filtration through 0, 2µm or 0, 1µm filter Millipore (Drummond). Autoclaving is not used because the sucrose will caramelize.

c) Method

Two 10 cm dishes containing HFF cells were infected with HCMV virus at a low m.o.i of 0, 1 and cultured for 10 to 14 days. The supernatant was collected from day 8 (by this time all cells displayed a cytopathic effect (CPE)), 5ml of fresh medium was added and the cells were cultured for three more days. The supernatant was collected daily and centrifuged for five minutes at 780 g and decanted into new tubes. The virus containing supernatant was stored at -80°C. It was not thawed until it was used for infection of new stock.

For infection of HFF cells in ten T175 cm² flasks, 20 ml of frozen virus supernatant (as prepared above) was added to 30 ml of fresh medium. From this mixture 5 ml per T175cm² flask was used for infection of the HFF cells and the flask incubated for 30 minutes in a CO₂ incubator. After 30 minutes 20ml of fresh medium was added to each flask to reach a total volume of 25ml per a flask. The supernatant was collected daily in 50 ml tubes from day 5 (90-100% of cells displayed CPE) to day 7 (up to day 9 if the cells were still viable and productive) and 15ml fresh medium was placed into the flask. The collected supernatant was centrifuged for five minutes at 780 g and decanted into a sterile SW28 tube. The supernatant was centrifuged for a further one hour at 4500 g at 4°C in a SW28 swinging bucket rotor. The supernatant was decanted and the SW28 tube was placed into a sterile 50ml tube and 500µl of sterile sucrose buffer was added to the pellet. The virus pellet was resuspended and the tubes were shaken upright over night on a rotation shaker at 4°C. After the overnight shaking the virus suspension from all the tubes was combined and 250µl aliquots were frozen in 2ml cryo-tubes at -80°C. The same procedure was repeated for each day that virus was harvested. After the virus harvesting was completed one aliquot from each harvesting day was titred using HFF cells.

4.5 Interleukin-6 Experiments

a) Materials:

- U373 MG (Human Glioblastoma cell line) cells-These cells are used as an *in vitro* model of human malignant glioma and for studies of tumourigenicity
- 6 well cell culturing plate (Greiner Bio-One)
- Eagle's Minimum Essential Medium (EMEM): supplement with
 - 10% FBS
 - 1% Glutamine
 - 1% NEAA
 - 1% Pen/Strep.
 - 1% sodium pyruvate
- PBS
- Human cytomegalovirus (HCMV) - Titan strain (Multiplicity of infection (m.o.i of 2))
 - Wild type (WT)
 - Delta US28 (Δ US28)
- Solid phase ELISA Kit : Quantikine® Human IL-6 Immunoassay

b) Method:

Three hundred thousand U373 MG cells/well were seeded into a six well plate with 2ml of supplemented EMEM medium. Serum starvation was done for 24 hours using serum-free medium (normal growth media that contains only 0.5% of FBS instead of 10%). The cells were infected with WT or Δ US28 virus with an m.o.i of 2 and the infected cells were placed for 2-3 hours in a 37°C CO₂ incubator. After this incubation the cells were washed twice with PBS, 1ml of fresh medium was added and the plate was placed back in the incubator. Collection of the cell medium supernatant of the samples took place at 1, 8, and 24 hour intervals. At each interval the cells were washed three times with PBS. The supernatant was collected from each well using a pipette and placed in separate chilled microreaction vials. As the supernatant was collected simultaneously harvesting of the cells was done. They were separately scraped off each well in cold PBS at the correct time intervals and placed in labelled chilled microreaction vials. These harvested cells were also placed in chilled

microreaction vials and centrifuged for one minute at 2700 g 14000rpm at 4°C. The excess PBS was removed without disturbing the pellet and the pellets were freeze dried at -20°C.

A solid phase Enzyme-Linked Immunosorbent Assay (ELISA) was performed on the supernatant samples for the quantitative determination of IL-6. The manufacturer's (*Quantikine*®) recommended procedure was followed with the minor changes.

c) ELISA Method:

A 100µl of the buffered protein base solution RD1W (*Quantikine* assay diluent), was added to each well of a 96 well plate that was pre-coated with anti-human IL-6 monoclonal antibody. This was done to block any non-specific binding sites in the wells. A sample of 100 µl supernatant from the infected U373 MG cells was added to each well and the plate incubated for 2 hours at room temperature. Each well was aspirated and washed 5 times with 200 µl of wash buffer solution to remove any unbound protein. The samples were incubated with 200 µl streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IL-6 antibody and incubated for 2 hours at room temperature. The wells were aspirated and washed at least 5 times to remove any unbound antibody-enzyme conjugates. Peroxidase activity was visualised after adding 200 µl of 3, 3', 5, 5' tetra -methylbenzidine and peroxide in buffered solution to each well. The plate was incubated in the dark for 20 minutes at room temperature (RT) (a blue colour in direct proportion to the amount of IL-6 present was observed). The reaction was terminated by adding 50µl of 2 N sulphuric acid stop solution to each well (solution should change colour from blue to yellow). The absorbance was read at 450 nm using a Powerwave x340 (Bio-TEK Instruments Inc) micoplate reader (with a specifically programmed IL-6 assay).

4.6 Western Blots

a) Materials

- Nonyl phenoxypolyethoxylethanol (NP-40)
- Sodium deoxycholate
- Sodium dodecyl sulphate (SDS)
- Potassium chloride (KCl)
- Sodium chloride (NaCl)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Sodium Phosphate (Na_2PO_4)
- α -complete protease inhibitor cocktail (Roche)
- Sodium orthovanadate (Na_3VO_4)
- Sodium fluoride (NaF)
- Phenylmethanesulfonylfluoride (PMSF)
- Sodium hydroxide (0,2M NaOH)
- 30% Acrylamide/bis-acrylamide mix
- Ammonium persulfate (APS, store at -20°C when dissolved)
- Tris(hydroxymethyl)aminomethane (Tris)
- Tween20
- Glycin
- Polyvinylidene fluoride membrane (PVDF)
- Ethanol
- Hydrochloric acid (HCl) (for setting pH)
- ELK (powdered milk)
- BCA Assay kit (Thermo Scientific Pierce)
- Powerwave x340 plate reader
- A Bio-Rad minigel system was used to perform SDS–polyacrylamide gel electrophoresis.

b) Preparation of Solutions and Gels

- Phosphate Buffered Saline (PBS)
- RIPA (RadioImmunoPrecipitation Assay) buffer: PBS containing
 - 1% NP-40
 - 0.5% Sodium desoxycholate
 - 0.1% SDS
 - 1 mM NaVO₄ (1/100)
 - 1 mM NaF (1/100)
 - 1 mM PMSF (1/100)

Dissolve one α -complete tablet in 1 ml H₂O to make a 25x stock solution, dilute accordingly in RIPA buffer.

- Running buffer (1L):
 - 3 g Tris
 - 18.8 g Glycin
 - 0.1% SDS
- Transfer buffer
 - 3 g Tris
 - 15 g Glycin
 - 20% Ethanol (only necessary when using PVDF membrane)
- Tris Buffered Saline with Tween (TBST) in 10L
 - 88 g NaCl
 - 2 g KCl
 - 30 g Tris
 - 50 ml 10% Tween20

Set pH to 7.4 with HCl (This is important for antibody binding)

- 6x Sample Buffer (10 ml final volume)
 - 7.0ml 0.5 M Tris-HCl (pH 6.8)
 - 1.0g SDS
 - 3.0ml Glycerol
 - 0.93g Dithiothreitol (DTT)
 - Spatula point (10 mg) of Bromophenolblue

- Resolving gel:
 - 4.9 ml H₂O
 - 6.0 ml 30% acrylamide mix
 - 3.8 ml 1.5 M Tris-HCl (pH 8.8)
 - 150µl 10% SDS
 - 150µl 10% APS
 - 9µl Tetramethylethylenediamine (TEMED)
- Stacking gel:
 - 2.1 ml H₂O
 - 0.5 ml 30% acrylamide mix
 - 0.38 ml 1.0 M Tris-HCl (pH 6.8)
 - 30µl 10% SDS
 - 30µl 10% APS
 - 6µl TEMED
- 6X Loading Dye:
 - 0.09% bromophenol blue
(just enough until a sufficient colour change is obtained)
 - 0.09% xylene cyanol FF
 - 60% glycerol
 - 60mM EDTA
 - 0.5M EDTA pH 8.0 (For 1L)
 - 186.1g Na₂EDTA.2H₂O
 - 800 ml 18MΦ H₂O

The pH was brought to 8 with NaOH pellets (about 20 g); the EDTA would not dissolve until the pH was about right. The volume was brought to 1 litre with dH₂O and autoclaved.

c) Method

Day 1

The cell pellets that were harvested from virally infected U373 MG cells and freeze dried at -20°C were used in this experiment. After the samples were thawed and kept on ice they were lysed in 50 μl of RIPA buffer, to solubilise the proteins. Each microreaction vial was then vortex mixed for ten seconds to make sure the pellet was dissolved, then placed on ice for 30 minutes. Cellular debris was removed by centrifuged for ten minutes at 2700 g at 4°C . The supernatants (50 μl) were transferred (without the precipitate being disturbed) to new chilled microreaction vials. Protein concentrations were assayed in duplicate using a BCA kit. A set of seven protein standards were prepared by diluting the 2.0 mg/ml BSA stock standard: with final concentrations used for the curve being 1500 $\mu\text{g/ml}$: 1000 $\mu\text{g/ml}$: 750 $\mu\text{g/ml}$: 500 $\mu\text{g/ml}$: 250 $\mu\text{g/ml}$: 125 $\mu\text{g/ml}$: 25 $\mu\text{g/ml}$. The plate was covered and placed in a 37°C incubate for 30 minutes. After incubation, the plate was cooled to RT and the final absorbance was measured at or near 562 nm on a Powerwave x340 using KC junior software.

Once the protein concentrations were determined 6x sample buffer was added in preparation of the samples for electrophoresis. The gel casting setup (7cm) was assembled and checked for leaks. The resolving gel was poured between the glass plates $\frac{3}{4}$ of the length using a pipette tip. Propanol was poured over the gel, this was done to stop the air from entering as well as to prevent oxidation. The gel had completely polymerized after 15 minutes. Once this had occurred the gel was inverted to remove the propanol. The gel was further rinsed three times with water to get rid of any excess propanol. Next the stacking gel was prepared and poured on top of the resolving gel and the comb inserted immediately. Once again it took 15 minutes for the gel to completely polymerise. The gel system was assembled and the gels were inserted with the combs facing into the inner chamber. The system was filled with running buffer and in the first well 5 μl of the mass calibration protein ladder (Page Ruler Prest Prot) was loaded. Twenty micrograms of protein (determined by the BCA kit) was loaded per a well. The gel was run for 90 minutes at 100V. While the gel was running appropriately sized PVDF membranes and Whatman paper were cut, taking care not to touch the membrane with ungloved hands. Before being used the PVDF membranes were first immersed for 30 seconds in 96% ethanol. Once the gel had finished running, a blotting sandwich was prepared using transfer buffer. It was composed of the following listed from

negative - black electrode to the positive: foam, Whatman paper, resolving gel (cut off the stacking gel), membrane (PVDF), Whatman paper, and foam. The prepared sandwich was blotted for 60 minutes at 300 mA with continuous ice pack cooling. Following this the system was dismantled and the membrane was blocked for 30 minutes in 5% ELK / TBST and incubated overnight at 4°C in a 50 ml tube with 5 ml primary antibody solution on a roller-bench.

Day 2

The membrane was washed 3 times for 5 to 8 minutes each in TBST to remove any unbound antibody then incubated in 10 ml secondary antibody solution (1:5000 antibody in 5% ELK/TBST) on a shaker plate for 90 minutes. Once again the membranes were washed three times for five minutes each in TBST to remove any unbound detection antibody. The membranes were then placed between two transparent overhead projector sheets that were taped to the inside of a cassette case. An appropriate amount (250µl) of ECL (GE and Western Lightning) was added. An x-ray film (GE Healthcare) was placed over the membrane in the dark room. Optimal exposure time was determined using the following method:

- The film was initially exposed for 5 minutes to check whether the band intensity was sufficient to detect minor differences.
- If the signal was too weak, a further expose for 15 minutes was done. If still too weak, another exposure for 30 minutes was done. If after 30 minutes no signal could be obtained then the initial signal was not strong enough and the membranes were disregarded.
- If the initial signal was too strong, an exposure for two minutes was done. If it was still too strong, then an exposure of 30 seconds was performed.

The film was placed in developer (Ilford) and once an image was clearly visible the film was placed into the fixer solution (Ilford). The same membrane was probed for several proteins in sequence. Between staining with different primary antibodies the membrane was stripped and washed with 0,2M NaOH solution for 15 minutes. The Western blot films were analysed to calculate the relative density of each band using the program ImageJ. ImageJ is a public domain Java image processing program. It is image analysis software for calculating area and pixel value statistics of user-defined selections. It can create density histograms and calculate line profile plots. It supports standard image processing functions such as contrast manipulation, sharpening, smoothing, edge detection and median filtering. Spatial calibration is available to provide real world dimensional measurements in units such as millimetres. Density or gray scale calibration is also available.

4.7 Human foreskin fibroblast back titrations

A back titration of the viral isolate was included for each experiment to allow calculation of the titre of virus that was actually present in that particular experiment. Back titrations act as a control for each experiment confirming that the number of virus particles used in that experiment corresponds to the correct virus titre for the experiment. If the virus titre in the experiment was not within the acceptable range – regarded as within half a log, the test was repeated with the dilutions adjusted (either higher or lower, as appropriate) to contain the correct amount of virus. This is because outside of this range is considered unreliable for the model.

a) Materials

- 96 well plate (Greiner Bio-One)
- HFF cells
- MEM (inositol)
- HCMV Titan and TB40-BAC4 strains:
 - WT
 - Δ US28
 - Δ US28/UL33
- Methanol/Acetone solution – (50:50)

- 12 channel pipette
- PBS
- Primary antibody (1/10) dilution of pp65 antibody in 1% BSA in PBS solution
- Secondary antibody - anti mouse-HRP conjugate diluted (1/500) in 1% BSA
- 3-amino-9-ethyl-carbazole (AEC) (Sigma) solution: It is a substrate for peroxidase.

This substrate produces an insoluble end product that is red in colour and can be observed visually.

- 1 AEC tablet dissolved in 4ml of dimethylformamide (DMF) (J.T.Bakker)
- 16ml 50mM sodium acetate buffer
- 60 μ L 30% H₂O₂
- A Motic AE21 inverted microscope

b) Methods

Two thousand HFF cells per well were seeded into a 96 well plate. The cells were grown to a uniform mono-layer using MEM medium. Titrations were carried out to determine the concentration of virus in triplicate in serial log dilutions from 2×10^1 - 2×10^8 starting with the lowest dilution in the top row. This was done for the back titration to make sure the same correct m.o.i was used when infecting the cells. The cells were infected with 100 μ l of Titan and TB40-BAC4 strains of WT, Δ US28 and Δ US28/UL33. The plate was incubated at 37°C and 5% CO₂ for five days post infection.

Fixation of the titration

The supernatant was removed with a 12 channel pipette, and the cells were left on the plate. The cells were then washed with a 100 μ l of PBS and subsequently fixed with 100 μ l of cold methanol/acetone solution. The plate was placed at -20°C for 20 minutes. The methanol/acetone was removed and the plate was knocked dry. The plate was further dried for 20 minutes under the fume hood. Once completely dried 100 μ l of PBS was added. The plate was then stored at 4°C.

Immunocytochemistry staining: Plaque Assay

The cells were washed three times with PBS and stained with the primary antibody against pp65. The primary antibody was left on the cells for one hour at RT with the plate being placed on a shaker at low speed. The cells were once again washed three times with PBS. The secondary antibody (anti-mouse) solution was added and incubated for one hour on the shaker. After incubation the cells were washed three times with PBS and 50 μ l/well of AEC solution was immediately pipetted onto the cells. After an hour, depending on the intensity of the staining viewed under the microscope, the AEC solution was removed and the cells were washed with PBS. To determine the titre as pfu, cells that appeared orange red (positive) were counted under the light microscope (lowest magnification: 4 x 0.10). A hundred cells were counted per well and a percentage positive was calculated.

4.8 Signal Transducer and Activation of Transcription 3 experiments

a) Materials

- U373 cells
- IBIDI slide
- HCMV TB40-BAC4 strain virus with an m.o.i of 1
 - WT
 - Δ US28
 - Δ US28/ Δ UL33
- 4 % paraformaldehyde (J.T.Baker)
- PBS
- Olympus FSX100 confocal microscope
- Primary Antibodies
 - Immediate early - (Millipore); (1:1000); diluted in 10% BSA
 - STAT3 - (Cell signaling); (1:100); diluted in 10% BSA
- Secondary antibodies :
 - Anti-rabbit 488nm IgG (STAT3 staining) , (1/1000) dilution, animal source : goat
 - Anti-mouse 555nm IgG (Immediate early staining) , (1/1000) dilution, animal source: rabbit
- 4',6-diamidino-2-phenylindole (DAPI)

b) Method:

Thirty thousand U373 cells were seeded onto an IBIDI slide and left overnight. Serum starvation using serum free medium was done for 24 hours. The cells were infected with virus (WT, Δ US28, Δ US28/ Δ UL33) with an m.o.i of 1. A second serum starvation for 24 hours was done and then the cells were fixed for 20 minutes with 4 % paraformaldehyde in PBS. The cells were permeabilised with cold methanol for 20 minutes at -20°C and blocked for a further 30 minutes in 10% BSA at 4°C . An immunofluorescence assay was done: Primary and secondary antibody staining were done in 10% BSA at 4°C and RT respectively. The dilution of the antibodies differed for each antibody. A double staining for immediate early and STAT3 proteins was performed to compare TB40-BAC4 Δ US28 and Δ US28/ Δ UL33 to the WT and mock cells. A counterstaining was done with DAPI (4', 6-diamidino-2-phenylindole) to visualise the nuclei of all cells – infected and uninfected cells, and to be able to count the total number of cells. Primary antibodies were left overnight, between the staining steps the cells were washed three times with wash buffer (0, 1% Triton and 1% BSA in PBS). The secondary antibodies were left on the cells for an hour. After the second incubation, the cells were counterstained with DAPI (diluted 1:1000) for 10 minutes at RT in the dark. The cells were washed five times with PBS and supplemented with 1:2 PBS and glycerol. The slide was analysed under a fluorescent microscope (Olympus FSX100). The image analysis software program used was Bio-imaging Navigator. Pictures were taken with FSX-BSW version 01.05. Statistical analyses of the pictures were done in adobe Photoshop (CS4) and in ImageJ (1.43u). In analysis regarding the STAT3 expression in U373 cells ImageJ was used to compare the intensity of the signals of the pictures. This was done by manually selecting the nuclei of the cells, in the DAPI picture, and saving as a region of interest (ROI). The ROI was then loaded upon the picture of the STAT3 channel and the intensity was assessed by the measure tool of ImageJ.

4.9 Determination of US28 in the HCMV viral particle

a) Materials

- HFF cells
- MEM supplemented with:
 - 10 % FBS
 - 1% Penicillin-Streptomycin
 - 5 ml Non essential amino acids
 - 5 ml Glutamine
- Cycloheximide (100ng/ml in 1ml of medium)
- HCMV TB40-BAC4 strain:
 - WT
 - Δ US28
- IBIDI slide
- Phosphate Buffered Saline (PBS)
- 4% Paraformaldehyde (PFA)
 - 100 ml PBS
 - 4 g paraformaldehyde
- Triton X-100 (Used to permeabilise eukaryotic cell membranes)
- 10 % Bovine serum albumin (BSA) solution:
 - BSA
 - Tris Buffered Saline with Tween 20
- Wash Buffer:
 - 1 ml Triton in 4ml PBS
 - 1 ml BSA in 9ml PBS
- Primary antibodies:
 - US28 (1:1000), diluted in 10% BSA
 - pp65 (1:1000), diluted in 10% BSA
- Secondary antibodies (Invitrogen):
 - Anti-rabbit 488nm IgG (US28 staining) , (1/1000) dilution, animal source : goat
 - Anti-mouse 555nm IgG (pp65 staining) , (1/1000) dilution, animal source: rabbit

b) Method

Day 1

HFF cells were cultured in T175 cm² culture flasks. The cells were trypsinised and 300000 cells/well were seeded onto an IBIDI slide. The cells were left overnight to attach and form a uniform monolayer.

Day 2

The cells were treated with cycloheximide solution (150 µl/well). Then left for 90 minutes in a CO₂ incubator at 37°C after which the cells were infected with the appropriate amounts of HCMV virus with an m.o.i of 50. The virus was left on the cells for one hour. The cells were then washed three times with 200µl of PBS and then placed in the incubator for two hours. The supernatant was removed and the cells washed once with 200 µl of PBS. For immunofluorescence staining, the cells first had to be fixed with 200 µl of chilled 4% PFA for 10 minutes. After this the PFA was removed, 200µl of PBS was added and the slide was placed overnight in the refrigerator at (4°C).

Day 3

The PBS was first removed and the cells were permeabilised with 200 µl of a 0.5% Triton X-100 detergent solution for five minutes at RT. The cells were blocked using 200 µl of 10% BSA solution per well and left in the refrigerator for 30 minutes. The BSA solution was removed and a total of 200 µl of both primary antibodies - US28 and pp65 was added per well. These primary antibodies were left on the cells for one hour at (RT). The cells were then washed three times with 200 µl of wash buffer each and incubated in the dark for one hour with the secondary anti-rabbit and anti-mouse antibodies. The excess secondary antibodies were removed by pipette and 200 µl of PBS containing DAPI (1:1000) was left on the cells for 10 minutes. The final wash with 200 µl of PBS was done and the cells were covered with 200 µl of (1:2) PBS/glycerol. The fluorescence was then measured using a (SFC) microscope.

4.10 Statistical analysis

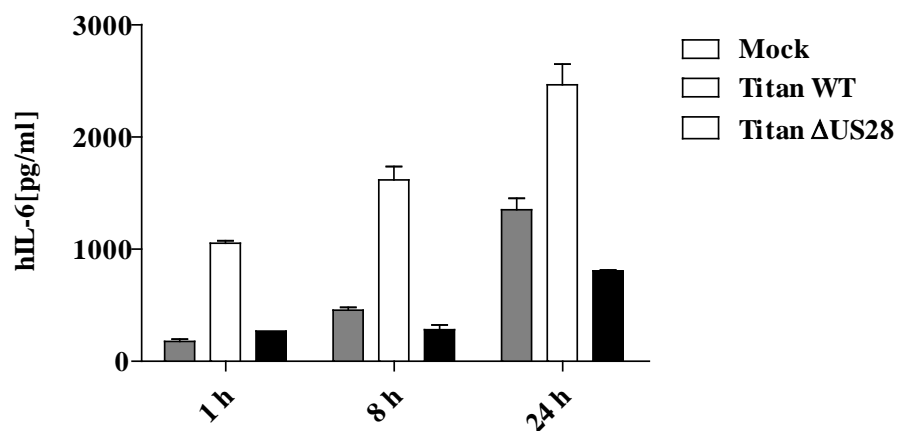
All experiments were performed at least three times, and where possible in duplicate or triplicate. For the statistical analysis of results a student's t-test and ANOVA were performed using GraphPad Prism software.

Chapter 5: Results

Experiments monitoring the presence and level of extracellularly secreted IL-6 and STAT3 were carried out. U373 cells were infected with HCMV the Titan strain, WT and mutants of the Titan strain Δ US28; all with an m.o.i of 2.

A)

Interleukin 6 concentrations from infected U373 supernatants



B)

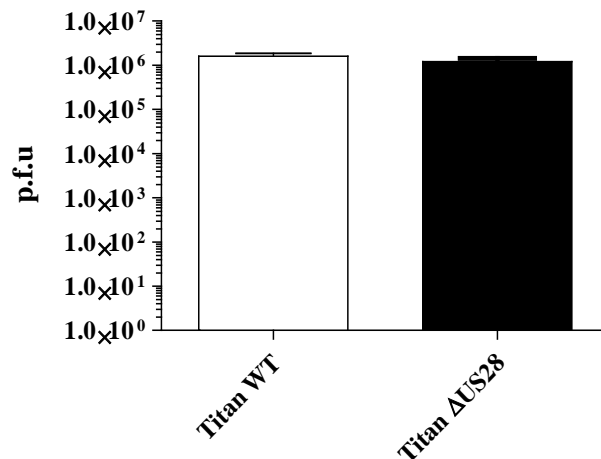


Figure 18: A) Enzyme-Linked Immunosorbent Assay (ELISA) assay for the quantitative determination of IL-6. 300000 U373 cells were seeded into a 6 well plate and infected with HCMV Titan strain WT or Δ US28 with an m.o.i of 2. IL-6 secretion was measured at time intervals of 1hr, 8hrs, and 24hrs respectively. **B) Back titration** done with HCMV Titan strain WT and Δ US28 to ensure that an m.o.i of 2 was used for the infection of the cells assayed by ELISA.

Supernatant samples were taken from infected U373 cells, and an ELISA for the quantitative determination of IL-6 was performed. IL-6 secretion was measured at different time intervals after infection with HCMV; 1hr, 8hrs, and 24hrs respectively. From the measured concentrations of IL-6 it could be seen that IL-6 was produced in greater concentrations by the cells expressing the US28 gene. This was already clearly noticeable 1 hour post infection ($495\% \pm 1\%$) and after 8 hours the IL-6 secretion was ($254\% \pm 1\%$). After 24 hours there was still an increase ($83\% \pm 1\%$). During these time intervals only low background increases could be detected in both mock and Δ US28 samples (Figure 18(A)). An ANOVA statistical analysis was done using GraphPad Prism software and a p-value equal to 0.004 was obtained proving the interaction to be very significant.

Figure 18(B) shows the back titration results to determine the number of viral particles in each of the samples (titre). The back titration was done using HFF cells that were infected with the same HCMV Titan strains, WT or mutant Δ US28 virus to ensure that the correct m.o.i was used. These particles were re-elevated from the virus solution that was used to infect the U373 cells used to determine the effect of viral infection on IL-6 expression using ELISA. Both WT and Δ US28 showed no significant difference in infectious viral particles to cell ratio. The WT sample had a value of $4,8 \times 10^6$ p.f.u, and the Δ US28 sample had a value of $3,6 \times 10^6$ p.f.u. These two values fall within an acceptable range of half a log.

STAT3 phosphorylation in infected U373 cells

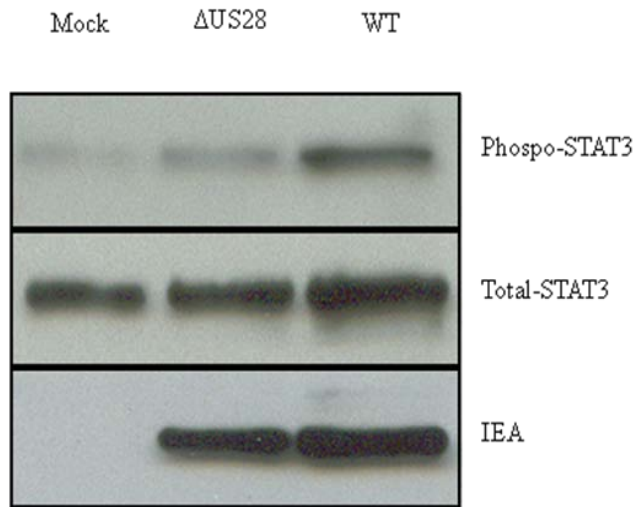
The phosphorylation status of STAT3 was assessed by Western blot analysis was done to investigate the contribution of US28 on the phosphorylation status of STAT3 in U373 MG cells during HCMV infection. U373 MG cells were infected with HCMV Titan WT and HCMV Titan mutant Δ US28 with an m.o.i of 2. The presence of phospho-STAT3, Total-STAT3 and immediate early antigen were assessed.

Figure 19(A) shows the results of a Western blot analysis for the phosphorylation of STAT3. Phospho-STAT3 was not detected in mock and Δ US28 infected cells. However a signal was generated for cells that contained US28. An antibody for Total-STAT3, which detects levels of phosphorylated and non-phosphorylated STAT3, was used as a control to show that equal amounts of all 3 samples were loaded onto the electrophoresis gel. The presence of the immediate early antigen which was detected in both the WT and Δ US28 samples proved these cells had been infected with the HCMV virus. This method of testing for active HCMV infection which could be diagnosed by the detection of HCMV immediate early antigen (IEA) was first reported by (Van der Bij et al. 1988).

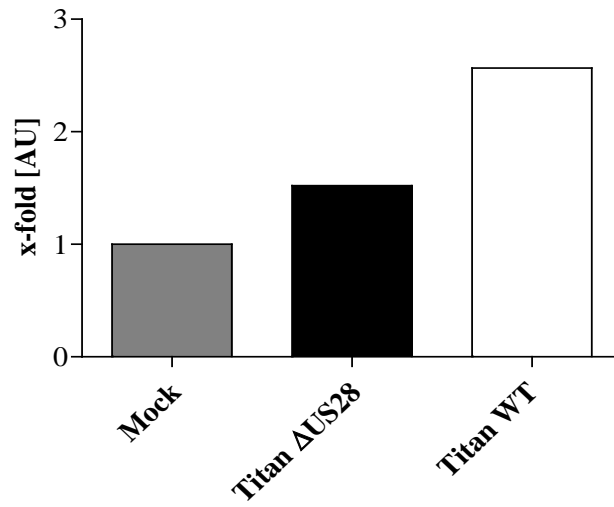
Figure 19(B) shows the quantification of phospho-STAT3 that was obtained from the Western blot shown in Figure 19(A). Phospho-STAT3 was corrected for Total-STAT3 in infected U373 MG cells and measured in AU units using the program ImageJ. In the WT sample there is a higher expression of phospho-STAT3 ($297.6\% \pm 1\%$) than in the mock and Δ US28 samples.

Figure 19(C) shows a back titration to determine the number of viral particles in the samples. As done in the back titration for the determine of IL-6 concentrations, HFF cells were infected with the same HCMV Titan strain, WT and mutant Δ US28 particles that were used in the Western blot. These particles were re-evaluated from the respective virus solutions used to infect the U373 MG cells. Both WT ($2,4 \times 10^6$ p.f.u) and Δ US28 ($1,15 \times 10^6$ p.f.u) showed less than half a log difference between both samples for infectious particle to cell ratio.

A)



B)



C)

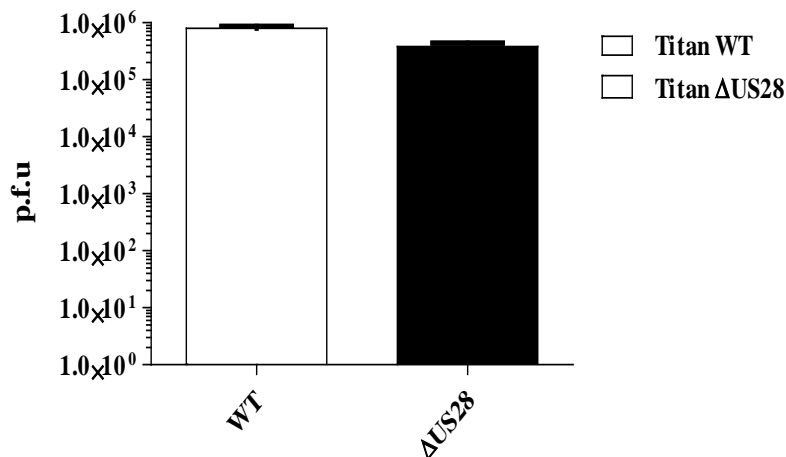


Figure 19: A) US28 expression triggers STAT3 phosphorylation in infected U373 cells. U373 cells were infected with HCMV titan strain WT and Δ US28 with an m.o.i of 2. A Western blot was done to detect the presence of phospho-STAT3, total-STAT3 and immediate early antigen. **B) Quantification of phospho-STAT3.** Phospho-STAT3 was corrected for total-STAT3 in infected U373 MG cells. **C) A back titration** done with the titan strains WT and Δ US28 of HCMV, to prove an m.o.i of 2 was used for the detection of phospho-STAT3 in the Western blots.

Further investigation into the phosphorylation of STAT3 was done using the HCMV strain TB40-BAC4. For quantitative STAT3 determination and location infected U373 cells were fixed and assayed by immunofluorescence. U373 cells were infected with an m.o.i of 1 of the respective viruses. Double staining for immediate early (green) and STAT3 proteins (red) was done to compare the two TB40-BAC4 mutant strains, Δ US28 and Δ US28 /UL33 to the WT and mock cells. The cells were counterstained with DAPI (blue). DAPI's selectivity for DNA and high cell permeability allows efficient staining of nuclei with little background from the cytoplasm.

Figure 20 illustrates that Δ US28/UL33 contributes to tSTAT3 translocation to the nucleus. This is important because unlike HIV which is an RNA virus, that replicates in the cytoplasm. HCMV is a DNA virus; and moves to the nucleus to be transcribed. The merged three colour images show an overlay of the STAT3 with the DAPI and the immediate early proteins. The different rows show cells with infection of the HCMV TB40-BAC4 strains WT, Δ US28, Δ US28/ Δ UL33 and the non-infected (mock) cells respectively. Infected cells all showed a positive IE protein staining. In the infected cells with the US28 gene, the

Total-STAT3 (active and non-active STAT3) staining was higher than that of the tSTAT3 in the Δ US28, Δ US28/UL33 and mock cells. This is also evident in the merged images of the figure. There was a decrease in the signal strength when US28 was deleted and an even more pronounced decrease in the signal strength when both US28 and UL33 were deleted.

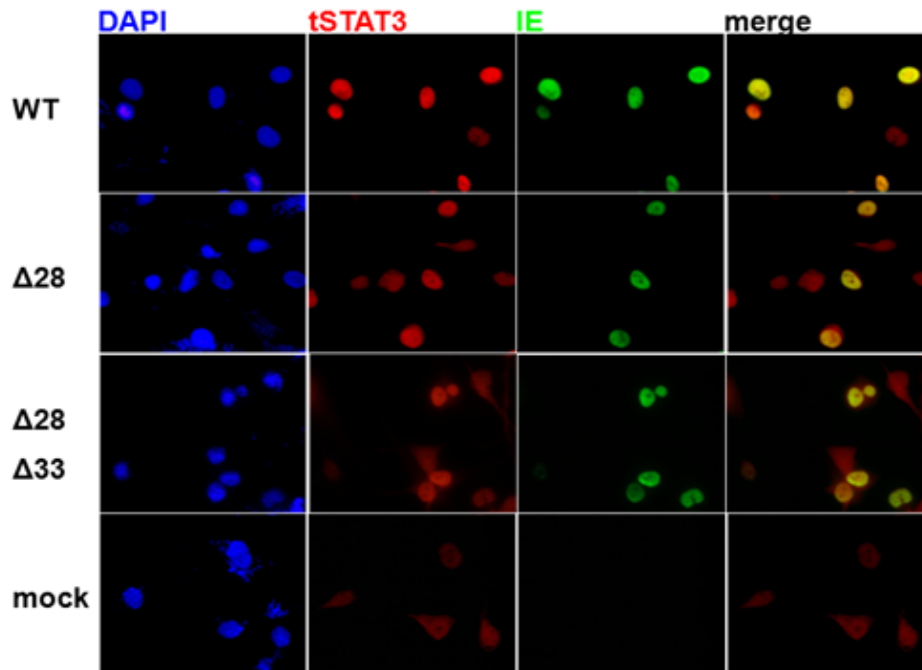


Figure 20: US28/UL33 contributes to tSTAT3 translocation to the nucleus. 30000 U373 cells were seeded and infected with an m.o.i of 1. Fixation was done 1 day post infection. The image shows the immunofluorescence staining of the nuclear staining of DAPI (blue), the Total-STAT3 proteins (red) and of the immediate early (IE) proteins (green). The merged images show an overlay of the STAT3. The different rows show cells infected with the respective HCMV TB40-BAC4 strain virus. WT, Δ US28, Δ US28/ Δ UL33 and the non-infected cells (mock).

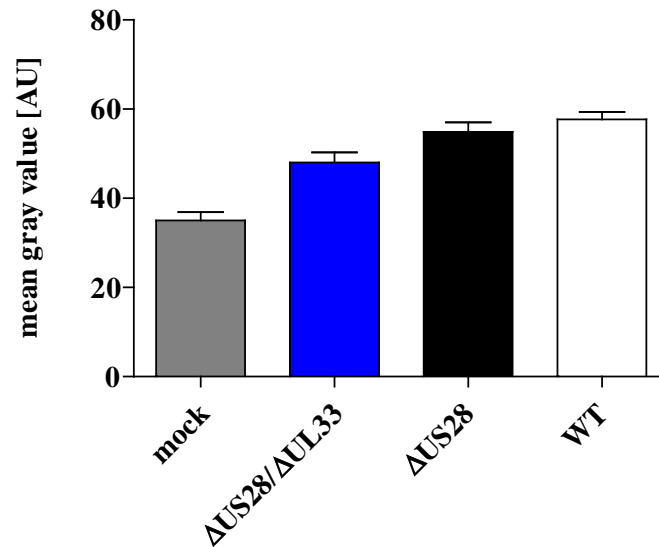


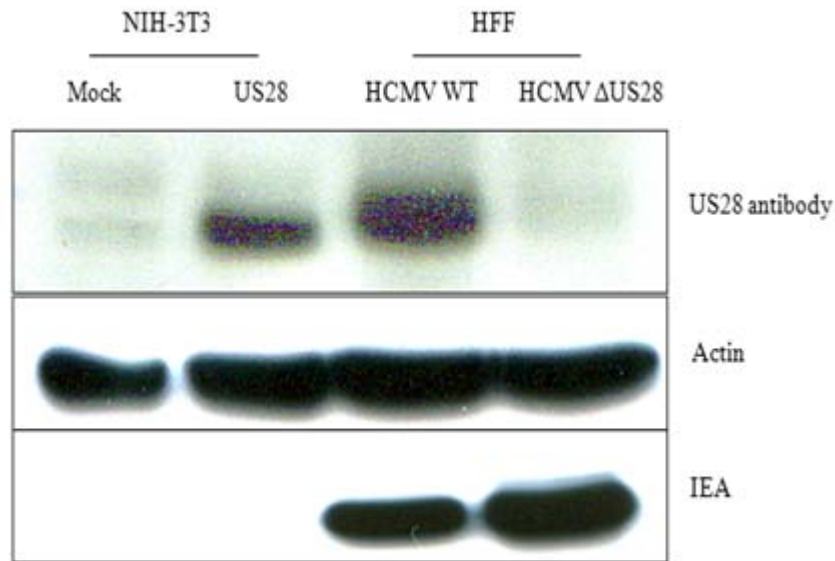
Figure 21: Quantification of nuclear STAT3 staining in infected U373 cells. 30000 U373 cells were seeded per a well and infected with an m.o.i of 1. The cells were infected with TB40-BAC4 WT, mutant's Δ US28, and Δ US28/ Δ UL33 and non-infected (mock). Fixation was done 1 day post infection. Intensities of the nuclear staining (Total-STAT3) in U373 cells were measured by ImageJ in AU units.

To quantify the intensities of the nuclear staining in U373 cells shown in Figure 20, intensities were measured in AU units using the program ImageJ. The results are shown in Figure 21. It shows the quantification of nuclear STAT3 staining in infected and non-infected (mock) U373 cells. The cells were infected with TB40-BAC4 WT, mutant's Δ US28, and Δ US28/ Δ UL33 with an m.o.i of 1. Due to the small number of measured points only the difference between WT and mock treatment showed statistically significant differences ($p < 0.01$).

Validation of the US28 antibody

The US28 antibody was made up in 5% BSA (2,5g BSA, 5ml TBST and 5 μ l of antibody in a 50ml tube). It was found that once the antibody was made up in this way it could not be frozen or reused as no signal was generated when using frozen antibody. Once the antibody was made up Western blotting was done to determine whether a signal for the US28 protein (H2N-SSDTLSDEVCRVSQIIP-CO₂H) could be detected.

A)



B)

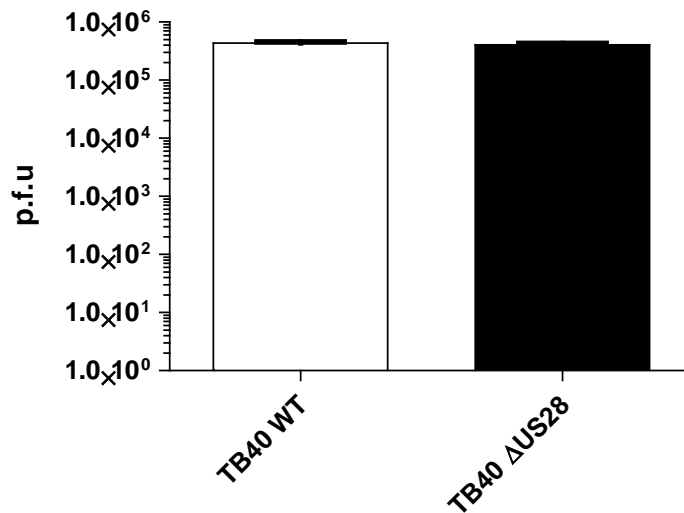


Figure 22: A) Validation of US28 antibody using NIH-3T3 expressing US28 and HFF infected HCMV TB40-BAC4 strain cells. Actin was used as a loading control, and the immediate early antigen was used to detect the presence of HCMV infection. **B) Back titration done for HCMV TB40-BAC4 samples used in validation of US28 antibody blots and for the detection of Phospho-STAT3.**

Figure 22(A) shows the validation of the US28 antibody for HCMV infection. To achieve this the TB40-BAC4 stain was used. A stable transfected mouse fibroblast cell line (NIH-3T3/US28) expressing US28 receptors was used as a positive control. HFF cells were infected with one of the three HCMV TB40-BAC4 strains. The protein actin was used as the gel loading control, and the immediate early antigen was used to detect positive HCMV infection. The NIH-3T3 cells expressing US28 and the HCMV WT infected HFF's cells were both positive for the presence of the US28 protein. The immediate early antigen could be observed in HFF cells that had been infected with either HCMV WT or Δ US28 with an m.o.i of 1. This also showed that the correct secondary antibody was used to recognize the primary antibody.

Immunofluorescent assay to determine the presence of US28 in the HCMV particle

A triple stained immunofluorescence assay (IFA) was used to detect the cell nucleus, the pp65 protein and US28 in HCMV infected cells. HFF cells were seeded on an IBIDI slide and treated with cycloheximide. The cells were then infected with HCMV TB40-BAC4 strain WT and mutant Δ US28 with an m.o.i of 50. Figure 23 shows images taken with a live scan swept field confocal (SFC) microscope in an attempt to determine whether the US28 protein was present in the viral particle. The merged images show the DAPI staining of the nucleus (blue), pp65 staining at an early time point (red), and US28 staining (green). The cells were counterstained with DAPI which is known to form fluorescent complexes with double-stranded DNA. This was done to locate the nucleus and because of DAPI's compatibility with antibodies. Pp65 is a tegument protein; used due to the fact that it is present in the viral particle and is one of the most abundant proteins and major constituent of the HCMV virion. It is also an ideal viral marker to detect the presence of HCMV viral proteins. Pp65 localises predominantly to the nucleus after virus penetration and accumulates in both nucleus and cytoplasm as the virus matures late in infection.

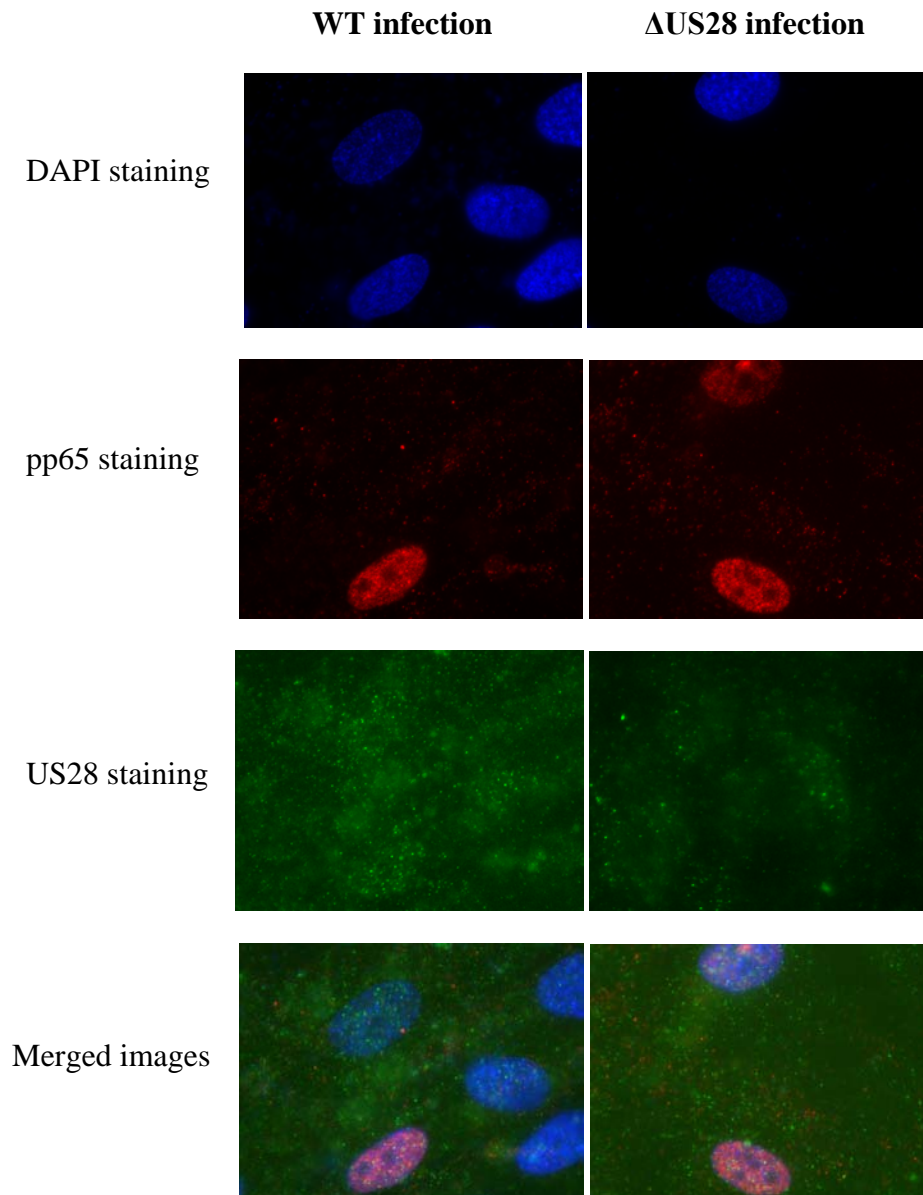


Figure 23: Determination if US28 is in the viral particle. 300000 HFF cells were seeded on an IBIDI slide and treated with cycloheximide to block cell protein synthesis. The cells were infected with HCMV TB40-BAC4 WT and Δ US28 strains, incubated and fixed with 4% paraformaldehyde. Slides were analysed using a Live scan Swept Field Confocal (SFC) Microscope from Nikon. Images of DAPI staining of the nucleus (blue), pp65 staining (red), and US28 staining (green) were taken individually and then merged.

Due to the early stages of the images being taken pp65 would still be localised to the nucleus. The presence of US28 is clear from the green staining due to the anti-US28 antibody. It was however still uncertain whether the US28 was in the viral particle or originated from protein synthesis by the fibroblasts.

Chapter 6: Discussion and Conclusion

Most of the HCMV and US28 data available in the Department of Medicinal Chemistry at Vrije Universiteit was the result of experiments in which the AD169 (a lab HCMV strain) and Titan (an isolated clinical HCMV strain) were used. Stable transformed cell lines expressing the US28 gene or virally transfected cells were used for these experiments. It was proven by (Cha et al., 1996) that HCMV clinical isolates carry at least 19 genes not found in laboratory strains. Due to this fact further characterisation of the TB40-BAC4 strain with respect to IL-6 and STAT3 manipulation were attempted. The TB40-BAC4 strain which has been found to infect more cell types than its common laboratory counter parts has been completely sequenced and has been found to be more complete regarding its genome compared to the AD169 and Titan strains. For future use the department was aiming to find a strain with more relevance for clinical use. The TB40-BAC4 was believed to be this strain.

Table 2: Differences between the HCMV Titan and TB40-BAC4 strains.

Titan Stain	TB40-BAC4
Expresses GFP (green fluorescence protein) this makes it easier to track	Infects endothelial cells – lab strains do not do this e.g. AD169
Not sequenced at all	Sequenced completely
A major concern is that it is already adapted to lab strain behaviour	Genome is more complete than its counterparts

These two viral strains were used to infect two mammalian cell lines: HFFs and U373 to see the effects on IL-6 secretion and STAT3 expression. The increase in IL-6 production and secretion in infected U373 cells (Figure 18 (A)) showed a strong association with an increase in STAT3 phosphorylation in the same U373 infected cells (Figure 19 (A) and (B)). This increase was only apparent in infected U373 cells expressing US28 and not in mock infected or Δ US28 cells. This increase in production and secretion of IL-6 is especially noticeable one hour post infection. In cells lacking US28 the low levels of secretion of IL-6 could be attributed to impaired constitutive activation. However IL-6 secretion still occurred in the mock infected cells due to the fact that these cells were astrocytes and still proliferating. (Van Meir et al., 1990) proved that glioblastoma cells constitutively produce IL-6 *in vivo* and *in*

vitro. In data not shown in this thesis a plateau effect was reached with IL-6 secretion at 48 hours.

Back titrations to determine the number of viral particles in samples were done for each experiment. The back titration acted as a control for the number of infectious viral particles. As long as the plaque forming units (pfu) were within a concentration difference of half a log it was viewed as acceptably close. This was obtained in all three back titrations done (Figure 18(B), 19(C) and 22(B)).

To characterise the HCMV TB40-BAC4 strain in U373 cells using Western blot analysis could not be achieved during this study. This was partially due to the small quantities of protein obtained from the cell pellets of the infected U373 cells. Further testing of the supernatants of the U373 infected with the TB40-BAC4 was not done due to limited funding. Also staining of the U373 cells infected with the TB40-BAC4 virus was not easily achieved. The staining assay required more time than its counter-part infected with the Titan strain. The AEC solution had to be left on the infected cells for at least one hour longer. The cause for this was not clear but could have been due to the pp65 epitope being mutated or hidden by folding differently or possibly a contamination.

From (Figures 20 and 21) it can be seen that both US28 and UL33 are important in the upregulation of the translocation of STAT3 into the nucleus after infection with HCMV. There appears to be a decreasing trend in the STAT3 signal developing in the deletion mutants. UL33 appears to have a stronger positive effect on the nuclear expression of STAT3 than US28. The increased intensities were however a measure of a small sample of cells (~20 for mock infected and 4-5 for the Δ US28 and Δ US28/UL33 assays). Moreover US28 was compared by its deletion out of the WT sample and UL33 was deleted out of Δ US28 sample. Therefore the results seen here do show a trend but not a true statistical validation. To establish whether this is a significant STAT3 activation effect would require further research. A staining for phospho-STAT3 was attempted, but this did not show clear results.

A model (Figure 24) for US28-induced STAT3 activation and subsequent proliferative signalling was suggested by (Slinger et al., 2010b). From this model and the above data it would indicate that HCMV-encoded chemokine receptor US28 mediates proliferative signalling by establishing a positive feedback loop involving activation of the IL-6-STAT3 axis.

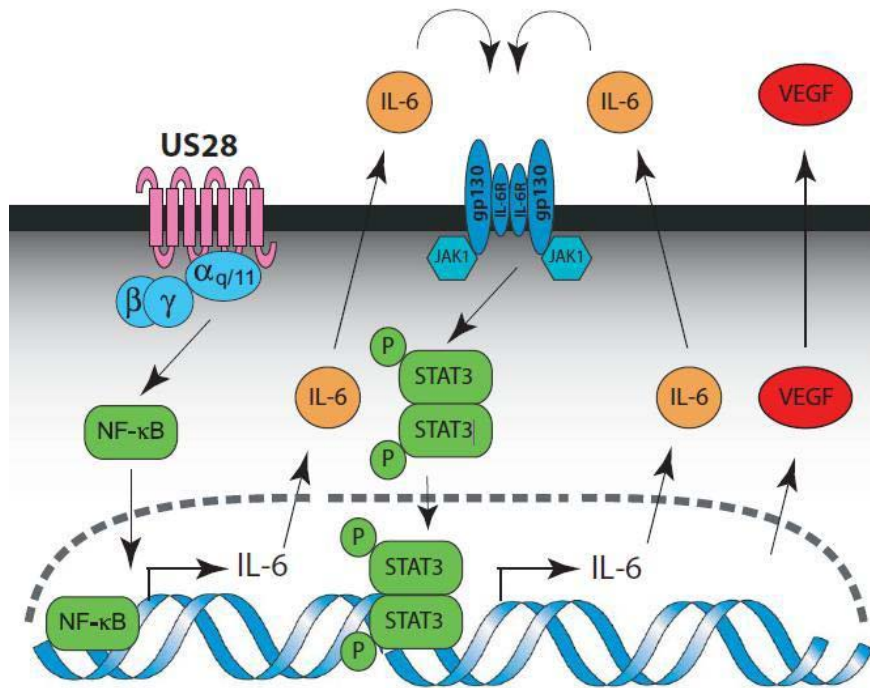


Figure 24: Model outlining the US28 positive feedback loop. This model suggests that US28 activates STAT3 through autocrine stimulation initiated by inducing IL-6 production via the NF- κ B pathway. IL-6 subsequently activates the IL-6 receptor, which results in STAT3 phosphorylation and activation of its target genes (among others, VEGF). IL-6 secreted by US28-expressing cells can also activate STAT3 in a paracrine fashion. In both cases, IL-6 is a target gene of STAT3 and activation of the IL-6–STAT3 axis may initiate in a positive feedback loop. This figure was taken with the authors approval from (Slinger et al., 2010b).

Considering that an increase in IL-6 production and secretion occurred so shortly after infection would suggest that US28 plays a major role in the upregulation of IL-6 and triggers STAT3 phosphorylation in HCMV infected U373 cells. The reason for this could be due to US28 incorporation into the viral particle. This led to the secondary research aim of this thesis. The question whether US28 is present in the viral particle has been raised countless times by researchers. Many scientists in the past decade have tried and failed to prove this (Fraile-Ramos et al., 2002, Varnum et al., 2004). The sequence of the HCMV genome has been known for over a decade, however the full set of viral and cellular proteins that compose the HCMV virion are still unknown.

To be able to determine US28 on the viral particle a method had to be designed and carried out. The validation of the US28 antibody was very important in the development of this method. A means of detecting US28 was needed. The US28 antibody was a polyclonal antibody which had not been used before. The characterisation was the first step in working with this antibody. The first Western blots done with the US28 antibody gave a very weak signal and it was found that the antibody could not be made up in ELK (milk powder) and could not be frozen to be reused; because of this a limited amount of the antibody was available for testing. In Figure 22 (A) clear bands are seen with the NIH-3T3 cells expressing US28 and HFF cells infected with the HCMV WT TB40-BAC4 strain. There are no bands seen for the mock and Δ US28 infected cells. This clearly showed that the antibody detects US28. It was also confirmed that anti-rabbit antibody was the best secondary antibody to use with this antibody. The US28 antibody is a polyclonal antibody and was suspected of cross reacting with US27 (from previous Western blots done and not shown). The potential of cross reactivity therefore had to be taken into account when trying to detect US8.

It is important to note that HCMV infection of cells in culture generates three different types of particles, including infectious mature virions, non-infectious enveloped particles (NIEPs), and dense bodies. NIEPs are composed of the same viral proteins as infectious virions but lack viral DNA. Dense bodies are uniquely characteristic of HCMV infection and are non-replicating enveloped particles composed primarily of the tegument protein pp65 (UL83). The quantities of these different HCMV particles are dependent on the viral strain and the multiplicity of infection (Varnum et al., 2004). Cycloheximide was used to treat the cells because it is widely used to inhibit protein synthesis in eukaryotic cells that are studied *in vitro*. It was used to distinguish between genes expressed in organelles and genes expressed in the nucleus. Genes expressed in the nucleus would therefore not be expressed in the presence of cycloheximide. This was important because HCMV is a DNA virus and moves to the nucleus to be transcribed. It was important to make sure the cells themselves were not producing the US28 gene but that it in fact was on the virion itself. After the cycloheximide treatment the cells were infected with the respective HCMV strains. The TB40-BAC4 strain was selected for this experiment because of the availability of the complete sequence of the viral genome. An m.o.i of 50 was used because unfortunately, as virions are purified, the particles tend to lose infectivity. This situation makes identification of essential virion proteins difficult to assess except by abundance (Varnum et al., 2004). Fusion of the virus and cell membranes is followed by entry into the host cytoplasm of the nucleocapsid and

tegument proteins, and their rapid translocation into the nucleus, where pp65 is detected less than one hour p.i. Interaction of HCMV glycoprotein's with their receptors is enough to trigger an intracellular signal transduction pathway, leading to the alteration of cellular gene expression (Landolfo et al., 2003). The presence of US28 in the samples was illustrated in Figure 23. However it was not clear whether US28 was carried in the viral particle or if it was already present in the infected cells. Modification to the experimental protocol was made but due to bacterial contamination these results had to be disregarded.

There are several reasons that the method that was developed for the determination of US28 in the HCMV viral particle did not give a conclusive answer. It could not have been that insufficient US28 antigen was present due to the fact that the sample was infected with an HCMV strain with US28 at an m.o.i of 50. However the outcome of the cycloheximide treatment in blocking the cell protein synthesis of the HFF cells was uncertain. Cycloheximide is also known to cause DNA damage. Besides inhibiting protein synthesis it was unclear if cycloheximide had any effects on the virus or the virus entry into the cells nucleus. The reason for using cycloheximide was that it was less toxic than other protein inhibitors, it was inexpensive, works rapidly and its effects are quickly reversed by simply removing it from the culture medium. A possibility of cross reactivity between the other primary probe antibodies and secondary antibodies could have occurred. The US28 antibody (of which not much is known) could have been diluted too far. The incubation time was estimated; initially it was increased from three hours to five hours to allow more time for trafficking of the virus. The viral particles had attached but longer trafficking times for the virus to reach the nucleus may be needed. Following entry the HCMV capsids must move to the nucleus prior to permissive replication of the viral genes. The viral particles attached, which was clear from the red staining of the pp65 IEA therefore to reach the nucleus and uncode more time appears to be required. Beside the trafficking time being increased the secondary antibody concentration should be increased from (1:1000) to (1:500) to provide stronger signal amplification. Initially the incubation time for the US28 antibody was one hour. This incubation time for the US28 antibody should be increased to overnight. This should decrease the background noise detected and increase the specificity of the antibody.

Conclusion

Herpes viruses use a variety of methods to evade host responses and to enhance viral infection. The role of the viral receptors encoded by HCMV during the viral life cycle and in viral pathogenesis is still poorly understood. Over the years it has been shown that virus infection induces profound changes in chemokine and chemokine receptor expression. In most cases US28 acts as a decoy receptor for many chemokines thereby removing them from the microenvironment of HCMV infected cells through rapid and constitutive internalisation (Billstrom et al., 1998, Bodaghi et al., 1998). Recent work on US28 provided a molecular link between HCMV and cancer. It was reported that in US28 stably transfected NIH-3T3 cells tumours formed when these cells were implanted into nude mice (Maussang et al., 2006, Alcami and Lira, 2010).

The main research question of this thesis was whether US28 unregulated IL-6 expression and STAT3 phosphorylation in constitutive signalling in HCMV infected HFF and U373 cells. A clear increase in IL-6 secretion ($495\% \pm 1\%$) was seen, and this was after only an hour in HCMV WT infected cells. IL-6 has been linked to numerous cancers as mentioned in the literature review. From the increase in IL-6 secretion a subsequent increase in STAT3 phosphorylation was detected in the same samples. A clear link has been established between IL-6 and STAT3 (Figure 24). This link is very important due to the fact that HCMV proteins and DNA have been detected in several tumours (Muñoz et al., 2003, Ganem, 1997). This link to cancer could be due to US28 expression and its constitutive activity with ligand-dependent and ligand independent signalling properties (Vischer et al., 2006). This is vital information in the future regarding drug development.

The secondary aim to determine whether US28 was present in the HCMV viral particle was not conclusive. A method was designed and preliminary results were obtained. Work is still on going and modifications to the method are being made.

Viral chemokine regulation can give more insight into the pathophysiology of virus infections. The molecular functions of most HCMV genes are not well understood, and to a large degree, is a result of the difficulty in constructing specific HCMV mutants. HCMV in clinical material cannot replicate efficiently *in vitro* until it has adapted by mutation. Consequently, wild-type HCMV differ fundamentally from the laboratory passaged strains used for research. Future studies exploring the function and biological roles of US28 *in vivo* may provide new insights regarding the molecular pathogenesis of herpesvirus infections.

Chapter 7: Limitations and Future work

One of the major limitations to this project was the time available due to the fact that the work was performed as part of a one year student exchange abroad. Because this study was done at a foreign university only a year was given in which all laboratory work and write up had to be completed. Due to limited funds available not all the aims could be accomplished as originally set out for this study. The HCMV strain TB40-BAC4 could not be characterised for IL-6 expression in infected U373 cells. Another limitation was viral stock contamination and the limited availability of the virus isolates. The virus was supplied to the Medicinal Department from the Virology Department of Universitätsklinikum Ulm, in Germany.

The ELISA procedure performed also had limitations; any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age could cause variation in binding. The assay was designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. But until all factors had been tested in the Quantikine Immunoassay, the possibility of interference could not be excluded.

Scientific data was limited for certain sections due to the fact that there was insufficient research on this topic, since this was relatively new work that was being done in the department. There was a great deal of trial and error with development of protocols and procedures, but many aspects were learnt about the methods and the potential problems.

Dr Andreas Schreiber was the only available Virologist in the department. The re-use of certain antibodies was not an option especially for US28 e.g. Western blotting.

Future work on the determination of US28 in the viral particles needs to be further addressed. Sufficient strains of HCMV need to be developed that do not show specific viral sequence deletions as well as the development of *in vivo* models for HCMV infection. Lastly the characterisation of the TB40-BAC4 strain for IL-6 needs to be completed.

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