University of Alberta

The Placenta as a Viral Reservoir:
Implications for Congenital Cytomegalovirus Infection

by

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Abstract

Human Cytomegalovirus (HCMV) is the most common cause of congenital infection in newborns. One mechanism for this virus to reach the fetus is to cross the placenta through the syncytiotrophoblast layer. Accumulation and protection of pathogens in the syncytiotrophoblast could affect the systemic distribution of pathogens and prolong maternal infections leading to increased incidence of fetal infections. Primary infections, reactivation or reinfection with another strain during pregnancy are risk factors for intrauterine HCMV transmission to the fetus. All lead to an active infection; however, viral load in blood or urine does not correlate with intrauterine transmission. I have shown that HCMV reversibly binds to the syncytiotrophoblast *in vitro,* protecting it from degradation. Furthermore, I demonstrated *in vivo* that HCMV is present in the placenta, even when cleared from maternal blood and urine. This evidence suggests increased potential for fetal transmission by virtue of continued virus localized at the maternal-fetal interface.

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Abbreviations

Abbreviation	Meaning
Br-cAMP	Bromoadenosine 3',5' –cyclic monophosphate
CaCo-2	Colon epithelial cell-derived cell line
CT(s)	Cytrotrophoblast(s)
ddH_2O	Double-distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EVT	Extravillous trophoblasts
FBS	Fetal bovine serum
gB	Glycoprotein B
gCMV	Guinea pig cytomegalovirus
HCMV	Human cytomegalovirus
HEL	Human embryonic lung fibroblasts
HIV-1	Human immunodeficiency virus type 1
HSPG(s)	Heparan sulfate proteoglycan(s)
ICW	In cell western
IE	Immediate early protein
IFN(s)	Interferon(s)
IFC	Immunofluorescence

IHC	Immunohistochemistry
IMDM	Iscoves's modified Dulbecco's medium
IL	Interleukin
IVIG	Intravenous immune globulin
LCMV	Lymphocytic choriomeningitis
MHC	Major Histocompatibility Compex
MOI	Multiplicity of infection
NF-κB	Nuclear factor kappa B ear factor kappa B
OCW	On cell western
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Real time quantitative PCR
RT	Room temperature
SEM	Standard error of the mean
SHL	Sensorineural hearing loss
siRNA	Small interfering RNA
ST	Syncytiotrophoblast
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Vim	Vimentin

Chapter 1

Introduction

1.1 Prologue

Human cytomegalovirus (HCMV) is the leading cause of congenital infection in the developed world, affecting up to 2% of the newborn population. When acquired *in utero* the disease manifests from mild or no apparent illness to severe sequelae or death. One potential route of transmission to the fetus is via the syncytiotrophoblast (ST) layer of the placenta, which functionally separates maternal and fetal circulations. The ST layer is microvilliated and represents a 12 m^2 surface area by term. In a guinea pig model, the placenta has been shown to function as a viral reservoir for guinea pig cytomegalovirus where even when cleared from maternal blood the virus persevered in the placenta. In humans, other pathogens, such as *Listeria monocytogenes*, and malaria parasitized red blood cells have also been shown to be harbored by the placenta. The placenta represents a strong physical barrier between maternal and fetal entities. However, it is subject to physical damage via systemic maternal and localized placental immune responses. The accumulation in or around the ST could increase the accessibility of HCMV to fetal tissues through breaks in the ST layer. I chose to investigate the relationship between HCMV and the ST as well as identify the seroprevalence of HCMV and distribution of viral load within our pregnant population. It is important to understand how HCMV functions and also how the placenta creates and could potentially harbor pathogens before defining the relationship between an HCMV infection during pregnancy.

1.2 Human Cytomegalovirus

1.2.1 Incidence

Human cytomegalovirus (HCMV) is endemic in the world and seroprevalence of HCMV varies greatly depending on geographical location and socioeconomic status, resulting in 40-100% seropositivity (Mocarski, Shenk et al. 2006; Cannon, Schmid et al. 2010). The highest seroprevalence is found in South America, Africa and Asia and this dramatically decreases in Western Europe and North America (Cannon, Schmid et al. 2010). The importance of HCMV as a pathogen has increased over the last 30 years, mainly due to the increase in immunosuppressed patients by way of AIDS, organ allografts and post-transplant therapies (de la Hoz, Stephens et al. 2002). A compromised immune system leaves people more susceptible to primary HCMV infections, or reactivation of latent infections (Mocarski and Courcelle 2001). As well, our knowledge of consequences in pregnancy associated with HCMV infection has increased. This is, in part, due to connections made between late presenting sequelae in babies such as, sensorineural hearing loss in infected infants, and the mothers having an active HCMV infection during pregnancy (Fowler, McCollister et al. 1997; Dollard and Schleiss 2010).

HCMV is the most common opportunistic viral infection in immunocompromised individuals, often resulting in severe sequelae such as retinitis, colitis and neurological disorders (Cheung and Teich 1999). For example a productive HCMV infection is known to occur in up to 40% of all AIDS patients (Cheung and Teich 1999). Furthermore, in recipients of solid organ transplants, HCMV is an important cause of morbidity that can result in severe immunosuppression (Rubin 1990) leading to other pathologies such as bacteremia (Falagas, Snydman et al. 1996) and invasive fungal disease (George, Snydman et al. 1997).

1.2.2 Pathology

1.2.2.1 Types of Infection

A productive HCMV infection can be defined as primary or secondary. Seroconversion, the *de novo* appearance of virus-specific IgG and/or IgM, defines a primary infection (Hagay, Biran et al. 1996). After a primary infection, HCMV establishes lifelong persistence within a host, where the virus enters into cycles of latency and reactivation (Sinclair and Sissons 2006). During latency, the virus remains present in the host, but in the absence of infectious virus production. HCMV can be reactivated from latency in the presence of stressful (Stowe, Mehta et al. 2001) or inflammatory (Hummel and Abecassis 2002) situations and often occurs in immunosuppressed individuals, such as transplant and HIV-infected persons (Sinclair and Sissons 2006). Interestingly, during pregnancy there is an increase in the rate of HCMV excretion recovered from the cervix (13.4%) when compared to the isolation rate (1%) in the general population (Reynolds, Stagno et al. 1973). This indicates that pregnancy may play a role in the reactivation of HCMV from latency.

A secondary infection via reinfection with a different strain of virus, or reactivation from latency can be diagnosed if there is a significant rise in HCMV-specific IgG and/or IgM antibodies in a person who was previously infected (Ornoy and Diav-Citrin 2006).

1.2.2.2 Viral Load

Primary infections, reactivation from latency or reinfection with another strain, all lead to an active HCMV infection. In high risk populations for HCMV infection, such as transplant patients, measuring HCMV by quantitative PCR in blood is useful for predicting and monitoring the progression of CMV disease (Humar, Gregson et al. 1999; Pang, Fox et al. 2009). HIV patients are another population largely at risk for developing HCMV disease (de la Hoz, Stephens et al. 2002). Proper prophylaxis can be given to prevent severe manifestations of HCMV disease in this immunocompromised group (Bowen, Sabin et al. 1997);

HCMV viremia levels detected by PCR have shown predictive value in identifying severe HCMV disease (Hansen, Ricksten et al. 1994; Bowen, Sabin et al. 1997).

1.2.2.3 Transmission

HCMV transmission can occur horizontally through the exchange of saliva, transplants, blood transfusions, or sexual fluids (de la Hoz, Stephens et al. 2002). While HCMV is not highly contagious, there are groups at high risk for acquiring it such as immunocompromised individuals, babies *in utero* and daycare workers (Adler 1985; de la Hoz, Stephens et al. 2002). Also, persons with sexual risk factors (multiple partners, acquired sexually transmitted infections, etc.) are more likely to be infected as HCMV is found in both semen and cervicovaginal secretions (Handsfield, Chandler et al. 1985; Stover, Smith et al. 2003).

Transmission of HCMV can also occur vertically from mother to child and is the most common cause of congenital infection (Kenneson and Cannon 2007). Congenitally-infected children excrete the virus in high quantities for long periods in urine and saliva (Noyola, Demmler et al. 2000). While transmission of HCMV *in utero* accounts for up to 2% of the newborn population being born HCMV positive, children can also be infected during delivery, through blood transfusions, breastfeeding, and contact with other children who are excreting HCMV (Peckham, Johnson et al. 1987; Staras, Dollard et al. 2006). The rate of transmission to infants varies from 20 – 60% (Dworsky, Yow et al. 1983). This contributes to transmission of HCMV from daycare centers to seronegative children and their parents as well as daycare workers (Adler 1985).

1.2.2.4 Cell Tropism

The pathogenesis of HCMV infections is greatly influenced by their broad target cell range (Sinzger, Digel et al. 2008). HCMV is species-specific and infects many different cell types such as: monocyte /macrophages, endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, stromal cells, neuronal cells, neutrophils, and hepatocytes (Compton 2004; Sinzger, Digel et al. 2008).

Each cell type has different susceptibility and productivity to HCMV. For example, some cell types are permissive to HCMV infection and therefore allow for a fully productive infection where new infectious virus is made (Knipe and Howley 2007). Other cell types, such as polymorphonuclear cells, may be infected but the infection is aborted and does not progress past the immediate early stage with no new infectious virus made (Knipe and Howley 2007). In monocytes, infection is aborted but also progresses to a latent stage. Latently infected cells express no viral proteins and are immunologically silent; however, the infection can become productive if the cells are stimulated by appropriate stressors (Knipe and Howley 2007).

Epithelial cells are a major target of HCMV and can be productively infected *in vivo* and *in vitro* (Sinzger, Grefte et al. 1995). *In vitro*, the importance of establishing permissive, productive infections in epithelial cells is necessary to allow for the investigation of the pathogenesis of HCMV. Epithelial cells such as human colorectal adenocarcinoma cells (CaCo2) are permissive for HCMV infection *in vitro* (Esclatine, Bellon et al. 2001). The mechanism for HCMV entry is also cell-type specific; HCMV enters into epithelial cells via endocytosis and low-pH fusion (Ryckman, Jarvis et al. 2006; Ryckman, Rainish et al. 2008). Alternatively, with fibroblasts, a type of connective tissue cell, HCMV can enter the cell via direct fusion of the viral envelope with the plasma membrane (Ryckman, Jarvis et al. 2006; Ryckman, Rainish et al. 2008). The ease of the initial virus-cell attachment could, in part, explain why fibroblasts are the most readily infected cell type *in vitro* and *in vivo* (Sinzger, Grefte et al. 1995; Mocarski, Shenk et al. 2006). *In vitro*, fibroblasts are used as a standard culture system to propagate HCMV to high titres because they are so easily infected and allow for efficient replication of the virus (Mocarski, Shenk et al. 2006).

Lastly, cells that are not productive for viral replication, but can undergo latency, are important in HCMV tropism. Monocytes are one example; these white blood cells greatly contribute to the systemic spread of the virus *in vivo* (Smith, Bentz et al. 2004). These cells can be latently infected and it has been

shown that once infected, circulating monocytes then differentiate into mature macrophages which are permissive for infection (Smith, Bentz et al. 2004). Monocytes, alongside other hematopoietic cells are also suggested to be a primary site of viral latency which potentially assists in the systemic spread of the virus (Sinclair and Sissons 2006).

1.2.2.5 Strain tropism

The capacity of a virus to attach, enter and infect a cell is dependent on the proteins it expresses and the presence of cell-surface receptors. Laboratory strains of HCMV such as Towne and AD169 have deletions, mutations and rearrangements in the virus genome due to repeated propagation in fibroblasts (Cha TA 1996; Sinzger, Schmidt et al. 1999; Murphy, Yu et al. 2003). Due to specific deletions, such as genes UL128-131 (Wang and Shenk 2005) and UL150, these commonly studied laboratory strains do not replicate well in cell cultures of epithelial or endothelial cells (Ryckman, Jarvis et al. 2006; Ryckman, Rainish et al. 2008). Clinical isolates of HCMV carry at least 19 genes that could be essential to cell-specific pathogenesis that are not found in laboratory strains Towne or AD169 (Cha TA 1996).

1.2.3 Innate and Acquired Immune Response

An individual's immune response to HCMV infection greatly depends on their immune status (Gandhi and Khanna 2004). In an immunocompetent individual a HCMV infection triggers establishment of both humoral and cell-mediated immunity, and can, but rarely, result in a mild mononucleosis-like illness (Mocarski, Shenk et al. 2006). However, in an immunocompromised individual, a CMV-specific T-cell response is delayed when compared to the immunocompetent population (Lilleri, Zelini et al. 2009). As the immune status of the host is inevitably linked to the pathogenesis of this virus, the delayed response could account for the morbidity that immunocompromised groups face with HCMV infection (Boehme, Guerrero et al. 2006).

Upon infection with HCMV, inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8) and interferons (IFNs) generated in response to nuclear factor kappa B (NF-κB) activation, function to activate natural killer (NK) cells which work to limit replication and spread of the virus (Juckem, Boehme et al. 2008). This innate response is the primary line of defense and is backed by development of adaptive immunity. Specific adaptive immunity via cytotoxic T-lymphocytes is responsible for the final clearance of the virus (Mocarski, Shenk et al. 2006).

1.2.4 Description

Human Cytomegalovirus (HCMV) is a large double-stranded DNA virus that belongs to the *Herpesviridae* family (Mocarski and Courcelle 2001). The virions are 150 to 200nm in size and the linear genome is surrounded by an icosahedral capsid which is enveloped by a lipid bilayer (Mocarski and Courcelle 2001). This virus is also known as Human Herpes virus 5 (HHV-5), belongs to the subgroup of the *β-herpesviruses* and shares several characteristics with other members such as virion structure and the ability to establish persistent and latent infections (Mocarski and Courcelle 2001).

1.2.5 Replication Cycle

Attachment and penetration of HCMV is only partially understood. However, these processes are known to be rapid and efficient, involving widely distributed HCMV cellular receptors (Mocarski and Courcelle 2001). Most notably, the virus has two envelope glycoproteins, B (gB) and H (gH) that interact with cellular surface receptors to initiate attachment and fusion with the host cell (Compton 2004; Boehme, Guerrero et al. 2006). This virus has a three-stage replication process: immediate early (IE), early, and late (Mocarski and Courcelle 2001). The process of replication is very slow, involving 48-72 hours for release of progeny from most cell types (Mocarski and Courcelle 2001). Upon entry into the cell, the immediate early phase begins within 4 hours, with expression of the α gene products (Mocarski and Courcelle 2001). The α genes are responsible for viral subversion of host cellular machinery and promote the expression of β and γ

viral gene products (Fortunato and Spector 1999). The early genes, also known as β genes, function to produce DNA replication proteins and some structural proteins (Fortunato and Spector 1999). The late gene products (γ) are produced approximately 24 hours after infection (Fortunato and Spector 1999). The γ genes encode mRNAs that are necessary for production of structural proteins that are involved in virion assembly and exit (Fortunato and Spector 1999; Mocarski, Shenk et al. 2006).

1.2.6 Cellular Receptors

HCMV tethering, docking, and entry into cells are processes that affect the broad cell tropism of the virus. It is known that the primary attachment of HCMV involves tethering of envelope glycoproteins to cell surface heparan sulfate proteoglycans (HSPGs) (Compton, Nowlin et al. 1993). *In vitro*, HCMV virions are initially dissociable from the cell surface of fibroblasts by soluble heparin; however, the virus rapidly becomes heparin-resistant and binds to the cell surface (Compton, Nowlin et al. 1993). This suggests that additional HCMV glycoproteins are binding to other cell surface receptors for secondary attachment which commits the virus for entry (Compton, Nowlin et al. 1993).

The secondary attachment, or docking step, of the virus has many proposed receptors. One strong candidate for this step is epidermal growth factor receptor (EGFR). Cross-linking experiments by Wang *et al*. showed that HCMV glycoprotein B interacts with EGFR (Wang, Huong et al. 2003). Furthermore, it has been demonstrated that when fibroblast cell surface EGFR is neutralized, both HCMV attachment and entry are inhibited (Wang, Huong et al. 2003; Wang and Shenk 2005). However, although EGFR is widely expressed on many cell types, it is not found on all cell types permissive for HCMV infection, such as hematopoietic cells (Real, Rettig et al. 1986; Isaacson, Juckem et al. 2008). This suggests that EGFR may be a receptor for some cell types infected by HCMV, but not a universal receptor. Other candidates for HCMV entry receptors are integrin heterodimers (Feire, Koss et al. 2004; Wang and Shenk 2005). In mouse fibroblasts when β1 integrin neutralizing antibodies were added, HCMV

infectivity was inhibited (Feire, Koss et al. 2004). Interestingly, antibodies to integrins inhibited HCMV entry and infectivity, but not cell binding (Feire, Koss et al. 2004). This indicates that integrins may be co-receptors for attachment and entry but do not function independently as such.

In conjunction with HCMV attachment and cellular entry, early events in HCMV infection cause host cell transcription and translation rates to increase (Mocarski and Courcelle 2001). Toll like receptors (TLRs), known as pattern recognition receptors, interact with pathogens to activate cells of the innate immune system and stimulate the adaptive immune system response (Finberg and Kurt-Jones 2007). By HCMV binding to cell surface TLRs there is an increased activation of NF-κB thereby promoting early transcription of HCMV (Browne, Wing et al. 2001). This encourages the activation of a robust innate immune response, via the NF-κB pathway (Compton, Kurt-Jones et al. 2003). Required for the transactivation of the HCMV IE promoter and IE proteins, NF-κB plays a necessary role for viral replication (DeMeritt, Milford et al. 2004). UV-inactivated HCMV can activate an innate immune response which indicates that the mere cell contact by HCMV causes a cellular response (Compton, Kurt-Jones et al. 2003).

1.3 The Placenta

1.3.1 Placental Development

The placenta is a transitory organ that allows for proper nutrient and gas exchange between the maternal source and fetus. Within a week of conception, implantation of a rapidly dividing human blastocyst is potentiated by the trophoblasts that completely surround the embryoblast (Aplin 2000). Thus begins the formation of a hemochorial placenta, where maternal blood is in direct contact with fetally-derived trophoblasts (Benirschke, Kaufmann et al. 2006c; Figure 1.1).

Trophoblasts are epithelial cells that are unique to the placenta. The process of placental development involves two pathways of trophoblast differentiation that lead to two distinct phenotypes: extravillous and villous trophoblast (Benirschke, Kaufmann et al. 2006b; Figure 1.1). Extravillous

trophoblasts (EVTs), invade the uterine wall and spiral arteries and are responsible for the anchoring of the placenta in the uterus (Tarrade, Lai Kuen et al. 2001). Two types of EVTs are interstitial and endovascular. Interstitial trophoblasts invade through the uterine decidua while the endovascular trophoblasts infiltrate the spiral arteries and begin replacing the endothelium and smooth muscle cells (Cartwright and Wareing 2006; Benirschke, Kaufmann et al. 2006b; Lash, Naruse et al. 2010). This process of arterial remodeling creates a high-flow and low-resistance circulation, allowing for an adequate supply of nutrients to reach the fetus (Aplin 1991; Benirschke, Kaufmann et al. 2006b; Figure 1.1). EVTs produce and/or secrete many factors (type IV collagenase, matrix metalloproteinases, β-glucuronidase, aminopeptidases, cathepsin B, urokinase-type plasminogen activator (uPA), uPA receptor and lamin) that promote the degradation of extracellular matrix and give rise to their invasive properties (Morrish, Dakour et al. 1998). The second pathway of trophoblast differentiation leads to the villous trophoblast, which includes both the underlying progenitor cells, cytotrophoblasts (CTs) and the mature syncytiotrophoblast (ST) layer. Villous trophoblasts form over the chorionic villi to create the primary site of placental transport, as well as the protective and endocrine functions between the mother and the fetus (Tarrade, Lai Kuen et al. 2001).

Figure 1.1

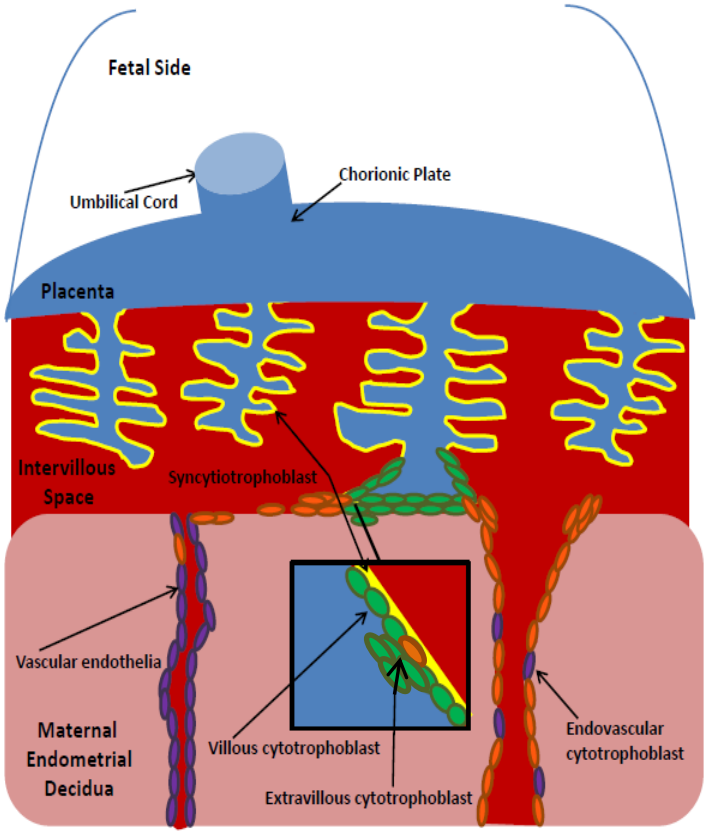

Figure 1.1 - The maternal-fetal interface

Fetal blood enters and exits the placenta via the umbilical cord. Trophoblast cells line all villous trees which contain fetal vessels in their core. Villous cytotrophoblast cells are the inner, mononuclear cells (green) that differentiate into the outer, multinuclear, syncytiotrophoblast layer (yellow). Extravillous trophoblast cells (orange) invade the maternal uterine arteries and become endovascular trophoblast cells (purple), allowing for remodeling of the uterine vasculature and adequate placental perfusion.

The trophoblasts in the embryoblast at the position of implantation eventually form the placenta and the rest of the trophoblasts surrounding the embryo transform into the chorion and membranes. Formation of the placenta begins with division of the trophoblastic covering of the blastocyst into three layers: the primary chorionic plate, the lacunar system and the trophoblastic shell (Benirschke, Kaufmann et al. 2006b). The primary chorionic plate faces the blastocytic cavity and is composed of embryoblast-derived mesenchymal cells

covered by layers of CTs and ST facing the lacunae (Enders and King 1988; Benirschke, Kaufmann et al. 2006b). The lacunae is a network of vacuoles, formed by pillars of ST called trabeculae, within the trophoblastic plate (Benirschke, Kaufmann et al. 2006b). Increasing trophoblast invasion causes the restructuring of spiral arteries which then allows for the lacunae to fill with maternal blood (Benirschke, Kaufmann et al. 2006b). The trabeculae are invaded by CTs from the primary chorionic plate and kept separate from the lacunae via the ST layer (Benirschke, Kaufmann et al. 2006b). When mesenchymal cells from the extraembryonic mesenchymal layer and fetal capillaries begin to invade these double-layered structures, the stems of the villous trees are formed (Boyd and Hamilton 1970).

The human placenta ultimately forms a discoid organ presenting two surfaces; the basal plate which borders the maternal endometrium and the chorionic plate that faces the baby and to which the umbilical cord is attached (Benirschke, Kaufmann et al. 2006c). As soon as an intervillous circulation is established, maternal and fetal blood are in close contact. However, in early pregnancy they remain separated by the placental barrier comprised of: syncytiotrophoblast, cytotrophoblast, basement membrane, connective tissue, and fetal endothelium (Benirschke, Kaufmann et al. 2006b). As pregnancy progresses, the number of these barriers is reduced and the distance across the barrier decreases from 50 to 100 µm in the second month to 4 to 5 µm at term (Benirschke, Kaufmann et al. 2006b).

1.3.2 The Villous Tree

There are five different types of placental villi (mesenchymal, immature intermediate, stem, mature intermediate and terminal) and all share the same basic structure (Castellucci, Scheper et al. 1990) (Benirschke, Kaufmann et al. 2006e). The structure of the villous tree, as described below, allows for the intact barrier function of the placenta thereby separating maternal and fetal circulations. Any pathogen must cross these layers to reach the fetus so I will discuss each layer that comprises all villous trees.

1.3.2.1 Villous Syncytiotrophoblast

The ST is an epithelial surface layer, in direct contact with maternal blood, that covers all villous trees as well as lining the chorionic and basal plates (Benirschke, Kaufmann et al. 2006e). The ST layer is composed of fused CTs and it lacks the ability to divide that the CT possess prior to fusion (Benirschke, Kaufmann et al. 2006b). Therefore, it forms a continuous, multinucleated layer with a characteristic microvilliated, brush-border membrane facing the maternal blood (Benirschke, Kaufmann et al. 2006e). The microvilli that cover the villous ST increases the area by a factor of 7.67, resulting in the $12m^2$ surface area (Teasdale and Jean-Jacques 1986). The STs microvilli normally form an uninterrupted covering over the chorionic villi, except where there is syncytial damage; fibrinoid fills in damaged and de-epithelialized areas which are normally susceptible to re-epithelialization (Burton 1987; Nelson, Crouch et al. 1990; Mayhew and Barker 2001).

1.3.2.2 Villous Cytotrophoblast

Cytotrophoblasts or Langhans cells act as stem cells and guarantee growth of the ST through continuously replenishing the ST layer (Benirschke, Kaufmann et al. 2006b). The CT are undifferentiated, mononucleated cells that underlie the ST and are supported by the basal lamina (Benirschke, Kaufmann et al. 2006e). As pregnancy progresses the CT becomes discontinuous, being found at term beneath only 20% of the villous syncytiotrophoblast (Benirschke, Kaufmann et al. 2006e; Mori, Ishikawa et al. 2007) .

1.3.2.3 Trophoblast Turnover

Trophoblast turnover is an essential task for placental maintenance (Huppertz and Kingdom 2004). The ST layer must be continuously regenerated by CTs to remain functionally active (Benirschke, Kaufmann et al. 2006b). It is known that syncytial fusion is necessary for growth and regeneration of the aging ST (Benirschke, Kaufmann et al. 2006b). This is supported by the observation that

without CT fusion to the ST there is a rapid decline in hormone production rates and death of the ST layer within days (Benirschke, Kaufmann et al. 2006b).

Along with syncytial fusion, the turnover of ST also involves syncytial knot formation at the surface of the ST. These syncytial knots contain a group of aggregated nuclei that are shed from the placenta into maternal circulation (Abumaree, Stone et al. 2006; Benirschke, Kaufmann et al. 2006e). The shedding of the ST is likely the loss of damaged or aged trophoblasts through an apoptotic process (Huppertz, Kingdom et al. 2003; Huppertz and Kingdom 2004). The balance between renewal of the ST by CT fusion and release of syncytial knots is essential for proper maintenance of the placenta (Huppertz and Kingdom 2004).

1.3.2.4 Basement membrane, connective tissue, and fetal endothelium

The trophoblastic basement membrane effectively separates the trophoblastic epithelium from the villous stroma (Benirschke, Kaufmann et al. 2006e). Spanning 20-50 nm under normal conditions, this basement membrane is thought to serve two main functions (Benirschke, Kaufmann et al. 2006e). The first is to support the trophoblastic epithelium by producing matrix proteins that allow CTs to detach and migrate for the necessary syncytial fusion (Aufderheide and Ekblom 1988; Benirschke, Kaufmann et al. 2006e). The second main function of the trophoblastic basement membrane is to act as a filtration barrier between maternal and fetal circulations (Benirschke, Kaufmann et al. 2006e). This is supported by the finding that in intrauterine fetal death, calcification of the villous trophoblastic basal lamina is a common pathology that is not found in placentas from living fetuses (Roberts, Sebire et al. 2000; Kasznica and Petcu 2003).

The villous stroma consists of fixed connective tissue cells that form a network, free connective tissue cells, and fetal vessels (Benirschke, Kaufmann et al. 2006e). Separated from the trophoblasts by the trophoblastic basement membrane, the fixed connective tissue cells vary in cell type depending on the age of the placenta and villous sample (Castellucci and Kaufmann 1982). The fixed connective tissue cells range from proliferating mesenchymal cells to differentiated myofibroblasts (Kohnen, Kertschanska et al. 1996; Demir, Kosanke

et al. 1997). These cells mainly function to assist in the production of connective tissue fibers and extracellular matrix components (Benirschke, Kaufmann et al. 2006e).

The free connective tissue cells are fetally-derived tissue macrophages known as Hofbauer cells. These cells are large vacuolated cells with granulated cytoplasm that are phagocytic and thought to play a role in immunological reactions as they express major histocompatibility (MHC) I and II antigens (Castellucci and Kaufmann 1982; Benirschke, Kaufmann et al. 2006e).

The fetal endothelium consists of mesenchymal-derived endothelial cells that line fetal blood vessels and later in pregnancy form a monolayer on the basal lamina (Demir, Kaufmann et al. 1989). In early gestation small vessels form within the stroma, but by term the endothelial cells are connected to each other by intercellular junctions thereby forming a continuous endothelium, located directly adjacent to the ST (Benirschke, Kaufmann et al. 2006e).

1.3.3 Pathogens and the Placenta

In pregnancy, the organ distribution of disease burden for some infections is dramatically shifted (Guilbert LJ 2006). Parasites, bacteria and viruses can be harbored in and around the placenta during infection of the maternal host. Some examples include *Plasmodium falciparum, Listeria monocytogenes*, and lymphocytic choriomeningitis virus in mice as well as guinea pig cytomegalovirus (gCMV).

The parasite *Plasmodium falciparum,* which causes malaria, parasitizes red blood cells and these are predominantly found in the intervillous space during pregnancy-associated malaria (Fried M 1996). A woman's peripheral blood can be free of malarial parasites, while the placenta has been demonstrated to incur parasite densities exceeding 50% of the total placental erythrocyte count (Flick K 2001). Parasitized red blood cells bind to a specific portion of a glycosaminoglycan, chondroitin sulphate A that is highly expressed on the ST lining. This tissue tropic binding is highly specific for chondroitin sulphate A and not seen in any other cell type (Alkhalil A 2000). As a consequence of the parasite

being sequestered in the placenta, the parasites avoid the clearance mechanisms of the spleen that are capable of recognizing and removing infected erythrocytes from circulation (Andrews KT 2002). Similarly, the intracellular bacteria *Listeria monocytogenes* continues to replicate in the placenta of mice even though it has been cleared from the maternal liver (Guleria I 2000). Furthermore, in the guinea pig, continuous placental infection with *Listeria monocytogenes* leads to continuous reinfection of the maternal organs until removal of the placenta (Bakardjiev AI 2006).

Persistent infection of the placenta has also been demonstrated with lymphocytic choriomeningitis virus in mice (Constantin, Masopust et al. 2007). This RNA virus was effectively removed from the maternal system through an active specific CD8 T-cell response and yet still remained present in the placenta (Constantin, Masopust et al. 2007). Another viral example is gCMV; guinea pigs were inoculated mid-gestation with gCMV and tested for gCMV infection at term by a plaque reduction neutralizing test for antibodies (Griffith, McCormick et al. 1985). The virus was found to persist in the placenta, even when cleared from maternal blood (Griffith, McCormick et al. 1985). It has been suggested that the guinea pig placenta acts as a viral reservoir for guinea pig CMV (Griffith, McCormick et al. 1985; Revello and Gerna 2002); here the virus is protected from circulating maternal antibodies and acts locally to maintain the infection throughout gestation (Goff, Griffith et al. 1987; Harrison and Myers 1990).

In humans, CMV specific lymphoproliferative response and cytokine production are not impaired in pregnant women when compared to non-pregnant subjects (Lilleri, Zelini et al. 2009). Therefore, if the placenta functions as a viral reservoir for pathogens in humans, it likely does so during a normal immune response.

1.3.4 Uterine and placental immune defense mechanisms

In all of the above listed interactions between pathogens and the placenta a normal immune response was established; despite this, parasites, bacteria and viruses still persist in and around the placenta. Medawar in 1953 suggested that

one potential explanation for this is that the placental surface is an immune privileged site (Medawar 1953; Trowsdale and Betz 2006). The placenta is immune privileged as it does not elicit an inflammatory immune response against the fetal derived antigens of paternal origin (Szekeres-Bartho 2002). There are multiple mechanisms hypothesized to underlie this tolerance, such as: the systemic and localized helper T cell (Th1, Th2) balance during pregnancy; the presence of regulatory T cells in the uterus (Tregs); and the unique expression of human leukocyte antigens by the placenta (Marzi, Vigano et al. 1996; Aluvihare, Kallikourdis et al. 2004; Hunt, Petroff et al. 2005).

The maternal immune system continues to be active during pregnancy; however, many components are altered to provide protection to the allogenic fetus (Hunt, Petroff et al. 2005). One component is helper T cells (Th), which function to signal the production of cytokines, and promote the activation of B cells and/or killer T cells. Th1 cells, which promote cell-mediated immunity by up-regulating IL-2, IL-6, interferon-γ (IFN- γ) and tumor necrosis factor-α (TNF-α), are expressed less during pregnancy when compared to their counterpart Th2 (Reinhard, Noll et al. 1998). It is also known that during pregnancy-associated complications, Th1 responses are up-regulated and Th2 are down-regulated, indicating a role for increased Th1 in pathological conditions (Saito, Umekage et al. 1999). It is well supported that Th2 cytokines, such as IL-4 and IL-10 are constitutively expressed at the maternal-fetal interface and enhance humoral immunity during pregnancy (Lin, Mosmann et al. 1993). Based on this altered balance of Th1/Th2 during pregnancy, there could be a dampened cell-mediated immunity that assists in immune tolerance of the placenta, whereas an otherwise normal cell-mediated response would normally produce an inflammatory response (Lin, Mosmann et al. 1993; Marzi, Vigano et al. 1996; Reinhard, Noll et al. 1998; Szekeres-Bartho 2002). In contrast, it has been reported that in cases of recurrent abortion, Th2 cytokines are up-regulated (Bates, Quenby et al. 2002). Both Th1 and Th2 cell responses have been demonstrated as altered within normal and pathological conditions during pregnancy (Matthiesen, Ekerfelt et al. 1998;

Reinhard, Noll et al. 1998; Bates, Quenby et al. 2002). Th2 dominance during pregnancy is controversial in being a sole paradigm that regulates immune tolerance during pregnancy (Chaouat 2007). However, in response to pathogens such as, *Listeria monocytogenes*, *Plasmodium falciparum*, and HIV, the Th1/Th2 shift is still used to identify the severity of disease (Krishnan, Guilbert et al. 1996; Chaouat 2007).

Another factor important to the immune privilege of the maternal-fetal barrier is the generation and expansion of regulatory T cells (Tregs) during pregnancy (Aluvihare, Kallikourdis et al. 2004). Tregs are normally involved in preventing autoimmunity and tolerating allogenic organ grafts by suppressing non-self T-cell responses (Waldmann, Graca et al. 2004). During pregnancy the amount of circulating Tregs increases (Somerset, Zheng et al. 2004) and a reduction in circulating Tregs has been associated with repeat miscarriages (Arruvito, Sanz et al. 2007). There is also an accumulation of Tregs in the uterus during pregnancy where they express a highly suppressive phenotype (Somerset, Zheng et al. 2004; Kallikourdis, Andersen et al. 2007). It has been suggested that Tregs assist at the maternal-fetal interface by producing several protective factors such as: IL-10, (an anti-inflammatory cytokine) transforming growth factor-β, (suspected to block the activation of lymphocytes) and heme oxygenase (which helps to mediate oxidative stress) (Waldmann, Graca et al. 2004).

A major component of the localized placental immune response is the unique, non-classical, non-polymorphic MHC molecules, including human leukocyte antigen C, E and G (HLA-C, HLA-E and HLA-G) expressed by the EVT (Le Bouteiller and Lenfant 1996; Hunt, Petroff et al. 2005). Normally, MHC molecules are necessary for stimulation of adaptive and or innate immune responses. In pregnancy, HLA-G has been implicated as a protective factor as it is strongly expressed in the placenta and has been shown to program cells into immunosuppressive phenotypes by inhibiting maternal immune responses to foreign antigens, particularly paternal antigens (Hunt, Petroff et al. 2005). While the villous trophoblast does not express any class I or class II MHC antigens it

does have high amounts of glycosaminoglycans associated with the microvilli, which potentially prevent the adherence of maternal T cells (Sunderland, Naiem et al. 1981; Jones and Fox 1991). This provides another mechanism by which the placenta is functioning as an immune privileged site.

1.4 Human Cytomegalovirus Infection and Pregnancy

1.4.1 Incidence

HCMV is the most common cause of congenital infection in North America (Demmler 1991). The rate of congenital cytomegalovirus (CMV) occurring in approximately 1% of all live births (Alford, Stagno et al. 1990; Boppana, Rivera et al. 2001) may be an underestimate as CMV is also found at a rate of 2.5% in the amniotic fluid of pregnancies terminated due to congenital anomalies, as compared to 0% for other viruses (adenoviruses and enteroviruses) (Petrikovsky, Lipson et al. 2003). HCMV vertical transmission occurs in approximately 30-40% of mothers with a primary infection (Raynor 1993; Kenneson and Cannon 2007). With reactivation from latency or reinfection with another strain, the risk of transmission to the fetus is up to 2% (Raynor 1993; Kenneson and Cannon 2007); however, this is not insignificant as HCMV is endemic in the population and over 60% neonates infected *in utero* are born to mothers with some kind of preconceptional immunity to the virus (Demmler 1991; Ornoy and Diav-Citrin 2006).

HCMV-positive infants born to mothers who were seropositive prior to pregnancy are typically born asymptomatic (Ornoy and Diav-Citrin 2006). Of these infants, 10-15% will go on to develop sensorineural hearing loss (SHL) during their first 6 years of life (Fowler, McCollister et al. 1997; Grosse, Dollard et al. 2009). This progressive development of hearing loss has been noted by Fowler *et al.* who reported that in a cohort of 338 congenitally HCMV-infected children SHL was found in 5.2% at birth, an additional 3.2% at 12 months and 15.4% of the total cohort had SHL by 6 years (Fowler, McCollister et al. 1997). Since most children born with congenital HCMV infection have no physical

abnormalities that suggest infection, neonatal screening of hearing is often missed because there are no clinical indications (Fowler, Dahle et al. 1999).

Infants with symptomatic congenital HCMV are often subject to severe neonatal morbidity with approximately 10% dying within the newborn period (Arav-Boger and Pass 2002). Clinical symptoms of symptomatic congenital CMV-infected neonates include: intrauterine growth restriction, hepatosplenomegaly microcephaly, thrombocytopenic purpura, and central nervous system abnormalities (Paixao, Almeida et al. 2005; Kylat, Kelly et al. 2006; Ornoy and Diav-Citrin 2006). In symptomatic congenital HCMV-infected infants, Kimberlin *et al.*, prevented hearing deterioration by treating newborn symptomatic CMV-infected infants with intravenous ganciclovir therapy (Kimberlin, Lin et al. 2003). This study indicates a possible treatment for HCMV disease involving the central nervous system if detected in a newborn (Kimberlin, Lin et al. 2003).

1.4.2 Systemic Immune Response to CMV in pregnancy

The engagement of the innate immune system is an occurrence during normal childbearing as well as in manifested inflammatory states of labor (Kim, Romero et al. 2005). Generally, intrauterine infections are associated with the exaggerated expression of the interleukin cytokines: IL-1, IL-6 and IL-8 (Chou, Ma et al. 2006; Kovacs, Hegedus et al. 2007). One function of IL-8 cytokines is that high expression within the ST may enhance productive infection of HCMV in the placenta (Kovacs, Hegedus et al. 2007). The increased accumulation of gene expression, stimulated by HCMV infection of the placenta, assists in the direct pathology of the virus.

1.4.3 Viral load during pregnancy

During pregnancy, there is no correlation between maternal viremia and the clinical course of infection (Revello, Zavattoni et al. 1998). This is in contrast to other at risk groups, as viremia is often used as a clinical tool for predicting patient outcomes (Pang, Fox et al. 2009). Furthermore, viral load in blood

(Revello, Zavattoni et al. 1998; Lazzarotto, Gabrielli et al. 2004) or urine (Stagno 2001) does not correlate with intrauterine transmission which occurs even when virus in these compartments is low or undetectable. During pregnancy, once a congenital infection is confirmed, predictors of fetal disease do exist (Arav-Boger and Pass 2002). For example, a high viral load in the amniotic fluid correlates to a more severe outcome for the neonate (Lazzarotto, Varani et al. 2000).

1.4.4 Routes of vertical transmission

Primary infections, reactivation or reinfection with another strain during pregnancy are risk factors for intrauterine HCMV transmission to the fetus (Fowler, Stagno et al. 1992). Intrauterine viral infections can ascend from the genital tract or spread through the hematogenous route, ultimately leading to congenital infection (Pereira, Maidji et al. 2005).

An ascending infection occurs when pathogens invade the lower genital tract of a pregnant women and move into the amniotic sac, resulting in what is known as aminotic sac infection syndrome (Pereira, Maidji et al. 2005; Benirschke, Kaufmann et al. 2006d). This syndrome begins with maternal leukocyte infiltration of the extraplacental membranes followed by fetal leukocyte involvement after 20 weeks, and causes severe chorioamnionitis (inflammation of the membranes) (Benirschke, Kaufmann et al. 2006d). In this route of transmission, the pathogen must physically bypass the intact membranes and then evade the antibacterial activity of the amniotic fluid (Schlievert, Johnson et al. 1977; Benirschke, Kaufmann et al. 2006d). There is some evidence demonstrating high rates of bacterial vaginosis in relation to an ascending HCMV infection. However, HCMV was not directly identified in the inflamed placental membranes or amniotic fluid (Coonrod, Collier et al. 1998).

HCMV may also be transmitted from maternal blood through the villous placenta to the fetus. This hematogenous transmission often manifests as chronic villitis (inflammatory lesions within the villous) and the villitis is associated with HCMV PCR-positive placental tissues (Syridou, Spanakis et al. 2008). There are many routes by which HCMV could pass through the placenta to the fetus from

maternal blood. One identified route of infection of HCMV is CT infection, void of ST infection, in sections of anchoring and floating first trimester chorionic villi (Fisher, Genbacev et al. 2000). Therefore, a fetal infection results from cell-to-cell infection of invasive CTs by infected uterine cells leading to infection of the anchoring chorionic villi (Fisher, Genbacev et al. 2000). Another possible route for CMV is via IgG transport across the placenta (Maidji, McDonagh et al. 2006). The FcRN, or neonatal Fc receptor functions to transport maternal antibodies across the placenta (Maidji, McDonagh et al. 2006). It has been demonstrated that IgG-CMV virion complexes with low neutralizing activity can be internalized by the placenta and lead to a productive CT infection (Maidji, McDonagh et al. 2006). This infection could spread to stromal fibroblasts, blood vessels in the villus core and leukocytes in the fetal bloodstream, and result in a fetal infection (Maidji, McDonagh et al. 2006). Lastly, HCMV could pass through the placenta via direct infection of the ST, or through physical breaks caused by damage to the ST.

1.4.5 The villous trophoblast and cytomegalovirus

We and others have previously shown that villous trophoblasts can be productively infected in culture (Halwachs-Baumann, Wilders-Truschnig et al. 1998; Hemmings, Kilani et al. 1998; Maidji, Percivalle et al. 2002). It has been demonstrated by the presence of RNA transcripts for viral proteins in each trimester and in all cell types that placental infection precedes fetal infection (Trincado, Munro et al. 2005; McDonagh, Maidji et al. 2006). However, progeny virus is mostly retained by these cells with <30% released apically (maternal side) and <1% released basally (fetal side) (Hemmings and Guilbert 2002). It is therefore unlikely that an ST infection leads directly to a fetal infection.

Accumulation in or around the ST would increase the accessibility of HCMV to fetal tissues. It has been shown that the interaction of HCMV with innate immune co-receptors CD14 and TLR2, on the surface of cultured ST cells, increases TNF-α expression (Chan, Hemmings et al. 2002; Chaudhuri, Lowen et al. 2009). Chan *et al.* used an *in vitro* model to show that HCMV infection of STs

induces TNF-α mediated apoptosis in neighbouring uninfected cells (Chan, Hemmings et al. 2002). The increased apoptosis accelerates trophoblast turnover, thereby decreasing the ST layer's capacity for renewal (Chan, Hemmings et al. 2002). This could lead to physical breaks in the ST layer thereby creating a route by which HCMV could directly cross the maternal-fetal interface.

1.4.6 Trophoblast Receptors for HCMV

Trophoblasts are epithelial cells that can be permissively infected by HCMV (Halwachs-Baumann, Wilders-Truschnig et al. 1998; Hemmings, Kilani et al. 1998; Hemmings DG 1998)). The initial tethering of HCMV to most cell types is to the ubiquitously expressed heparan sulfate proteoglycans (HSPGs), and is followed by stronger binding to cell surface receptors that allow viral entry (Compton, Nowlin et al. 1993; Feire, Koss et al. 2004). It has previously been shown that HSPGs are a vital first step in HCMV infection in fibroblasts (Compton, Nowlin et al. 1993). The placenta expresses all three major types of HSPGs: syndecans, glypicans and perlecans (Kirn-Safran, D'Souza et al. 2008). However, only certain syndecans and glypicans are reported to be expressed by the ST (Crescimanno, Marzioni et al. 1999). While expressed in the ST, most syndecans and glypicans are also expressed elsewhere in the utero-placental unit throughout gestation. However, Syndecan-1 (Syn-1) is the only HSPG to be constitutively expressed only in the ST throughout pregnancy (Jokimaa V 1998; Crescimanno, Marzioni et al. 1999). The stronger, secondary binding to cell surface receptors on the trophoblast is yet to be characterized. As mentioned above, EGFR and integrins are possible candidates (Compton 2004). Both EGFR and integrins are expressed in the ST and CT in early and term pregnancy (Bulmer, Thrower et al. 1989; Burrows, King et al. 1993).

UV-inactivated HCMV can activate an innate immune response which indicates that the mere cell contact by HCMV causes the expression of cellular genes such as inflammatory cytokines and interferons (Compton, Kurt-Jones et al. 2003). Placental recognition of pathogens is also receptor mediated via TLRs (Abrahams, Visintin et al. 2005). TLRs have a major role in the interaction

between self and non-self of the immune system (Finberg, Wang et al. 2007). TLR-2 is expressed by the trophoblast and is a cell surface, transmembrane protein that functions as a co-receptor for HCMV through means of another pattern recognition protein, CD14 (Finberg, Wang et al. 2007; Rindsjo, Holmlund et al. 2007). Therefore, co-receptors TLR-2 and CD14 play a central role in triggering inflammatory cytokine production in fibroblasts via the TLR2-dependent activation of NF-κB (Compton 2004).

1.5 References

Abrahams, V. M., I. Visintin, et al. (2005). "A role for TLRs in the regulation of immune cell migration by first trimester trophoblast cells." J Immunol **175**(12): 8096-8104.

Abumaree, M. H., P. R. Stone, et al. (2006). "An in vitro model of human placental trophoblast deportation/shedding." Mol Hum Reprod **12**(11): 687-694.

Adler, S. P. (1985). "The molecular epidemiology of cytomegalovirus transmission among children attending a day care center." J Infect Dis **152**(4): 760-768.

Alford, C. A., S. Stagno, et al. (1990). "Congenital and perinatal cytomegalovirus infections." Rev Infect Dis **12 Suppl 7**: S745-753.

Alkhalil A, A. R., Valiyaveettil M, Ockenhouse CF, Gowda DC (2000). "Structural requirements for the adherence of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulphate proteoglycans of human placenta." Journal of Biological chemistry **275**(51): 40357-40364.

Aluvihare, V. R., M. Kallikourdis, et al. (2004). "Regulatory T cells mediate maternal tolerance to the fetus." Nat Immunol **5**(3): 266-271.

Andrews KT, L. M. (2002). "Maternal malaria: *Plasmodium falciparum* sequestration in the placenta." Parasitology **88**(715-723).

Aplin, J. D. (1991). "Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro." J Cell Sci **99 (Pt 4)**: 681-692.

Aplin, J. D. (2000). "The cell biological basis of human implantation." Baillieres Best Pract Res Clin Obstet Gynaecol **14**(5): 757-764.

Arav-Boger, R. and R. F. Pass (2002). "Diagnosis and management of cytomegalovirus infection in the newborn." Pediatr Ann **31**(11): 719-725.

Arruvito, L., M. Sanz, et al. (2007). "Expansion of CD4+CD25+and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction." J Immunol **178**(4): 2572-2578.

Aufderheide, E. and P. Ekblom (1988). "Tenascin during gut development: appearance in the mesenchyme, shift in molecular forms, and dependence on epithelial-mesenchymal interactions." J Cell Biol **107**(6 Pt 1): 2341-2349.

Bakardjiev AI, T. J., Portnoy DA (2006). "Listeria monocytogenes traffics from maternal organs to the placenta and back." PLos Pathog **2**(6): e66.

Bates, M. D., S. Quenby, et al. (2002). "Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss?" Hum Reprod **17**(9): 2439-2444.

Benirschke, K., P. Kaufmann, et al. (2006b). Early development of the human placenta. Pathology of the Human Placenta. K. Benirschke, P. Kaufmann and R. Baergen. New York, New York, Springer Verlag**:** 42-49.

Benirschke, K., P. Kaufmann, et al. (2006c). Placental Types. Pathology of the Human Placenta. K. Benirschke, P. Kaufmann and R. Baergen. New York, New York, Springer Verlag**:** 30-40.

Benirschke, K., P. Kaufmann, et al. (2006d). Infectious diseases. Pathology of the Human Placenta. K. Benirschke, P. Kaufmann and R. Baergen. New York, New York, Springer Verlag**:** 657-732.

Benirschke, K., P. Kaufmann, et al. (2006e). Basic Structure of Villous Trees. Pathology of the Human Placenta. K. Benirschke, P. Kaufmann and R. Baergen. New York, New York, Springer Verlag**:** 50-120.

Boehme, K. W., M. Guerrero, et al. (2006). "Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells." J Immunol **177**(10): 7094-7102.

Boppana, S. B., L. B. Rivera, et al. (2001). "Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity." N Engl J Med **344**(18): 1366-1371.

Bowen, E. F., C. A. Sabin, et al. (1997). "Cytomegalovirus (CMV) viraemia detected by polymerase chain reaction identifies a group of HIV-positive patients at high risk of CMV disease." AIDS **11**(7): 889-893.

Boyd, J. and W. Hamilton (1970). The Human Placenta. Cambridge, Heffer.

Browne, E. P., B. Wing, et al. (2001). "Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs." J Virol **75**(24): 12319-12330.

Bulmer, J. N., S. Thrower, et al. (1989). "Expression of epidermal growth factor receptor and transferrin receptor by human trophoblast populations." Am J Reprod Immunol **21**(3-4): 87-93.

Burrows, T. D., A. King, et al. (1993). "Expression of integrins by human trophoblast and differential adhesion to laminin or fibronectin." Hum Reprod **8**(3): 475-484.

Burton, G. J. (1987). "The fine structure of the human placental villus as revealed by scanning electron microscopy." Scanning Microsc **1**(4): 1811-1828.

Cannon, M. J., D. S. Schmid, et al. (2010). "Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection." Rev Med Virol **20**(4): 202-213.

Cartwright, J. E. and M. Wareing (2006). "An in vitro model of trophoblast invasion of spiral arteries." Methods Mol Med **122**: 59-74.

Castellucci, M. and P. Kaufmann (1982). "Evolution of the stroma in human chorionic villi throughout pregnancy." Bibl Anat(22): 40-45.

Castellucci, M. and P. Kaufmann (1982). "A three-dimensional study of the normal human placental villous core: II. Stromal architecture." Placenta **3**(3): 269-285.

Castellucci, M., M. Scheper, et al. (1990). "The development of the human placental villous tree." Anat Embryol (Berl) **181**(2): 117-128.

Cha TA, T. E., Kemble et al (1996). "Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains." Journal of Virology **70**(1): 78-83.

Chan, G., D. G. Hemmings, et al. (2002). "Human cytomegalovirus-caused damage to placental trophoblasts mediated by immediate-early gene-induced tumor necrosis factor-alpha." Am J Pathol **161**(4): 1371-1381.

Chaouat, G. (2007). "The Th1/Th2 paradigm: still important in pregnancy?" Semin Immunopathol **29**(2): 95-113.

Chaudhuri, S., B. Lowen, et al. (2009). "Human cytomegalovirus interacts with toll-like receptor 2 and CD14 on syncytiotrophoblasts to stimulate expression of TNFalpha mRNA and apoptosis." Placenta **30**(11): 994-1001.

Cheung, T. W. and S. A. Teich (1999). "Cytomegalovirus infection in patients with HIV infection." Mt Sinai J Med **66**(2): 113-124.

Chou, D., Y. Ma, et al. (2006). "Cytomegalovirus infection of trophoblast cells elicits an inflammatory response: a possible mechanism of placental dysfunction." Am J Obstet Gynecol **194**(2): 535-541.

Compton, T. (2004). "Receptors and immune sensors: the complex entry path of human cytomegalovirus." Trends in Cell Biology **14**(1): 5-8.

Compton, T., E. A. Kurt-Jones, et al. (2003). "Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2." J Virol **77**(8): 4588-4596.

Compton, T., D. M. Nowlin, et al. (1993). "Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate." Virology **193**(2): 834-841.

Constantin, C. M., D. Masopust, et al. (2007). "Normal establishment of virus-specific memory CD8 T cell pool following primary infection during pregnancy." J Immunol **179**(7): 4383-4389.

Coonrod, D., A. C. Collier, et al. (1998). "Association between cytomegalovirus seroconversion and upper genital tract infection among women attending a sexually transmitted disease clinic: a prospective study." J Infect Dis **177**(5): 1188-1193.

Crescimanno, C., D. Marzioni, et al. (1999). "Expression pattern alterations of syndecans and glypican-1 in normal and pathological trophoblast." J Pathol **189**(4): 600-608.

de la Hoz, R. E., G. Stephens, et al. (2002). "Diagnosis and treatment approaches of CMV infections in adult patients." J Clin Virol **25 Suppl 2**: S1-12.

DeMeritt, I. B., L. E. Milford, et al. (2004). "Activation of the NF-kappaB pathway in human cytomegalovirus-infected cells is necessary for efficient transactivation of the major immediate-early promoter." J Virol **78**(9): 4498-4507.

Demir, R., P. Kaufmann, et al. (1989). "Fetal vasculogenesis and angiogenesis in human placental villi." Acta Anat (Basel) **136**(3): 190-203.

Demir, R., G. Kosanke, et al. (1997). "Classification of human placental stem villi: review of structural and functional aspects." Microsc Res Tech **38**(1-2): 29-41.

Demmler, G. J. (1991). "Infectious Diseases Society of America and Centers for Disease Control. Summary of a workshop on surveillance for congenital cytomegalovirus disease." Rev Infect Dis **13**(2): 315-329.

Dollard, S. C. and M. R. Schleiss (2010). "Screening newborns for congenital cytomegalovirus infection." JAMA **304**(4): 407-408; author reply 408.

Dworsky, M., M. Yow, et al. (1983). "Cytomegalovirus infection of breast milk and transmission in infancy." Pediatrics **72**(3): 295-299.

Enders, A. C. and B. F. King (1988). "Formation and differentiation of extraembryonic mesoderm in the rhesus monkey." Am J Anat **181**(4): 327-340.

Esclatine, A., A. Bellon, et al. (2001). "Differentiation-dependent redistribution of heparan sulfate in epithelial intestinal Caco-2 cells leads to basolateral entry of cytomegalovirus." Virology **289**(1): 23-33.

Falagas, M. E., D. R. Snydman, et al. (1996). "Exposure to cytomegalovirus from the donated organ is a risk factor for bacteremia in orthotopic liver transplant recipients. Boston Center for Liver Transplantation CMVIG Study Group." Clin Infect Dis **23**(3): 468-474.

Feire, A. L., H. Koss, et al. (2004). "Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain." Proc Natl Acad Sci U S A **101**(43): 15470-15475.

Finberg, R. W. and E. A. Kurt-Jones (2007). "Tolls: you pay them on the way in and on the way out!" J Infect Dis **196**(4): 497-498.

Finberg, R. W., J. P. Wang, et al. (2007). "Toll like receptors and viruses." Rev Med Virol **17**(1): 35-43.

Fisher, S., O. Genbacev, et al. (2000). "Human cytomegalovirus infection of placental cytotrophoblasts in vitro and in utero: implications for transmission and pathogenesis." J Virol **74**(15): 6808-6820.

Flick K, S. C., Chen Q, Fernandez V, Pouvelle B, Gysin J, Wahlgren M (2001). "Role of nonimmune IgG bound to PfEMP1 in placental malaria." Science **293**: 2098-2100.

Fortunato, E. A. and D. H. Spector (1999). "Regulation of human cytomegalovirus gene expression." Adv Virus Res **54**: 61-128.

Fowler, K. B., A. J. Dahle, et al. (1999). "Newborn hearing screening: will children with hearing loss caused by congenital cytomegalovirus infection be missed?" J Pediatr **135**(1): 60-64.

Fowler, K. B., F. P. McCollister, et al. (1997). "Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection." J Pediatr **130**(4): 624-630.

Fowler, K. B., S. Stagno, et al. (1992). "The outcome of congenital cytomegalovirus infection in relation to maternal antibody status." N Engl J Med **326**(10): 663-667.

Fried M, D. P. (1996). "Plasmodium falciparum to chondroitin sulphate A in the human placenta." Science **272**(5267): 1502-1504.

Gandhi, M. K. and R. Khanna (2004). "Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments." Lancet Infect Dis **4**(12): 725-738.

George, M. J., D. R. Snydman, et al. (1997). "The independent role of cytomegalovirus as a risk factor for invasive fungal disease in orthotopic liver transplant recipients. Boston Center for Liver Transplantation CMVIG-Study Group. Cytogam, MedImmune, Inc. Gaithersburg, Maryland." Am J Med **103**(2): 106-113.

Goff, E., B. P. Griffith, et al. (1987). "Delayed amplification of cytomegalovirus infection in the placenta and maternal tissues during late gestation." Am J Obstet Gynecol **156**(5): 1265-1270.

Griffith, B. P., S. R. McCormick, et al. (1985). "The placenta as a site of cytomegalovirus infection in guinea pigs." J Virol **55**(2): 402-409.

Grosse, S. D., S. Dollard, et al. (2009). "Newborn screening for congenital cytomegalovirus: Options for hospital-based and public health programs." J Clin Virol **46 Suppl 4**: S32-36.

Guilbert LJ, H. D. (2006). Pregnancy related changes to maternal host defences Inflammation and Pregnancy. M. L. Peebles DM, Informa Healthcare.

Guleria I, P. J. (2000). "The trophoblast is a component of the innate immune system during pregnancy." Nat Med **6**(5): 589-593.

Hagay, Z. J., G. Biran, et al. (1996). "Congenital cytomegalovirus infection: a long-standing problem still seeking a solution." Am J Obstet Gynecol **174**(1 Pt 1): 241-245.

Halwachs-Baumann, G., M. Wilders-Truschnig, et al. (1998). "Human trophoblast cells are permissive to the complete replicative cycle of human cytomegalovirus." J Virol **72**(9): 7598-7602.

Handsfield, H. H., S. H. Chandler, et al. (1985). "Cytomegalovirus infection in sex partners: evidence for sexual transmission." J Infect Dis **151**(2): 344-348.

Hansen, K. K., A. Ricksten, et al. (1994). "Detection of cytomegalovirus DNA in serum correlates with clinical cytomegalovirus retinitis in AIDS." J Infect Dis **170**(5): 1271-1274.

Harrison, C. J. and M. G. Myers (1990). "Relation of maternal CMV viremia and antibody response to the rate of congenital infection and intrauterine growth retardation." J Med Virol **31**(3): 222-228.

Hemmings, D. G. and L. J. Guilbert (2002). "Polarized release of human cytomegalovirus from placental trophoblasts." J Virol **76**(13): 6710-6717.

Hemmings, D. G., R. Kilani, et al. (1998). "Permissive cytomegalovirus infection of primary villous term and first trimester trophoblasts." J Virol **72**(6): 4970-4979.

Hemmings DG, K. R., Nykiforuk C, Preiksaitis J, Guilbert LJ (1998). "Permissive cytomegalovirus infection of primary villous term and first trimester trophoblasts." Journal of Virology **72**(6): 4970-4979.

Humar, A., D. Gregson, et al. (1999). "Clinical utility of quantitative cytomegalovirus viral load determination for predicting cytomegalovirus disease in liver transplant recipients." Transplantation **68**(9): 1305-1311.

Hummel, M. and M. M. Abecassis (2002). "A model for reactivation of CMV from latency." J Clin Virol **25 Suppl 2**: S123-136.

Hunt, J. S., M. G. Petroff, et al. (2005). "HLA-G and immune tolerance in pregnancy." FASEB J **19**(7): 681-693.

Huppertz, B., J. Kingdom, et al. (2003). "Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation." Placenta **24**(2-3): 181-190.

Huppertz, B. and J. C. Kingdom (2004). "Apoptosis in the trophoblast--role of apoptosis in placental morphogenesis." J Soc Gynecol Investig **11**(6): 353-362.

Isaacson, M. K., L. K. Juckem, et al. (2008). "Virus entry and innate immune activation." Curr Top Microbiol Immunol **325**: 85-100.

Jokimaa V, I. P., Kujari H, Hirvoren O, Ekholm E, Antila L (1998). "Expression of Syndecan-1 in human placenta and decidua." Placenta **19**(2-3): 157-163.

Jones, C. J. and H. Fox (1991). "Ultrastructure of the normal human placenta." Electron Microsc Rev **4**(1): 129-178.

Juckem, L. K., K. W. Boehme, et al. (2008). "Differential initiation of innate immune responses induced by human cytomegalovirus entry into fibroblast cells." J Immunol **180**(7): 4965-4977.

Kallikourdis, M., K. G. Andersen, et al. (2007). "Alloantigen-enhanced accumulation of CCR5+ 'effector' regulatory T cells in the gravid uterus." Proc Natl Acad Sci U S A **104**(2): 594-599.

Kasznica, J. M. and E. B. Petcu (2003). "Placental calcium pump: clinical-based evidence." Pediatr Pathol Mol Med **22**(3): 223-227.

Kenneson, A. and M. J. Cannon (2007). "Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection." Rev Med Virol **17**(4): 253-276.

Kim, Y. M., R. Romero, et al. (2005). "Toll-like receptor 4: a potential link between "danger signals," the innate immune system, and preeclampsia?" Am J Obstet Gynecol **193**(3 Pt 2): 921-927.

Kimberlin, D. W., C. Y. Lin, et al. (2003). "Effect of ganciclovir therapy on hearing in symptomatic congenital cytomegalovirus disease involving the central nervous system: a randomized, controlled trial." J Pediatr **143**(1): 16-25.

Kirn-Safran, C., S. S. D'Souza, et al. (2008). "Heparan Sulfate Proteoglycans and Their Binding Proteins in Embryo Implantation and Placentation." Semin Cell Dev Biol. **19**(2): 187–193.

Knipe, D. and P. Howley, Eds. (2007). Pathogenesis of viral infection. Fields Virology. Philadelphia, PA, Lippincott Williams & Wilkins.

Kohnen, G., S. Kertschanska, et al. (1996). "Placental villous stroma as a model system for myofibroblast differentiation." Histochem Cell Biol **105**(6): 415-429.

Kovacs, I. J., K. Hegedus, et al. (2007). "Production of proinflammatory cytokines by syncytiotrophoblasts infected with human cytomegalovirus isolates." Placenta **28**(7): 620-623.

Krishnan, L., L. J. Guilbert, et al. (1996). "Pregnancy impairs resistance of C57BL/6 mice to Leishmania major infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines." J Immunol **156**(2): 644-652.

Kylat, R. I., E. N. Kelly, et al. (2006). "Clinical findings and adverse outcome in neonates with symptomatic congenital cytomegalovirus (SCCMV) infection." Eur J Pediatr **165**(11): 773-778.

Lash, G. E., K. Naruse, et al. (2010). "Secretion of angiogenic growth factors by villous cytotrophoblast and extravillous trophoblast in early human pregnancy." Placenta **31**(6): 545-548.

Lazzarotto, T., L. Gabrielli, et al. (2004). "Congenital cytomegalovirus infection: recent advances in the diagnosis of maternal infection." Hum Immunol **65**(5): 410-415.

Lazzarotto, T., S. Varani, et al. (2000). "Prenatal indicators of congenital cytomegalovirus infection." J Pediatr **137**(1): 90-95.

Le Bouteiller, P. and F. Lenfant (1996). "Antigen-presenting function(s) of the non-classical HLA-E, -F and -G class I molecules: the beginning of a story." Res Immunol **147**(5): 301-313.

Lilleri, D., P. Zelini, et al. (2009). "Human cytomegalovirus-specific CD4+ and CD8+ T cell responses in primary infection of the immunocompetent and the immunocompromised host." Clin Immunol **131**(3): 395-403.

Lin, H., T. R. Mosmann, et al. (1993). "Synthesis of T helper 2-type cytokines at the maternal-fetal interface." J Immunol **151**(9): 4562-4573.

Maidji, E., S. McDonagh, et al. (2006). "Maternal antibodies enhance or prevent cytomegalovirus infection in the placenta by neonatal Fc receptor-mediatedtranscytosis." Am J Pathol **168**(4): 1210-1226.

Maidji, E., E. Percivalle, et al. (2002). "Transmission of human cytomegalovirus from infected uterine microvascular endothelial cells to differentiating/invasive placental cytotrophoblasts." Virology **304**(1): 53-69.

Marzi, M., A. Vigano, et al. (1996). "Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy." Clin Exp Immunol **106**(1): 127-133.

Matthiesen, L., C. Ekerfelt, et al. (1998). "Increased numbers of circulating interferon-gamma- and interleukin-4-secreting cells during normal pregnancy." Am J Reprod Immunol **39**(6): 362-367.

Mayhew, T. M. and B. L. Barker (2001). "Villous trophoblast: morphometric perspectives on growth, differentiation, turnover and deposition of fibrin-type fibrinoid during gestation." Placenta **22**(7): 628-638.

McDonagh, S., E. Maidji, et al. (2006). "Patterns of human cytomegalovirus infection in term placentas: a preliminary analysis." J Clin Virol **35**(2): 210-215.

Medawar, P. (1953). "Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates." Symp Soc Exp Biol **7**: 320-338.

Mocarski, E. and C. Courcelle (2001). Cytomegaloviruses and their replication. Fields Virology. D. Knipe, P. Howley, D. Griffin and R. Lamb. Philadelphia, Lippincott Williams & Wilkins**:** 2629–2673.

Mocarski, E., T. Shenk, et al. (2006). Cytomegaloviruses. Fields Virology. D. Knipe. Philadelphia, Lippincott Williams and Wilkins**:** 2701-2772.

Mori, M., G. Ishikawa, et al. (2007). "The cytotrophoblast layer of human chorionic villi becomes thinner but maintains its structural integrity during gestation." Biol Reprod **76**(1): 164-172.

Morrish, D. W., J. Dakour, et al. (1998). "Functional regulation of human trophoblast differentiation." J Reprod Immunol **39**(1-2): 179-195.

Murphy, E., D. Yu, et al. (2003). "Coding potential of laboratory and clinical strains of human cytomegalovirus." Proc Natl Acad Sci U S A **100**(25): 14976-14981.

Nelson, D. M., E. C. Crouch, et al. (1990). "Trophoblast interaction with fibrin matrix. Epithelialization of perivillous fibrin deposits as a mechanism for villous repair in the human placenta." Am J Pathol **136**(4): 855-865.

Noyola, D. E., G. J. Demmler, et al. (2000). "Cytomegalovirus urinary excretion and long term outcome in children with congenital cytomegalovirus infection. Congenital CMV Longitudinal Study Group." Pediatr Infect Dis J **19**(6): 505-510.

Ornoy, A. and O. Diav-Citrin (2006). "Fetal effects of primary and secondary cytomegalovirus infection in pregnancy." Reprod Toxicol **21**(4): 399-409.

Paixao, P., S. Almeida, et al. (2005). "Diagnosis of congenital cytomegalovirus infection by detection of viral DNA in urine pools." J Virol Methods **128**(1-2): 1-5.

Pang, X. L., J. D. Fox, et al. (2009). "Interlaboratory comparison of cytomegalovirus viral load assays." Am J Transplant **9**(2): 258-268.

Peckham, C. S., C. Johnson, et al. (1987). "Early acquisition of cytomegalovirus infection." Arch Dis Child **62**(8): 780-785.

Pereira, L., E. Maidji, et al. (2005). "Insights into viral transmission at the uterine-placental interface." Trends Microbiol **13**(4): 164-174.

Petrikovsky, B. M., S. M. Lipson, et al. (2003). "Viral studies on amniotic fluid from fetuses with and without abnormalities detected by prenatal sonography." J Reprod Med **48**(4): 230-232.

Raynor, B. D. (1993). "Cytomegalovirus infection in pregnancy." Semin Perinatol **17**(6): 394-402.

Real, F. X., W. J. Rettig, et al. (1986). "Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation." Cancer Res **46**(9): 4726-4731.

Reinhard, G., A. Noll, et al. (1998). "Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes." Biochem Biophys Res Commun **245**(3): 933-938.

Revello, M. G. and G. Gerna (2002). "Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant." Clin Microbiol Rev **15**(4): 680-715.

Revello, M. G., M. Zavattoni, et al. (1998). "Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy." J Infect Dis **177**(5): 1170-1175.

Reynolds, D. W., S. Stagno, et al. (1973). "Maternal cytomegalovirus excretion and perinatal infection." N Engl J Med **289**(1): 1-5.

Rindsjo, E., U. Holmlund, et al. (2007). "Toll-like receptor-2 expression in normal and pathologic human placenta." Hum Pathol **38**(3): 468-473.

Roberts, L., N. J. Sebire, et al. (2000). "Histomorphological features of chorionic villi at 10-14 weeks of gestation in trisomic and chromosomally normal pregnancies." Placenta **21**(7): 678-683.

Rubin, R. H. (1990). "Impact of cytomegalovirus infection on organ transplant recipients." Rev Infect Dis **12 Suppl 7**: S754-766.

Ryckman, B. J., M. A. Jarvis, et al. (2006). "Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion." J Virol **80**(2): 710-722.

Ryckman, B. J., B. L. Rainish, et al. (2008). "Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates entry into epithelial and endothelial cells." J Virol **82**(1): 60-70.

Saito, S., H. Umekage, et al. (1999). "Increased T-helper-1-type immunity and decreased T-helper-2-type immunity in patients with preeclampsia." Am J Reprod Immunol **41**(5): 297-306.

Schlievert, P., W. Johnson, et al. (1977). "Amniotic fluid antibacterial mechanisms: newer concepts." Semin Perinatol **1**(1): 59-70.

Sinclair, J. and P. Sissons (2006). "Latency and reactivation of human cytomegalovirus." J Gen Virol **87**(Pt 7): 1763-1779.

Sinzger, C., M. Digel, et al. (2008). "Cytomegalovirus cell tropism." Curr Top Microbiol Immunol **325**: 63-83.

Sinzger, C., A. Grefte, et al. (1995). "Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues." J Gen Virol **76 (Pt 4)**: 741-750.

Sinzger, C., K. Schmidt, et al. (1999). "Modification of human cytomegalovirus tropism through propagation in vitro is associated with changes in the viral genome." J Gen Virol **80 (Pt 11)**: 2867-2877.

Smith, M. S., G. L. Bentz, et al. (2004). "Human cytomegalovirus induces monocyte differentiation and migration as a strategy for dissemination and persistence." J Virol **78**(9): 4444-4453.

Somerset, D. A., Y. Zheng, et al. (2004). "Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset." Immunology **112**(1): 38-43.

Stagno, S. (2001). Infectious diseases of the fetus and newborn infant. K. J. Remington JS. Philadelphia, Pa, W.B. Suanders Co.**:** 389-424.

Staras, S. A., S. C. Dollard, et al. (2006). "Seroprevalence of cytomegalovirus infection in the United States, 1988-1994." Clin Infect Dis **43**(9): 1143-1151.

Stover, C. T., D. K. Smith, et al. (2003). "Prevalence of and risk factors for viral infections among human immunodeficiency virus (HIV)-infected and high-risk HIV-uninfected women." J Infect Dis **187**(9): 1388-1396.

Stowe, R. P., S. K. Mehta, et al. (2001). "Immune responses and latent herpesvirus reactivation in spaceflight." Aviat Space Environ Med **72**(10): 884-891.

Sunderland, C. A., M. Naiem, et al. (1981). "The expression of major histocompatibility antigens by human chorionic villi." J Reprod Immunol **3**(6): 323-331.

Syridou, G., N. Spanakis, et al. (2008). "Detection of cytomegalovirus, parvovirus B19 and herpes simplex viruses in cases of intrauterine fetal death: association with pathological findings." J Med Virol **80**(10): 1776-1782.

Szekeres-Bartho, J. (2002). "Immunological relationship between the mother and the fetus." Int Rev Immunol **21**(6): 471-495.

Tarrade, A., R. Lai Kuen, et al. (2001). "Characterization of human villous and extravillous trophoblasts isolated from first trimester placenta." Lab Invest **81**(9): 1199-1211.

Teasdale, F. and G. Jean-Jacques (1986). "Morphometry of the microvillous membrane of the human placenta in maternal diabetes mellitus." Placenta **7**(1): 81-88.

Trincado, D. E., S. C. Munro, et al. (2005). "Highly sensitive detection and localization of maternally acquired human cytomegalovirus in placental tissue by in situ polymerase chain reaction." J Infect Dis **192**(4): 650-657.

Waldmann, H., L. Graca, et al. (2004). "Regulatory T cells and organ transplantation." Semin Immunol **16**(2): 119-126.

Wang, D. and T. Shenk (2005). "Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism." Proc Natl Acad Sci U S A **102**(50): 18153-18158.

Wang, X., S. M. Huong, et al. (2003). "Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus." Nature **424**(6947): 456-461.

Chapter 2
Rationale and Hypothesis

2.1 Rationale

HCMV is the leading cause of congenital infection, affecting up to 2% of the newborn population. Congenital CMV infection can result in a broad range of symptoms including long-term neurological damage and hearing loss which often have a delayed onset. Caring for and supporting these children is costly. To reach the fetus, this blood-borne virus must cross the placenta. One area where this likely occurs is through the ST layer which separates maternal and fetal circulations. Defining the interactions of HCMV with ST will increase our understanding of viral transmission from mother to fetus and could ultimately give rise to successful prophylactic and therapeutic strategies, reduce high annual treatment costs and lead to healthier babies. Both *in vitro* studies of viral mechanisms and clinical studies relating to viral load of CMV in our population will assist in understanding the events that lead to intrauterine transmission.

2.1.1 *In vitro* studies

The placenta has been identified as a potential viral reservoir for HCMV during pregnancy (Griffith, McCormick et al. 1985; Revello and Gerna 2002). Little is known about how HCMV interacts with the villous trophoblast, specifically the ST layer which is in direct contact with maternal blood. Interestingly, term ST can be productively infected in culture (Halwachs-Baumann, Wilders-Truschnig et al. 1998; Hemmings, Kilani et al. 1998); however, progeny virus is mostly retained by these cells with <30% released apically (maternal side) and <1% released basally (fetal side) (Chan, Hemmings et al. 2002; Hemmings and Guilbert 2002). Given that the ST layer *in vivo* is likely much more efficient than a cell culture model, it is therefore unlikely that a ST infection leads directly to a fetal infection. However, progeny virus released into the local placental environment on the maternal side from either intact or

sloughed infected ST could interact back with the ST and accumulate. This concept is supported by a study showing that mouse CMV binds to fibroblasts in a stable but reversible manner leading to an equilibrium of bound and free virus (Hodgkin, Scalzo et al. 1988). A similar interaction with trophoblasts would suggest that virus generated during maternal viremia or virus released from infected ST could be sequestered and protected by the large surface area of the ST layer and increase the viral load in the placenta. I defined that reversible binding of HCMV occurred with ST and CT and further investigated, the nature of the receptors that the virus used for reversible binding. This information further increases our understanding of how HCMV interacts with the placenta.

2.1.2 Clinical Collections

A positive association of placental viral load to the incidence of newborn congenital infection *in vivo* would further support the concept of HCMV reversible binding to the ST, leading to accumulation in the placenta and protection from maternal immune responses. It is estimated that 50% of women in the Edmonton region will be seropositive for HCMV at the time of delivery (Dr. J. Preiksaitis, Dept. Medicine; University of Alberta). HCMV is transmitted *in utero* to up to 40% of fetuses from mothers who develop a primary infection during pregnancy (Stagno, Pass et al. 1986). While the most severe adverse neonatal outcomes are reported in this group, congenital infections are also found in approximately 1% of newborns from women with prepregnancy immunity (Ahlfors K 1999; Boppana, Rivera et al. 2001). Interestingly, viral load in maternal blood (Revello, Zavattoni et al. 1998; Lazzarotto, Gabrielli et al. 2004) or urine (Stagno 2001) does not correlate with intrauterine transmission. Furthermore, CMV infection, in guinea pigs, during pregnancy has been associated with an established viral load in the placenta while the virus is absent from maternal blood (Griffith BP 1985). This study examined the seroprevalence of HCMV, seroconversion during pregnancy, and compared viral load in maternal blood, maternal urine, placenta and neonatal saliva. This information gives

insight into HCMV prevalence in our population as well as viral distribution of HCMV during pregnancy and associated neonatal outcomes.

2.2 Hypothesis

My hypothesis is that HCMV reversibly binds to and is protected by the ST which acts as a viral reservoir. Furthermore, I hypothesize the virus is reversibly binding to heparan sulfate proteoglycans (HSPGs) on the ST cell surface. In addition, I hypothesize that viral load in the placenta will be higher than the viral load in maternal blood or urine and will correlate with the incidence of seroconversion, adverse pregnancy outcomes and HCMV-positive neonates.

2.3 References

Ahlfors K, I. S., Harris S (1999). "Report on a long-term study of maternal and congenital cytomegalovirus infection in Sweden. Review of prospective studies available in the literature." Scand J Infect Dis. **31**(5): 443-457.

Boppana, S. B., L. B. Rivera, et al. (2001). "Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity." N Engl J Med **344**(18): 1366-1371.

Chan, G., D. G. Hemmings, et al. (2002). "Human cytomegalovirus-caused damage to placental trophoblasts mediated by immediate-early gene-induced tumor necrosis factor-alpha." Am J Pathol **161**(4): 1371-1381.

Griffith, B. P., S. R. McCormick, et al. (1985). "The placenta as a site of cytomegalovirus infection in guinea pigs." J Virol **55**(2): 402-409.

Griffith BP, M. S., Fong CK, et al (1985). "The placenta as a site of cytomegalovirus infection in guinea pigs." J Virol **55**(2): 402-409.

Halwachs-Baumann, G., M. Wilders-Truschnig, et al. (1998). "Human trophoblast cells are permissive to the complete replicative cycle of human cytomegalovirus." J Virol **72**(9): 7598-7602.

Hemmings, D. G. and L. J. Guilbert (2002). "Polarized release of human cytomegalovirus from placental trophoblasts." J Virol **76**(13): 6710-6717.

Hemmings, D. G., R. Kilani, et al. (1998). "Permissive cytomegalovirus infection of primary villous term and first trimester trophoblasts." J Virol **72**(6): 4970-4979.

Hodgkin, P. D., A. A. Scalzo, et al. (1988). "Murine cytomegalovirus binds reversibly to mouse embryo fibroblasts: implications for quantitation and explanation of centrifugal enhancement." J Virol Methods **22**(2-3): 215-230.

Lazzarotto, T., L. Gabrielli, et al. (2004). "Congenital cytomegalovirus infection: recent advances in the diagnosis of maternal infection." Hum Immunol **65**(5): 410-415.

Revello, M. G. and G. Gerna (2002). "Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant." Clin Microbiol Rev **15**(4): 680-715.

Revello, M. G., M. Zavattoni, et al. (1998). "Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy." J Infect Dis **177**(5): 1170-1175.

Stagno, S. (2001). Infectious diseases of the fetus and newborn infant. K. J. Remington JS. Philadelphia, Pa, W.B. Suanders Co.**:** 389-424.

Stagno, S., R. F. Pass, et al. (1986). "Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome." Jama **256**(14): 1904-1908.

Chapter 3
Materials and Methods

3.1 Materials and Methods Specific for Chapter 4

3.1.1 Cell preparations and culture

3.1.1.1 Term villous cytotrophoblasts

Placentas were obtained with patient consent as approved by the Human Ethics Research Board at the University of Alberta and Alberta Health Services, after normal term delivery or elective cesarean section from uncomplicated pregnancies. Isolation of villous cytotrophoblasts (>99.99% pure) was by trypsin/DNase digestion of minced chorionic tissue and immunoabsorption onto Ig-coated glass bead columns (Yui, Garcia-Lloret et al. 1994). To begin, 50g pieces were cut from the cotyledons of the placenta and rinsed 2x with Iscove's Modified Dulbecco's Media (IMDM; GIBCO, Grand Island, NY) containing 2% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON). The pieces were transferred to a culture dish where large blood clots and the film from the maternal surface were removed. A dull scalpel was used to scrape tissue off of the underlying vasculature; the tissue was transferred to a sieve and rinsed, with IMDM containing 2% FBS, while being stirred with forceps. The rinsed tissue was transferred into 50 mL conical collection tubes, containing 50 g each (50 g≈50 mL); noting that 50 g of tissue will produce approximately 200-300 x 10^6 purified trophoblast cells.

Each 50 g of tissue was transferred into a 250 mL glass screw-top bottle and 100 mL of Locke Ringers (9.0g sodium chloride, 0.42g potassium chloride, 0.2g sodium bicarbonate, 1.5g glucose, in 1 L ddH_20) containing 300 mg of trypsin was prepared. Fifty mL of Locke Ringers with trypsin was added to each 250 mL bottle, gently swirled, and incubated for 7 minutes at 37^{o}C. The bottles were then removed from the incubator and the supernatant was carefully decanted

into the waste for each sample (repeat 1x). Next, 50 mL of Locke Ringers with trypsin was added to each bottle and mixed thoroughly by inverting the bottles several times. The bottles were again incubated for 7 minutes at 37^{o}C while being mixed (2-3 strong swirls) at approximately 5 and 3 minutes. The bottles were removed from the incubator and the supernatant from each bottle was decanted through gauze into a 50 mL conical tube containing 1.5 mL of 1X phosphate buffered saline (PBS) with 2% FBS. The filtered supernatant was then centrifuged for 7 minutes at 1500 rpm at 4^{o}C (model G5-6R centrifuge; Beckman Instruments Inc., Mississauga, ON, Canada). The tubes were removed from the centrifuge and the supernatant was aspirated down to the start of the conical portion of the tube, the tubes were knocked to dislodge and resuspend the pellet. Then, 5 mL of ice cold PBS with 2% FBS was added to each tube, incubated on ice and transferred to a stock tube. The above steps were repeated 4 times (5 iterations in total). Lysis buffer (2.075g ammonium chloride, 210mg sodium bicarbonate, 9.3mg disodium EDTA, in 250 mL dd$H_2$0) was then added to a final volume of 35-37 mL and mixed gently by inverting the tubes 2-3 times at room temperature (RT). The tubes were then incubated for 8 minutes at room temperature in a horizontal position and rocked gently every 2 minutes. The tubes were centrifuged and the supernatant was aspirated leaving only the pellet. The cells were resuspended in cold PBS with 2% FBS to a final volume of 35 mL and centrifuged. The supernatant was aspirated and the pellets were resuspended in 5 mL cold PBS with 2% FBS and pooled into one tube; cold PBS with 2% FBS was added to get a final volume of 40 mL. The tubes were then inverted (2-3 times) and 50 µl of cells were diluted in 450 µl PBS with 2% FBS for a hemacytometer cell count. For example, if the count was 50 x 10^4 /mL then:

1) 50 x 10^4 /mL = 0.5 x 10^6 (1:10 dilution) x 10 = 5 x 10^6 /ml
2) 5 x 10^6 /mL x (the total amount you sampled from; 40mL) = 200 x 10^6 cells total

Finally, the supernatant was centrifuged for 7 minutes at 1500 rpm at 4^{o} C and aspirated.

Cells were then purified by immuno-elimination with anti-CD9 (Clone 50H.19) (Yui, Garcia-Lloret et al. 1994), anti-MHC class I (W6/32, Harlan Sera-Lab, Crawley Down, Sussex, England) and anti-MHC class II (Clone 7H3) antibodies. The pellet was resuspended in 3 mL cold PBS containing 2% FBS with 10% normal goat serum and incubated for 20 minutes on ice. PBS with 2% FBS was then added for a total volume of 50 mL and the cells were centrifuged for 7 minutes at 1500 rpm at 4^{o} C. The supernatant was aspirated and 3 mL of primary antibody solution (PBS^{+} with 8 µl mouse antihuman α-MHC I, 12 µl α-MHC II, and 4 µl α-CD9 mouse antibodies) was then added. The antibody mixture was incubated for 45 minutes on ice; during this time Ig-coated glass bead columns were washed with 4-5 volumes of cold PBS with 2% FBS and the drip rate on the columns was set to ~1drop every 6 seconds. After the incubation PBS with 2% FBS was added to reach a final volume of 50 mL and the cells were centrifuged, aspirated, and the pellet was resuspended in cold PBS with 2% FBS to less than 100 x 10^{6} cells per 5 mL. The solution was then loaded onto the columns, while maintaining the drip rate of ~1 drop every 6 seconds. 15 mL from each column was collected and placed in 15 mL conical tubes, on ice. The contents of each tube were pooled to a total of 50 mL and 50 µl was diluted from that into 450 µl PBS with 2% FBS for a cell count. The 50 mL tube was then centrifuged for 7 minutes at 1500 rpm at 4^{o}C. Next, ice-cold calf serum + 10% DMSO were added to produce 1 x 10^{7} cells per mL. Lastly, the cells were aliquoted into cryotubes at 10^{7} (1mL), 5 x 10^{6} (500µl) and 2.5 x 10^{6} (250µl).

All trophoblast preparations contained fewer than five vimentin-positive cells (non-trophoblasts) per well in a 96-well culture plate (seeded with 1 x 10^{5} cells per well) as assessed by immunohistochemistry with vimentin antibody (Vim, Clone V9, Dako Corporation) therefore, yielding cultures of >99.99% cytotrophoblast (Appendix A, Figure 1) were produced and placed in $N_{2(l)}$ for long term storage.

Trophoblasts were isolated from five different placentas and cryopreserved for use in this study. Isolated trophoblasts cultured in IMDM

supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin in the absence of recombinant human epidermal growth factor (rhEGF; Pepro-Tech, Rocky Hill, NJ) or Br-cAMP (Sigma-Aldrich) are termed cytotrophoblasts (CT) (Appendix A, Figure 2).

3.1.1.2 Syncytiotrophoblast

The syncytiotrophoblast (ST) culture is defined as trophoblasts which were syncytialized by treatment with 10 ng/mL rhEGF for five days (Appendix A, Figure 2A) (Morrish, Dakour et al. 1997; Hemmings, Kilani et al. 1998) or the cell permeable cAMP analog Br-cAMP (Appendix A, Fig 2B) used at 10 mM for three days (Guilbert, Riddell et al. 2010).

Syncytialization was confirmed by using immunohistochemistry (see detailed method below) to visualize desmosome-containing tight junctions with anti-desmoplakin monoclonal antibody, (10 µg/mL) (Appendix A) (Sigma-Aldrich) as previously described (Douglas and King 1990).

3.1.1.3 HEL Fibroblasts

Human embryonic lung fibroblasts (HEL fibroblasts, a gift from Dr. J. Preiksaitis, Dept. Medicine; University of Alberta) are highly sensitive to HCMV infection and were used in two ways for this study. First, confluent cultures in T-75 flasks were used to propagate virus. Secondly, HEL fibroblasts were used as an indicator cell line to quantify infectious virus (viral titer) of stock virus and experimental virus released into supernatants or remaining cell adherent (Hemmings, Kilani et al. 1998). Lastly, they were used as a comparison to trophoblast cultures in the binding and release studies. HEL fibroblasts (4×10^4 cells/well) were plated in 100 µL Eagle's Minimum Essential Medium (MEM), supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin in 96-well tissue culture wells and cultured to confluency. All cultures were used within 48 hrs of plating.

3.1.1.4 CaCo2 epithelial cells

Human epithelial colorectal adenocarcinoma cells (CaCo2 cells; a gift from Dr. T. Hobman, Dept. Cell Biology; University of Alberta) represent another tight junctioned, microvilliated, epithelial cell type (Collett, Walker et al. 1997). They were used as a comparison to trophoblast cultures in the binding and release studies. CaCo2 cells (2 x 10^5 cells/well) were plated in 100 µL Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin in 96-well tissue culture wells and cultured to confluency. All cultures were used within 48 hrs of plating.

3.1.2 Staining

3.1.2.1 Immunohistochemistry

Infected and uninfected cultures were washed twice with PBS, fixed in ice-cold methanol for 10 min at -20°C and washed three times with PBS. Endogenous peroxidase activity was neutralized by 30-min incubation at RT with 3% H_2O_2 and followed by a 1 hr blocking incubation at RT in 10% non-immune goat serum (Zymed/Intermedico, Markham, CA). Primary antibodies detecting HCMV IE used at 1 µg/mL (p68-72, Millipore, Billerica, MA) or its isotype control, IgG_{2a} (Zymed/Intermedico) were added, the plates sealed with parafilm and incubated overnight at 4°C. After thorough washing with PBS, secondary antibody (biotinylated goat anti-mouse IgG) was added for 60 mins. Following a PBS wash streptavidin-peroxidase conjugate was then added for 30 mins (Streptavidin Biotin System, Histostain-SP Kit, Zymed) according to the manufacturer's instructions. Following another PBS wash, amino-ethyl carbazole substrate was added for 2 to 5 mins, yielding a red precipitate. The wells were then washed with ddH_20 and the number of IE-positive nuclei assessed per microwell. The percent of infectious virions was calculated as the number of infectious virions / number of nuclei per well. The number of nuclei per well was calculated in parallel wells stained with hematoxylin (Sigma-Aldrich) by multiplying the mean number of nuclei counted in five 0.25 mm^2 fields by the total number of fields per well (123 fields at 200X

magnification).

3.1.2.2 Immunofluorescence

Infected and uninfected cultures were washed twice with PBS, fixed in ice-cold methanol for 10 min at -20°C and washed three times with PBS. Then, 100 µl of 10% non-immune goat serum was added to each well to block for 1 hr (Zymed/Intermedico, Markham, CA). Primary antibodies detecting HCMV IE (p68-72, Millipore, Billerica, MA) or its isotype control, IgG_{2a} (Zymed/Intermedico), or anti-desmoplakin monoclonal antibody (Sigma-Aldrich) were added, the plates sealed with parafilm and incubated overnight at 4°C. After thorough washing with PBS, secondary antibody (FITC conjugated goat anti-mouse IgG; Alexa Flour 488, 1 µg/mL) was added at 50 µl per well for 1 hr at RT Following a PBS wash, 100 µl of 1.4 µg /mL 4',6-diamidino-2-phenylindole (DAPI) to identify nuclei was added to the wells for 10 mins at RT. The wells were then washed with PBS and fluorescent images were obtained using an Olympus IX2-UCB motorized inverted fluorescent microscope equipped with a Lambda DG-4 high-speed filter changer and a Cascade 16 bit digital monochrome camera (Olympus; Melville, NY). Digital Images of each well were obtained with DAPI (blue) and Alexa Fluor 488 (green) filters. Slidebook 3.0 (Carsen; Markham, ON, Canada) served as capture software and Image Pro-Plus (Media Cybernetics; Del Mar, CA) for analysis. The number of IE-positive nuclei observed per microwell was taken as an average of five fields per well from triplicate wells.

3.1.3 Virus stock preparations

HCMV laboratory strain, AD169 and a low-passage (maximum 7 passages) HCMV clinical isolate Kp7 (a gift from Dr. J. Preiksaitis, Dept. Medicine; University of Alberta) were propagated in HEL fibroblasts (Hemmings, Kilani et al. 1998). Infected cells and supernatant were removed from the flask with a cell scraper (Becton Dickinson; Bedford, MA) and sonicated on ice with a Vibracell sonicator (Sonics and Materials Inc., Danbury, CT, USA). The solution was then

centrifuged at 15 000 rpm for 1 hr at 4^{o} C (model J6-MI centrifuge; Beckman Instruments Inc.). The supernatant was removed and the pellet was re-suspended in plain IMDM. The virus preparation was purified by passage through a 0.45 μm filter (MILLEX-HV, Millipore Products Division, Bedford, MA) and stored in liquid nitrogen until use.

3.1.4 Quantification of viral titer

Stock virus titer was assessed by serial dilution onto confluent HEL fibroblast microcultures which were then centrifuged for 30 minutes at 2500 rpm in a GCL-2 Sorvall centrifuge to increase infectivity (Osborn and Walker 1968) and cultured for a further 18 hrs. IE antigen was detected using immunohistochemistry as previously described. Infectious virions were quantified by counting nuclei positive for HCMV IE antigen (Chou and Scott 1988; Hemmings, Kilani et al. 1998). The titer was determined within a dose-response concentration range as number of infectious virus/mL.

3.1.5 Addition of Inoculum virus

Inoculum virus was added at various amounts of virions per cell or multiplicity of infection (MOI), as described in each figure legend, in 2% FBS/IMDM at 37°C in 5% CO_2 in the presence (10 ng/mL; ST cultures) or absence (CT, CaCo2, and HEL fibroblast cultures) of rhEGF. ST, CT and CaCo2 were challenged with 1, 10 or 20 MOI and HELs, which are more readily infected in culture, were challenged with 0.2 MOI. The plates were immediately centrifuged at 2500 rpm for 30 mins in a GCL-2 Sorvall centrifuge (Osborn and Walker 1968) followed by incubation for 4 or 24 hrs prior to additional treatment as outlined below and in the individual figure legends. The amount of virus added in each experiment was calculated as the total number of nuclei per well multiplied by the total number of wells multiplied by the MOI, divided by the viral titer of the stock virus.

3.1.6 Culture Treatments

After initial incubation with inoculum virus, prior to any treatment listed below, all cultures were thoroughly washed five times with serum-free IMDM to remove any loosely and non-adherent virus.

3.1.6.1 Cultures with adherent inoculum virus

After washing, the cultures were refed with 2% FBS/IMDM and further incubated as noted in individual figure legends.

3.1.6.2 Removal of adherent inoculum virus

(1) Low pH treatment: After washing, cultures were incubated with a PBS solution at a pH of 3.0 for 2 mins to remove adherent virus (Compton, Nepomuceno et al. 1992), washed 3x with IMDM, refed fresh IMDM and further incubated as noted in individual figure legends.

(2) IVIG treatment: After washing, cultures were incubated with intravenous immune globulin (IVIG; a gift from Talecris Biotherapeutics, NC, USA) which contains neutralizing antibodies to HCMV (Zaia 1993), at a concentration of 10 mg/mL for 48 hr (Figs. 4.1-4.2) or for varying periods of time (0-360 mins) followed by a thorough wash and further 6 hr incubation (Figure 4.6). 10 mg/mL was the lowest concentration of this batch of IVIG that gives >95% inhibition of infection on HEL fibroblasts after a 10-min preincubation.

3.1.6.3 Polyethylene glycol treatment

Following addition of inoculum virus and centrifugation, some cultures were treated with 40% polyethylene glycol for 30 seconds and then extensively washed to increase adherence of virus to cells (Ryckman, Jarvis et al. 2006).

3.1.6.4 Treatments at 4^0 C

The reversible binding experiments were repeated at 4^0C to prevent viral entry (Compton, Nowlin et al. 1993). Cell culture plates were placed at 4^0C for 30 mins prior to incubation with or without IVIG and the remainder of the experiment was carried out at this temperature.

3.1.6.5 Treatments with Heparin

Virus was pre-treated for 30 mins at 37^0 C with 20 µg/mL Heparin Sodium (Sigma-Aldrich, Saint Louis, Missouri; H9399) before addition to cell cultures. Heparin sodium concentration was optimally determined by dose response using 0.1, 1, 10 and 20 µg/mL concentrations.

3.1.6.6 Cycloheximide treatment

Some cultures were treated with cycloheximide (1 µg/mL) to prevent viral replication 30 min prior to addition of inoculum virus and throughout the culture period.

3.1.6.7 Cell fixation

Some cultures were fixed in 4% paraformaldehyde prior to addition of inoculum virus to provide evidence that protection of HCMV by reversible binding is an active cellular process.

3.1.7 Quantification of inoculum virus remaining cell adherent or released into supernatants

Culture supernatants were removed at various times after the above treatment protocols and frozen at -80°C until assessed for viral titer. Cell cultures were washed three times with PBS, lysed in 100 µL 2% FBS/IMDM by freezing and thawing three times and stored at -80°C until assessment of viral titer (cell adherent virus). Viral titers were quantified on confluent HEL fibroblast cultures as described above.

3.1.8 Assessment of IE antigen production in ST, CT, HEL or CaCo2 cultures after viral challenge and treatments

Evidence for infection of all cell cultures after the above treatments was determined by the presence of IE-positive nuclei using immunohistochemistry. The average percent IE-positive nuclei from five 0.25 mm^2 fields in each microwell was assessed and reported as the mean ± SD of three microwells. All primary trophoblast preparations were assessed for a preexisting HCMV infection

by staining control cultures unchallenged by virus for IE antigen expression as described above. Additionally, all trophoblast preparations were negative for HCMV glycoprotein B as assessed by real-time PCR. None of the trophoblast preparations used in this study stained positive for IE antigen prior to addition of inoculum virus. Productive infection (production of progeny virus) in ST or CT cultures has previously been shown in our lab to be slower than that found in fibroblast cultures (96 hrs compared to 48 hrs) (Hemmings, Kilani et al. 1998).

3.1.9 Statistics

Cryopreserved trophoblasts from five different placentas were used in these experiments. While the ability of HCMV to infect a given trophoblast preparation is consistent in experiments repeated on different days, the fraction of infected cells in different preparations can be considerably variable even if examined at the same time. However, the trends remain consistent between different trophoblast preparations: regardless of what the infected fraction of a cell preparation is on day 2, it is 20 to 50% higher on day 8. However, the large variability in absolute number between placental preparations prevents pooling results from multiple experiments done with cells isolated from different placentas. Thus, the results presented are triplicate experiments performed with a single representative trophoblast preparation. Where statistics are carried out, Students T test is used to compare the error between two groups and one-way ANOVA with Fisher post-hoc test is used for multiple comparisons ($p<0.05$).

3.2 Materials and Methods Specific for Chapter 5

3.2.1 Collections

From January 2010 to December 2010, pregnant women who delivered at The Royal Alexandra Hospital, Edmonton, AB, were enrolled in this study with their informed consent (Appendix B). The study cohort consisted of 175 pregnant women with or without complications, excluding multiple births. For each enrolled pregnant woman we obtained maternal blood, maternal urine, neonatal

throat swab and placenta. There were a total of 175 samples in each category. Data for the study was collected by the research nurse via maternal chart review (Appendix B). Some data points for patients were not available and this is reflected in the n numbers as shown in figures and tables.

25, 6 mm x 6 mm punch biopsies were obtained from each placenta: the placenta was separated into five approximately equal sections and five biopsies were taken per section. Each section was washed three times with PBS and stored at -80^{o} C. Maternal blood, plasma (BD® Vacutainer EDTA spray coated tube) and serum (BD® Vacutainer SST™ tube) were collected within 24 hrs of delivery. Within 1 hr of collection, the blood was processed via centrifugation at 3000 rpm for 20 minutes at 4^{o} C (model G5-6R centrifuge; Beckman Instruments Inc.). The serum and plasma were then aliquoted into sterile 2 mL tubes and stored at -80^{o} C. Maternal urine was collected within 24 hrs of delivery either from a catheter tube or midstream urine collection from the patient. Within 1 hr of collection, the urine was aliquoted into 2 mL tubes and stored at -80^{o} C. A neonatal throat swab was obtained within 24 hrs of delivery and in most cases prior to breast-feeding. The swabs were stored at 4^{o} C for up to 2 weeks prior to DNA extraction.

3.2.2 DNA Extractions

DNA extraction was only performed on samples from women whose serum samples were seropositive for HCMV with the exception of the neonatal throat swabs. All 175 neonatal throat swabs were analyzed within 1 week of collection due to the importance of identifying a positive infant within the first 3 weeks of delivery to implement appropriate clinical treatment. Therefore placentas, plasma and urine samples from 105 study participants were processed for DNA extraction.

3.2.2.1 Maternal urine, plasma, neonatal throat swab

The preparation of DNA from plasma, urine, and neonatal saliva was carried out as described below. Double-stranded DNA was extracted from 200 µl of sample by using a QIAamp DNA mini kit (Qiagen, Mississauga, ON). 200 µl

of sample, 200 µl of Lysis Buffer AL and 20 µl of proteinase was added to a 1.5 mL tube, vortexed for 15 secs and incubated at 56^{o} C for 10 mins. The tubes were then picofuged for 3 secs, 200 µl of 95% ethanol was added and tubes were vortexed. The mixture was then added to a QIAamp spin column and centrifuged at 9000 rpm for 1 min. The column was then placed in a clean 2 mL collection tube, 500 µl of wash buffer AW1 added and micro-centrifuged at 9000 rpm for 1 min. The column was then placed in a clean 2 mL collection tube, 500 µl of wash buffer AW2 added and centrifuged in a micro-centrifuge at 14,000 rpm for 3 min. Then the column was placed in a clean 2 mL collection tube and spun at 14,000 rpm for 1 minute to remove any residual AW2. The DNA was eluted from the column with 100 µl of AE buffer. All DNA was stored at -80^{o} C until use.

3.2.2.2 Placental tissue

Placental samples were thawed and homogenized (PRO200 homogenizer; PRO Scientific Inc., Oxford, CT, USA) for 10-15 secs. DNA was then extracted from 200 mg of homogenized tissue using the DNeasy Blood & Tissue kit (Qiagen, Mississauga, ON). 200 µl of tissue lysis buffer buffer ATL and 60 µl of proteinase K (20 µg/mL) was added to each homogenized tissue, vortexed and incubated at 56^{o} C for 4 hrs. 400 µl of lysis buffer AL was then added to each sample, vortexed and incubated at 70^{o} C for 10 mins. The tubes were then picofuged for 3 secs, 400 µl of 95% ethanol was added and tubes were vortexed. The mixture was then added to a QIAamp spin column and micro-centrifuged at 9000 rpm for 1 min. The column was then placed in a clean 2 mL collection tube, 500 µl of wash buffer AW1 added and micro-centrifuged at 9000 rpm for 1 min. The column was then placed in a clean 2 mL collection tube, 500 µl of wash buffer AW2 added and centrifuged at 14,000 rpm for 3 min. Then the column was placed in a clean 2 mL collection tube and spun at 14,000 rpm for 1 minute to remove any residual AW2. The DNA was eluted from the column with 200 µl of AE buffer. DNA concentration was determined (NanoDrop 1000 Spectrophotometer; Thermo Fisher Scientific, Nepean, ON) and all samples were diluted to 25 ng/µl for use in PCR. All DNA was stored at -80^{o} C until use.

3.2.3 ELISA for CMV IgG

All serology was performed by the Virology – Serology program unit at the Provincial Public Health Laboratory and Alberta Health Services Microbiology (University of Alberta Site, Edmonton, AB). Prenatal blood was available for serological assessment for the majority of study participants through the Provincial Public Health Laboratory (Edmonton, AB).

3.2.3.1 Maternal Serum

Serology for CMV IgG was performed on all 175 maternal serum samples collected at the time of delivery. For women who tested positive for CMV IgG prenatal blood samples, stored in the Provincial Laboratory, where available, were then also assessed for CMV IgG to determine seroconversion during pregnancy. CMV IgG was measured according to the instructions of the manufacturer (Enzygnost® Anti-CMV IgG ELISA; Dade Behring, Newark, USA). The BEP® 2000 system (Siemens, Marburg, Germany) was used and is a fully automated process. Briefly, the reagents, controls and bar coded samples are placed in the instrument. Subsequent set-up steps performed by the machine include: dilution of controls and samples with blue-violet dyed sample buffer; the addition of 200 µl of non-dyed sample buffer to each well of a plate coated with inactivated CMV antigen, and lastly, 20 µl of each sample added to the appropriate wells. The samples were incubated for 60 mins at 37^{o} C and then were washed four times. Conjugate working solution was then added at 100 µl/well and incubated for 60 mins at 37^{o} C, and then washed four times. Chromogen working solution was then added at 100 µl/well and incubated for 30 mins at 37^{o} C, and washed four times. Lastly, 100 µl/well of stopping solution was added and absorbance was measured as 450 nm versus 650 nm.

3.2.4 Real Time Quantitative Polymerase Chain Reaction (qPCR) for HCMV

Real-time qPCR for detection and quantification of HCMV was performed using methods previously described (Pang, Chui et al. 2003). The primers selected in the glycoprotein B gene were expected to yield a 254-bp product (Figure 5.1A).

The hybridization donor probe with a fluorescein 3'end label and the acceptor probe with a LC-Red 640 5' end label were used for real-time detection during the LC-PCR reaction. Real-time PCR was carried out in a closed tube system using the LightCycler instrument (Roche Diagnostics, Laval, QB). 20 µl of PCR reaction mixture containing 10 µl of DNA solution, 4 mM $MgCl_2$, 0.5 µM concentrations of each primer, 0.2 µM concentrations of each probe, and 2 µl of the reagent from a LC-FastStart DNA Master hybridization probe kit (Roche Diagnostics) was added to the capillaries. The capillaries were centrifuged, mounted onto the carousel, and loaded into the LightCycler. The thermal cycles were as follows: an initial 10 min at 95°C, followed by 45 cycles of 15 sec of denaturing at 95°C, 10 sec of annealing at 55°C, and a 10 sec extension at 72°C. During the annealing period the data were collected in the single mode, with channel setting F2/F1. The data was automatically analyzed using LightCycler software 4.05 (Roche Diagnostics). Specimens with fluorescence signal higher than the background were considered positive. The specificity of the fluorescence signal was checked by a melting curve analysis after each run. Note: if a neonate was identified as positive by qPCR a notification letter and recommended follow-up requisition were sent to the neonate's attending physician by the research nurse (Appendix B).

3.2.5 Quantification of Viral Load

A known copy number of plasmid DNA containing CMV DNA fragment (320 base pairs) was diluted in a 10-fold series ($2.0x10^2$ to $2x10^6$) genome copies (Figure 5.2) and used to set up an internal standard curve for the quantification of CMV viral load in different sample types. The reporting unit for CMV viral load was copies/mL of plasma, urine and saliva samples and copies/µg of DNA of placental samples. The limit of detection was 50 copies/mL and the limit of quantification was 500 copies/mL for plasma, urine and saliva samples. The limit of detection was 4 copies/ug DNA and the limit of quantification was 60 copies/ug DNA for placenta samples.

3.2.6 Statistics

Statistical analysis was performed with SigmaStat statistical software, version 11.0, for windows. The Mann-Whitney U test was used to determine differences between the groups. Chi-squared or Fishers exact test were used as appropriate to determine differences between categorical data. Results are displayed as median with inter-quartile range.

3.3 References

Chou, S. W. and K. M. Scott (1988). "Rapid quantitation of cytomegalovirus and assay of neutralizing antibody by using monoclonal antibody to the major immediate-early viral protein." J Clin Microbiol **26**(3): 504-507.

Collett, A., D. Walker, et al. (1997). "Influence of morphometric factors on quantitation of paracellular permeability of intestinal epithelia in vitro." Pharm Res **14**(6): 767-773.

Compton, T., R. R. Nepomuceno, et al. (1992). "Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface." Virology **191**(1): 387-395.

Compton, T., D. M. Nowlin, et al. (1993). "Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate." Virology **193**(2): 834-841.

Douglas, G. C. and B. F. King (1990). "Differentiation of human trophoblast cells in vitro as revealed by immunocytochemical staining of desmoplakin and nuclei." J Cell Sci **96 (Pt 1)**: 131-141.

Guilbert, L. J., M. Riddell, et al. (2010). "Caspase activation is not required for villous cytotrophoblast fusion into syncytiotrophoblasts." Placenta **31**(11): 982-988.

Hemmings, D. G., R. Kilani, et al. (1998). "Permissive cytomegalovirus infection of primary villous term and first trimester trophoblasts." J Virol **72**(6): 4970-4979.

Morrish, D. W., J. Dakour, et al. (1997). "In vitro cultured human term cytotrophoblast: a model for normal primary epithelial cells demonstrating a spontaneous differentiation programme that requires EGF for extensive development of syncytium." Placenta **18**(7): 577-585.

Osborn, J. E. and D. L. Walker (1968). "Enhancement of infectivity of murine cytomegalovirus in vitro by centrifugal inoculation." J Virol **2**(9): 853-858.

Pang, X. L., L. Chui, et al. (2003). "Comparison of LightCycler-based PCR, COBAS amplicor CMV monitor, and pp65 antigenemia assays for quantitative measurement of cytomegalovirus viral load in peripheral blood specimens from patients after solid organ transplantation." J Clin Microbiol **41**(7): 3167-3174.

Ryckman, B. J., M. A. Jarvis, et al. (2006). "Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion." J Virol **80**(2): 710-722.

Yui, J., M. Garcia-Lloret, et al. (1994). "Functional, long-term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells." Placenta **15**(3): 231-246.

Zaia, J. A. (1993). "Prevention and treatment of cytomegalovirus pneumonia in transplant recipients." Clin Infect Dis **17 Suppl 2**: S392-399.

Chapter 4

Human Cytomegalovirus is Protected from Inactivation by Reversible Binding to Villous Trophoblast***

4.1 Introduction

Human cytomegalovirus (HCMV), from the *Herpesviridae* family is transmitted *in utero* to ~40% of fetuses from mothers who develop a primary infection during pregnancy (Demmler 1991). While the most severe adverse neonatal outcomes are reported in this group, congenital infections are also found in ~1% of newborns from women with prepregnancy immunity (Stagno, Pass et al. 1986; Boppana, Rivera et al. 2001). Neonatal sequelae range from no symptoms to significant neurodevelopmental problems including sensorineural hearing loss and in some cases can also lead to death. Importantly, asymptomatic congenital infections can also lead to late-onset long-term complications (Williamson, Demmler et al. 1992; Ahlfors, Ivarsson et al. 1999). The importance of preventing HCMV congenital infections is exemplified in the considerable societal economic burden of caring for and supporting these children; in the 1990`s the United States estimated an annual cost of $1.86 billion (Porath, McNutt et al. 1990). Seale et al reported that the Advisory Committee on Immunization Practices in the year 2000 estimated that implementation of an effective and fully utilized HCMV vaccine program could equate to a savings of $4 billion US dollars in health care costs (Seale, Booy et al. 2009).

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Primary infections, reactivation or reinfection with another strain during pregnancy are risk factors for intrauterine HCMV transmission to the fetus (Fowler, Stagno et al. 1992). These types of infections can in part be differentiated by the presence of IgM (primary, reactivation, reinfection) or detection of multiple viral strains (reinfection). All lead to an active infection; however, viral load in blood (Revello, Zavattoni et al. 1998; Lazzarotto, Gabrielli et al. 2004) or urine (Stagno 2001) does not correlate with intrauterine transmission which occurs even when virus in these compartments is low or undetectable.

In pregnancy, the organ distribution of disease burden for some infections is dramatically shifted (Griffith, McCormick et al. 1985). For example, the intracellular bacteria *Listeria monocytogenes* continues to replicate in the placenta of resistant female mice even though it is effectively cleared from the livers of these same mice (Guleria and Pollard 2000). In guinea pig, a continuous placental infection with *L. monocytogenes* leads to repeated reinfection of the maternal organs until removal of the placenta (Bakardjiev, Theriot et al. 2006). Similarly, the intracellular parasite *Plasmodium falciparum*, which causes malaria, parasitizes red blood cells which are predominantly found in the villous placenta of pregnant women. They bind to chondroitin sulphate A, which is highly expressed in the placental syncytiotrophoblast layer, where they cause placental damage associated with low birth weight babies (Fried and Duffy 1996; Lucchi, Koopman et al. 2006). Furthermore, lymphocytic choriomeningitis virus, a single-stranded RNA virus in the mouse is rapidly cleared from all tissues except for the placenta (Constantin, Masopust et al. 2007). The above-mentioned are striking examples of possible adverse consequences of the 12 m^2 surface of placental trophoblast present in term pregnancies that has been characterized as an immune privileged site (Medawar 1953; Trowsdale and Betz 2006). Whether the placenta sequesters HCMV even when cleared from maternal blood and urine, as happens with *L. monocytogenes* and thereby facilitates fetal infection or prolongs maternal disease has not been explored. It has been suggested that the guinea pig placenta

acts as a viral reservoir for guinea pig CMV (Goff, Griffith et al. 1987; Revello and Gerna 2002). Here, the virus is protected from circulating maternal antibodies thereby maintaining the infection throughout gestation (Harrison and Myers 1990).

In the placenta, maternal blood is separated from fetal blood vessels and stromal tissue by trophoblasts. The villous trophoblast consists of a mature, multinucleated syncytiotrophoblast (ST) layer that faces maternal blood and an underlying layer of replicative mononuclear cells called cytotrophoblasts (CT) that replenish the ST by fusion to its basal surface. Using sensitive polymerase chain reaction (PCR), viral DNA has been detected in all cell types of the placenta at much higher rates than initially believed: 69% of first trimester, 74% of second trimester (Kumazaki, Ozono et al. 2002) and 9.8% - 62% of term placentas (Muhlemann, Miller et al. 1992; Kumazaki, Ozono et al. 2002; Pereira, Maidji et al. 2003; McDonagh, Maidji et al. 2006). However, evidence for a widespread productive infection as detected by viral gene expression, particularly in the ST layer, is limited (Hemmings, Kilani et al. 1998; Fisher, Genbacev et al. 2000), even though mature virus is often found associated with ST. Collectively, these animal models and human studies suggest that the placenta acts as a repository to collect and protect mature and infectious virus, which increases the risk of fetal infection (Ziyaeyan, Alborzi et al. 2007). The ST layer is highly microvilliated and functions as a barrier between maternal and fetal circulations, in part through several mechanisms which contribute to immune privilege. It is these characteristics which suggest that it is in and around the ST layer of the placenta that HCMV is being sequestered and thereby protected from inactivation. We tested this hypothesis using a model of highly purified villous CT differentiated with epidermal growth factor (EGF) into ST-like cultures (Morrish, Dakour et al. 1997; Hemmings, Kilani et al. 1998).

4.2 Methods

All methods of this chapter are described above in 3.2.

4.3 Results

4.3.1 Inoculum virus strongly adheres to ST, is released back into culture supernatants and remains infectious

AD169, a laboratory strain of HCMV, was added to syncytiotrophoblast (ST) cultures at an MOI of 10 for 24 hrs. The cultures were extensively washed with IMDM to remove loosely adherent virus and further incubated with fresh 2% FBS/IMDM for an additional 48 hrs. Equivalent amounts of infectious inoculum virus were detected in both culture supernatants (released virus) and washed cell lysates (cell adherent virus) (Figure 4.1). Treatment of cultures with PBS at a pH of 3.0 for two mins prior to the extensive washing step to remove surface-bound virus dramatically reduced cell adherent and released virus after 48 hrs (Figure 4.1). Treatment with neutralizing antibodies to HCMV (10 mg/mL IVIG) after the extensive washing step to capture any released virus also reduced cell adherent and released virus after 48 hrs of additional incubation. Neither a 2-min pH 3.0 treatment nor a 2-day IVIG treatment affected the total number of nuclei in any culture (results not shown).

To determine if inoculum virus could remain infectious over a similar time period in the presence of other types of cells, we repeated these experiments in less differentiated trophoblasts (cytotrophoblasts; CT), fibroblasts (HEL) and CaCo2 (Figure 4.2). Both CT and CaCo2 showed similar results to that found with ST. In contrast, the remaining inoculum virus levels in HEL cultures were approximately 100 fold less (Figure 4.2). HEL cultures were incubated for 24 hr rather than 48 hr to ensure no progeny virus would be produced.

4.3.2 Infectious virus detected in supernatants and cell lysates is not due to progeny virus produced by infected cells

It is important to note that infectious virus recovered at this time point in ST cultures is likely inoculum virus (that added at the beginning of the

experiment) and not progeny virus (that produced by infected cells) since we have previously shown in these cells that production of progeny virus requires at least 4 days (Hemmings, Kilani et al. 1998). Two further experiments were done to confirm this. First, a shorter time for initial viral contact with the cells (4 hrs) prior to extensive washing was followed by further incubation in a time course from 0.5 hr to 24 hrs to ensure the total length of time was less than that required for production of progeny virus in these cultures. In these and other experiments, we used a low passage clinical isolate (Kp7) rather than the laboratory strain, AD169, to confirm that this effect was not due to the many gene deletions found in AD169 (Cha, Tom et al. 1996). Both cell adherent and released virus was detected at all time points (Figure 4.3) and was significantly reduced by low pH treatment as seen in Figure 4.1 and 4.2. However, the total amount of Kp7 detected was significantly less than that of AD169, likely because of the reduced viral challenge time in the Kp7 experiments. As well, only 25.2% of total Kp7 recovered 24 hrs after extensive washing was found in the supernatants (Figure 4.3) compared to 69.2% of total AD169 (Figure 4.1). The second experiment to show that virus detected in these experiments was not due to progeny virus was to treat the ST cultures with cycloheximide to inhibit viral protein synthesis. This treatment had no effect on released or cell adherent virus (Figure 4.4). Taken together, the results from AD169 and Kp7 demonstrate that strongly adherent virus (remains after extensive washing step) is released into the supernatant quickly (less than 30 mins) with equilibration occurring between released and adherent infectious inoculum virus for at least 48 hrs after initial exposure of the cultures to virus.

4.3.3 Low infection levels in ST are not affected by low pH or IVIG treatments

Further support for detection of inoculum rather than progeny virus detected in these studies is the finding that ST are poorly infected as previously seen (Hemmings, Kilani et al. 1998). The total amount of recovered virus from extensively washed but otherwise untreated cultures was 3.55×10^4 infectious

virions. The total number of nuclei in these cultures is approximately 4 x 10^4. Thus, the maximum expected infection efficiency would be approximately 90%. The finding of a low level of infection in these cultures 48 hrs after the extensive washing step (2.6 ± 0.5%) that is not affected by low pH treatment (2.4 ± 0.3%) or treatment with IVIG (1.9 ± 0.2%) suggests that the majority of strongly adherent virus in ST does not enter and infect the cells. However, increasing the contact time of virus to cells or centrifugation of virus onto cell cultures significantly increases infection efficiency in all cell types tested (Figure 4.5). Likewise, increasing adherence and fusion of the virus to the cells using polyethylene glycol treatment (Compton T 1993) also increased infection efficiency (data not shown). Overall, ST cultures showed the poorest infection efficiency, followed by that in CT cultures with the highest infection efficiency in HEL cultures (Figure 4.5A). The infection efficiency in CaCo2 cultures was similar to that of CT cultures (Figure 4.5A, B).

4.3.4 Neutralizing HCMV antibody treatment time dependently reduces cell adherent and released virus in ST cultures

One mechanism for the equilibration of cell adherent inoculum virus and that found in supernatants over time is through repeated binding and release or reversible binding as previously shown over a much shorter time frame in mouse fibroblasts (Hodgkin, Scalzo et al. 1988). To evaluate this possibility in ST cultures, neutralizing antibodies to HCMV were used to inactivate any virus reaching the supernatant (Figure 4.6). The half-life of supernatant virus was 6.91 ± 0.10 mins and half-life of cell adherent virus was 9.24 ± 1.24 mins over a 60-min time frame (no significant difference), showing that equilibrium had been reached (Figure 4.6A, B). Released virus is inactivated by IVIG and thus is unable to rebind to the ST resulting in a continual decrease in the amount of cell adherent virus compared to the amount found with no IVIG treatment over the same time (Figure 4.6A, B). By treating the cultures with IVIG for various time points after the extensive washing step followed by another thorough wash and further

incubation, we found approximately 4.98% of the inoculum was released per min (Figure 4.6C). The rate of release was calculated from the slope of the natural log of the bound virus versus time of IVIG treatment (Figure 4.6C). This experiment was repeated twice at 4^0C with equivalent results (Figure 4.7).

4.3.5 Pretreatment of virus with heparin significantly reduces reversible binding

Heparan sulfate proteoglycans (HSPGs) are likely the initial receptors for HCMV on a variety of cell types (Compton, Nowlin et al. 1993; Feire, Koss et al. 2004). To block the binding of HCMV to HSPGs, we pre-treated virus with 20 µg/mL of heparin for 30 mins at 37^0 C and then challenged ST cultures with pre-treated or untreated virus at an MOI of 10 for 4 hrs. The cultures were extensively washed with IMDM to remove loosely adherent virus and further incubated with fresh 2% FBS/IMDM for 30 mins, 2 hrs, 4hrs or 24 hrs. Equivalent amounts of infectious inoculum virus were detected in both culture supernatants (released virus) and washed cell lysates (cell adherent virus) from cultures receiving untreated virus (Figure 4.8A). However, the amount of virus detected in the supernatants and cell lysates from cultures receiving the heparin-treated virus was dramatically less than that detected in cultures receiving untreated virus (Figure 4.8B).

4.3.6 Maintenance of infectious cell adherent and supernatant virus over time is dependent on the presence of live cells

To determine if the maintenance of cell adherent virus and that found in the supernatants in ST cultures over time was an active process requiring live cells, experiments were repeated either in the absence of cells or in the presence of cells fixed with paraformaldehyde. The rate of loss of infectious virus over a 24-hr time period was significantly greater in cell-free (Figure 4.9A) or fixed ST cultures (Figure 4.9B) compared to live ST cultures ($p<0.05$).

Figure 4.1

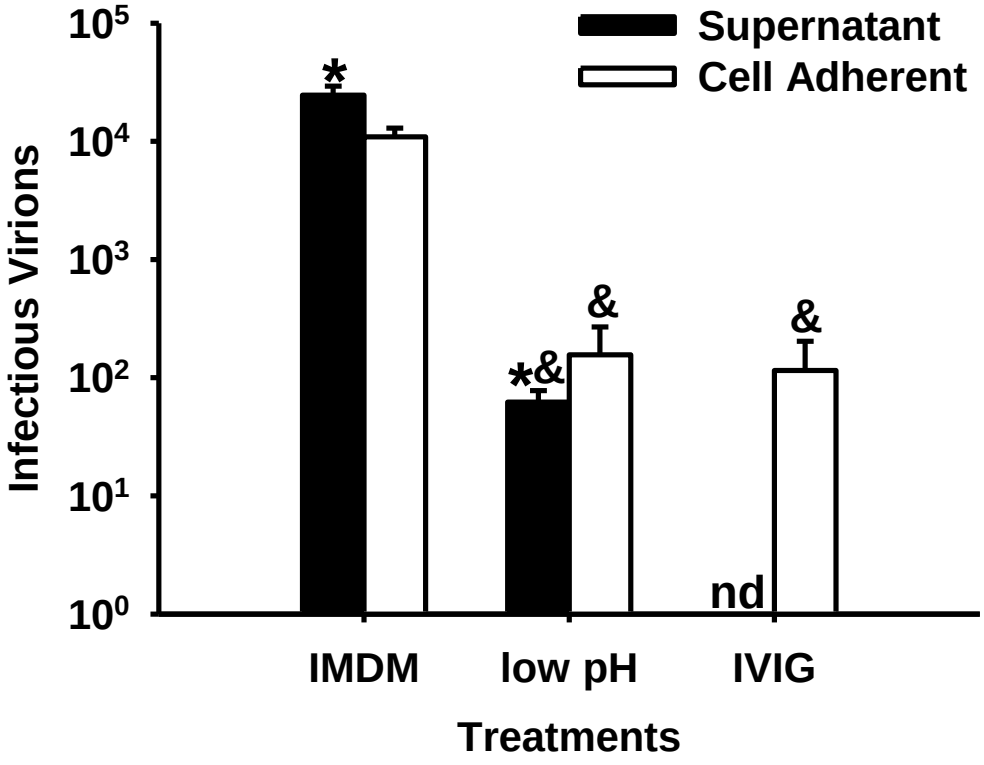


Figure 4.1 - Strongly adherent inoculum virus that is released into ST culture supernatants and remains infectious over 48 hrs is dramatically reduced by low pH or neutralizing antibody treatment

An HCMV laboratory strain, AD169 was added to ST cultures at an MOI of 10 for 24 hrs, then washed five times with IMDM (IMDM). Some cultures were then incubated for two mins in phosphate-buffered saline at pH 3.0 (low pH), washed well and further incubated for 48 hr. Other cultures were incubated in 10 mg/mL of IVIG for 48 hr following the initial IMDM wash (IVIG). Cell adherent and supernatant infectious virus was assessed as described in the Methods. Results are presented as the mean ± SEM from three independent experiments. & = significantly different from IMDM ($p<0.05$), * = significantly different from cell adherent virus ($p<0.05$), nd = not detected.

Figure 4.2

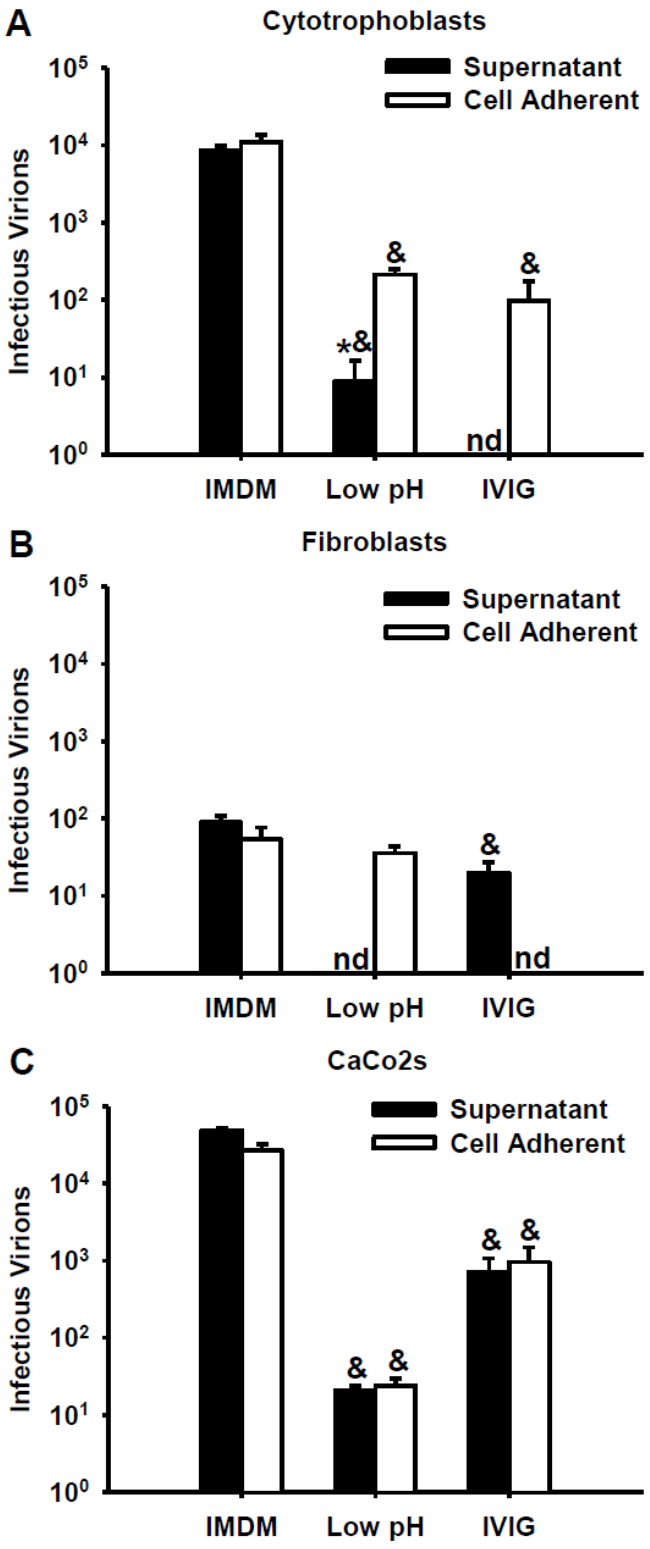

A
Cytotrophoblasts
Supernatant
Cell Adherent
Infectious Virions
10^5
10^4
10^3
10^2
10^1
10^0
*&
&
&
nd
IMDM
Low pH
IVIG
B
Fibroblasts
Supernatant
Cell Adherent
Infectious Virions
&
nd
nd
IMDM
Low pH
IVIG
C
CaCo2s
Supernatant
Cell Adherent
Infectious Virions
& &
& &
IMDM
Low pH
IVIG

Figure 4.2 - Release of strongly adherent infectious inoculum virus in CT, HEL and CaCo2 culture supernatants

AD169 was added to CT **(A)** and HEL **(B)** cultures and KP7 was added to CaCo2 cultures **(C)**, at an MOI of 10 for 24 hrs, then extensively washed five times with IMDM (IMDM). Some cultures were then incubated for two mins in phosphate-buffered saline at pH 3.0 (low pH) and again washed well. After a further 48 hr **(A, C)** or 24 hr **(B)** incubation in 2% FBS/IMDM, cell adherent and supernatant infectious virus was assessed as described in the Methods. Other cultures were incubated in 10 mg/mL of IVIG for 48 hr **(A, C)** or 24 hr **(B)** following the initial IMDM wash. Results are presented as the mean ± SEM from three independent experiments. & = significantly different from IMDM ($p<0.05$), * = significantly different from cell adherent virus ($p<0.05$), nd = not detected.

Figure 4.3

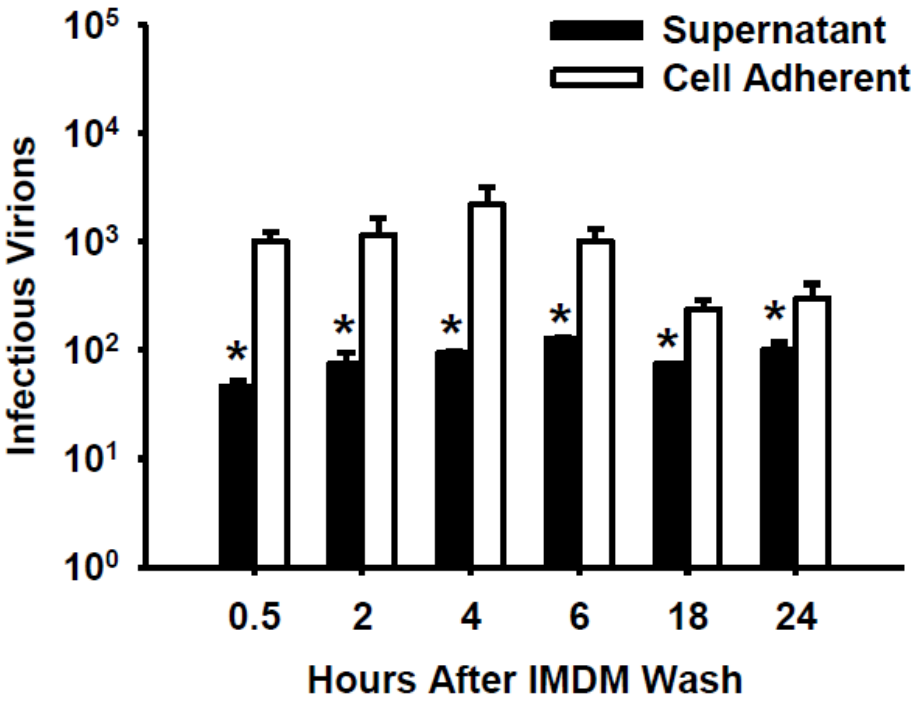


Figure 4.3 - Time course of cell adherent and released virus in ST cultures

An HCMV clinical isolate, Kp7, was added to ST cultures at an MOI of 10 for 4 hrs, then washed five times with IMDM. The cultures were then further incubated in 2% FBS/IMDM for various periods of time (0.5 to 24 hrs). Cell adherent and supernatant infectious virus was assessed as described in the Methods. Results are presented as the mean ± SEM from three independent experiments. * = significantly different from cell adherent virus ($p<0.05$).

Figure 4.4

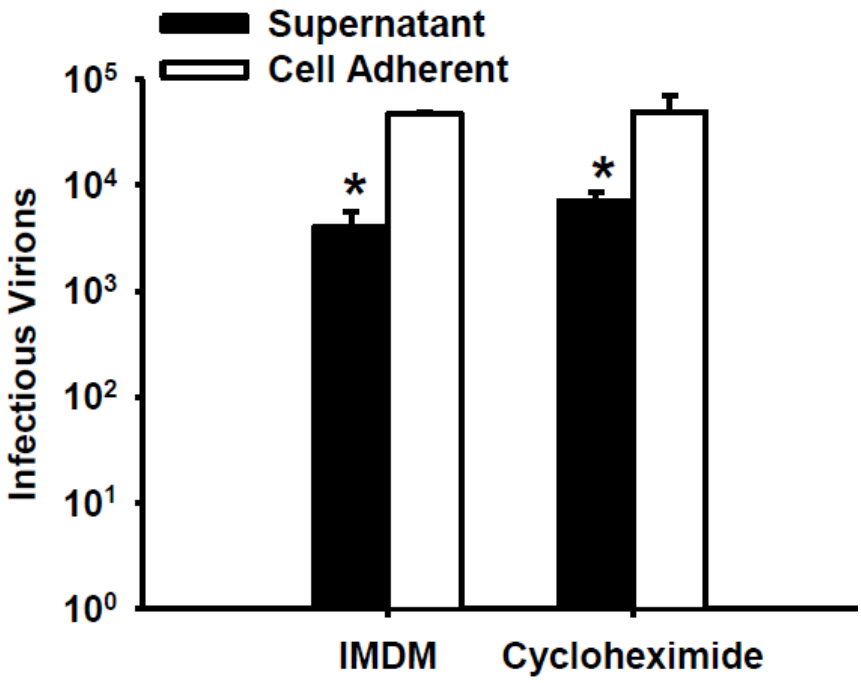


Figure 4.4 - Strongly adherent inoculum virus and its release in ST cultures is not affected by cycloheximide treatment

An HCMV clinical isolate, Kp7 was added to ST cultures at an MOI of 10 for 4 hr followed by further incubation for 24 hrs in the presence and absence of 1 μg/mL cycloheximide. Results are presented as the mean ± SEM from three independent experiments. * = significantly different from cell adherent virus ($p<0.05$).

Figure 4.5

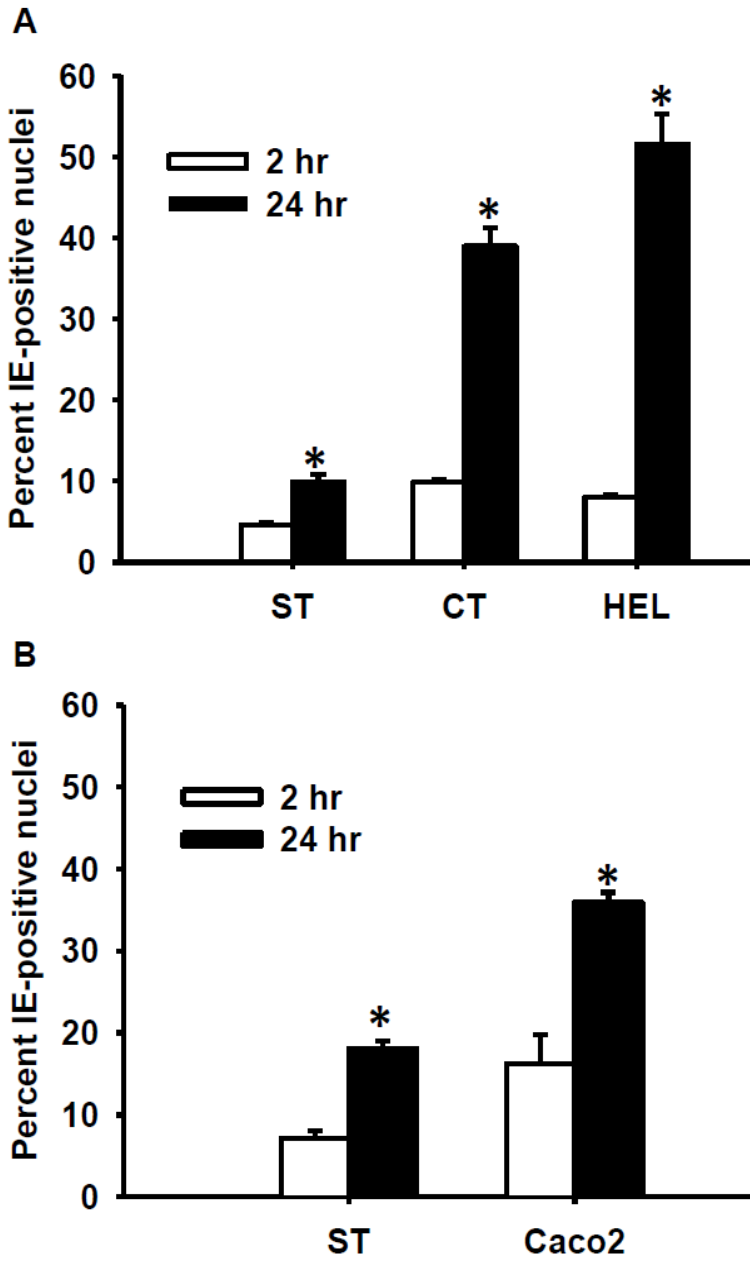

A
Percent IE-positive nuclei
60
50
40
30
20
10
0
2 hr
24 hr
*
*
*
ST
CT
HEL
B
Percent IE-positive nuclei
60
50
40
30
20
10
0
2 hr
24 hr
*
*
ST
Caco2

Figure 4.5 - HCMV infection efficiency in cell cultures as a function of initial virus exposure time

AD169 was added to ST and CT cultures **(A)** and KP7 was added to ST and CaCo2 cultures **(B)** at an MOI of 10. AD169 was added to HEL fibroblast cultures **(A)** at an MOI of 0.2. After a 2 or 24-hr challenge time, each culture was washed five times in IMDM, incubated for a further 48 hrs (ST, CT or CaCo2) or 18 hrs (HEL) and immunohistochemically stained for HCMV immediate early (IE) antigen. Percentages were calculated as described in the Methods and reported as the mean ± SEM of triplicate wells from one * = significantly different from 2 hr time point ($p<0.05$).

Figure 4.6

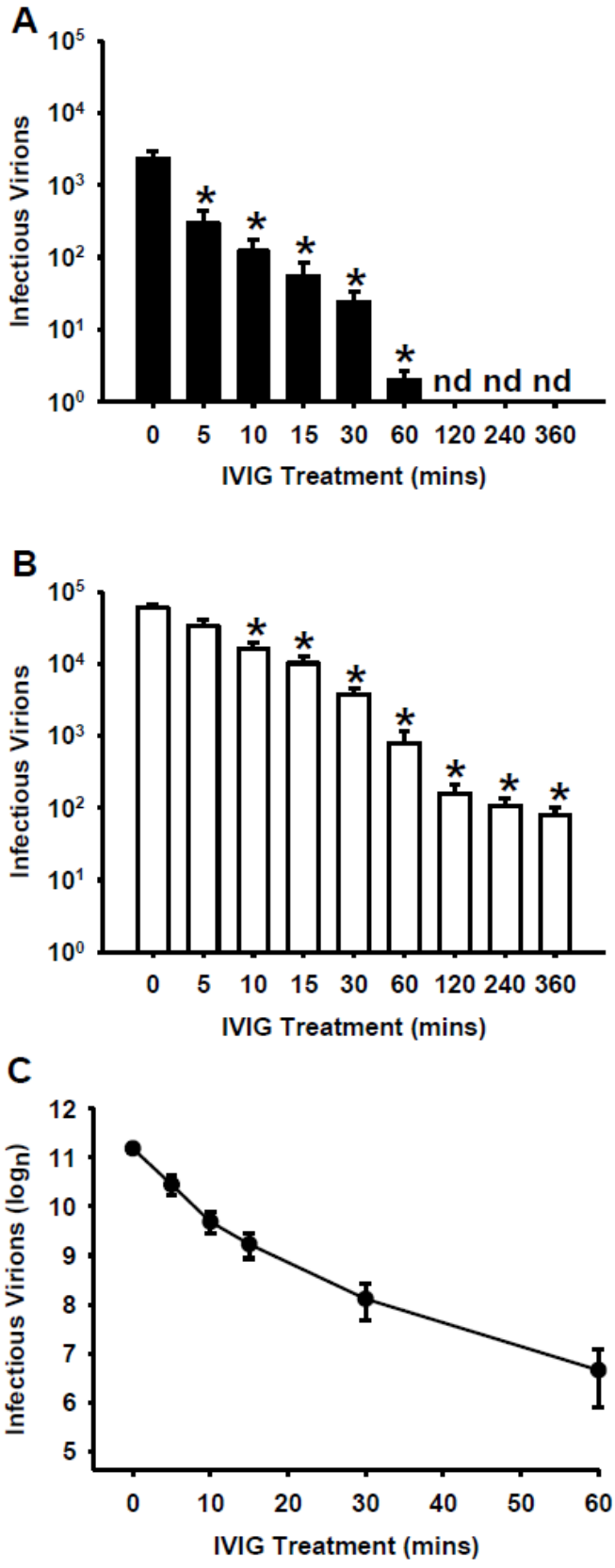

A
Infectious Virions
10^5
10^4
10^3
10^2
10^1
10^0
nd nd nd
0 5 10 15 30 60 120 240 360
IVIG Treatment (mins)
B
Infectious Virions
10^5
10^4
10^3
10^2
10^1
10^0
0 5 10 15 30 60 120 240 360
IVIG Treatment (mins)
C
Infectious Virions (log_n)
12
11
10
9
8
7
6
5
0 10 20 30 40 50 60
IVIG Treatment (mins)

Figure 4.6 - Neutralizing antibody treatment time dependently reduces the amounts of released and cell adherent virus

After addition of Kp7 for 4 hrs at MOI of 10 followed by five IMDM washes, IVIG was added to ST cultures for varying periods of time (x-axis) followed by a thorough wash and further 6-hr incubation in 2% FBS/IMDM. Supernatants **(A)** and cell lysates **(B)** were assessed for infectious virus as described in the Methods. **(C)** Total virus after IVIG treatment. Rate of release is presented as calculated by slope of the line. Results are presented as the mean ± SEM from three independent experiments. * = significantly different from zero time point ($p<0.05$), nd = not detected.

Figure 4.7

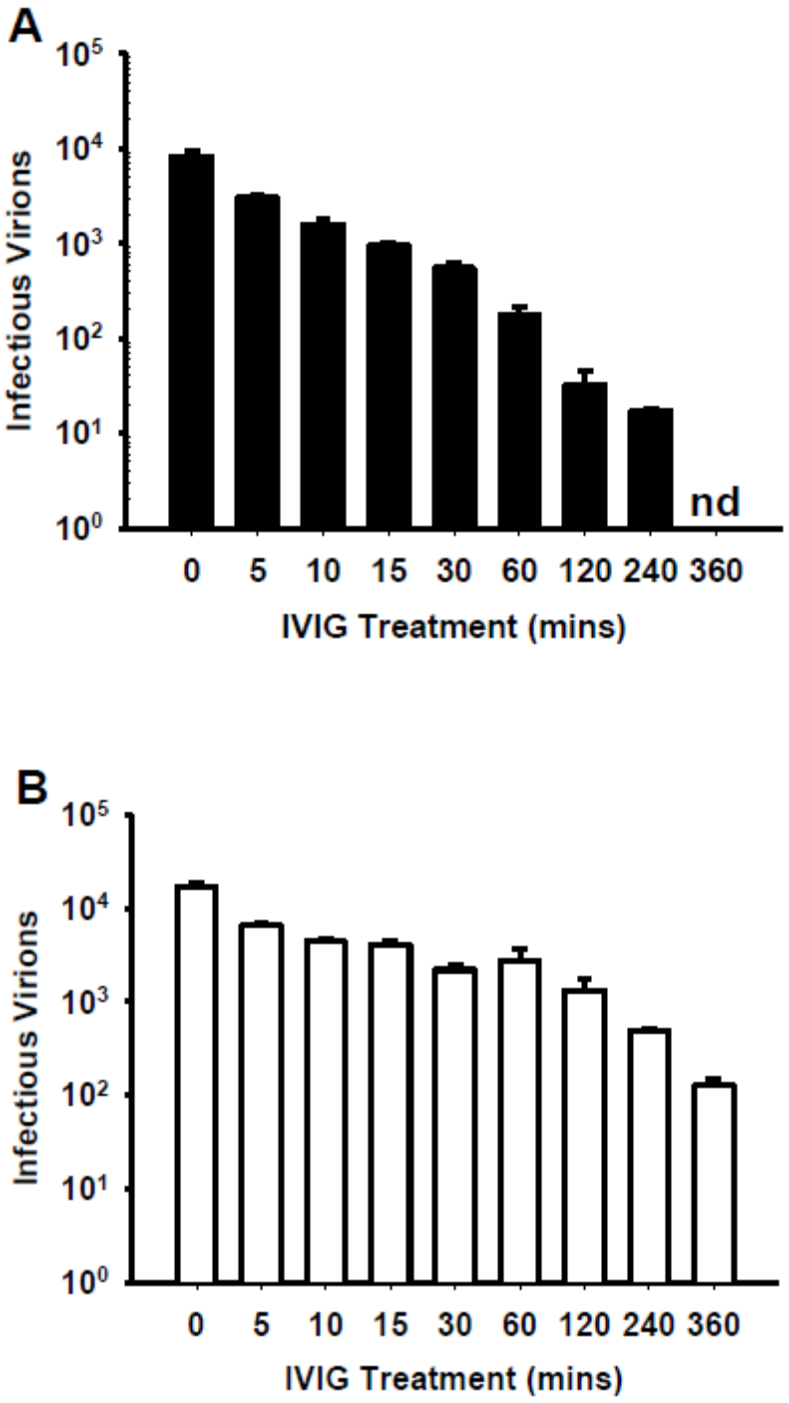

A
Infectious Virions
10^5
10^4
10^3
10^2
10^1
10^0
nd
0 5 10 15 30 60 120 240 360
IVIG Treatment (mins)
B
Infectious Virions
10^5
10^4
10^3
10^2
10^1
10^0
0 5 10 15 30 60 120 240 360
IVIG Treatment (mins)

Figure 4.7 - Neutralizing antibody time dependently reduces the amounts of released and cell adherent virus at 4^{o} C

After addition of Kp7 at an MOI of 10 plates were incubated at 4^{o} C for 4 hrs, followed by five IMDM washes, IVIG was added to ST cultures for varying periods of time (x-axis) followed by a thorough wash and a further 6-hr incubation in 2% FBS/IMDM at 4^{o} C. Supernatants **(A)** and cell adherent virus **(B)** were assessed for infectious virus as described in the methods. No statistical analysis was performed due to an n of 2.

Figure 4.8

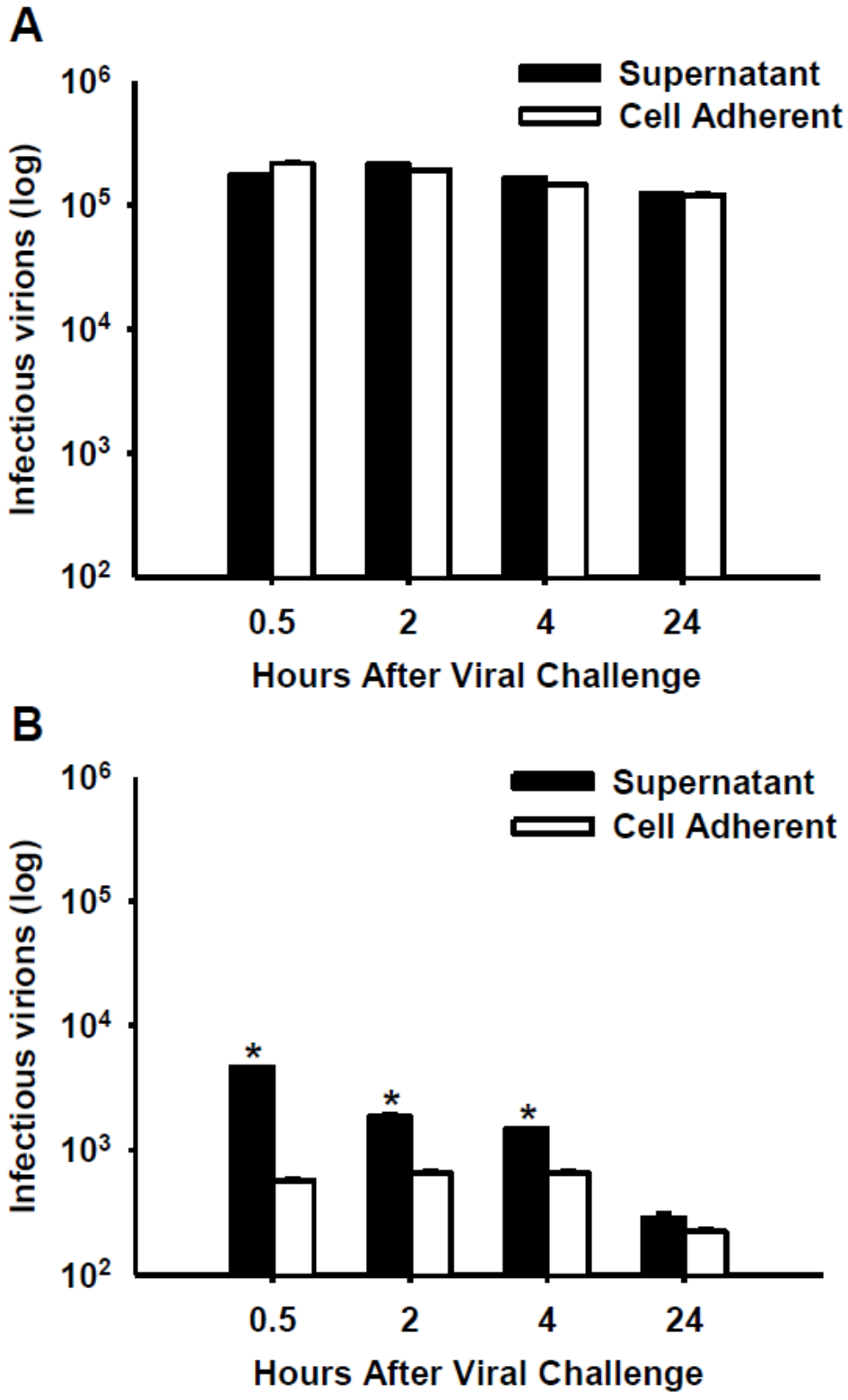

A
Supernatant
Cell Adherent
Infectious virions (log)
10⁶
10⁵
10⁴
10³
10²
0.5
2
4
24
Hours After Viral Challenge
B
Supernatant
Cell Adherent
Infectious virions (log)
10⁶
10⁵
10⁴
10³
10²
*
*
*
0.5
2
4
24
Hours After Viral Challenge

Figure 4.8 - Pre-treatment of virus with heparin dramatically reduces reversible binding of HCMV to ST

ST cultures were challenged for 4 hours at an MOI of 10 with untreated KP7 or KP7 pre-treated with 20 µg/mL heparin for 30 mins. Cultures were then extensively washed and further incubated as described in the Methods. Infectious virus was then isolated from supernatants and cell lysates at the respective time points. **(A)** Untreated virus **(B)** Virus pre-treated with heparin. Results are presented as the mean ± SEM from three independent experiments. * = significantly different from cell adherent virus ($p<0.05$). The lack of detectable error bars is due to very little inter-experimental error.

Figure 4.9

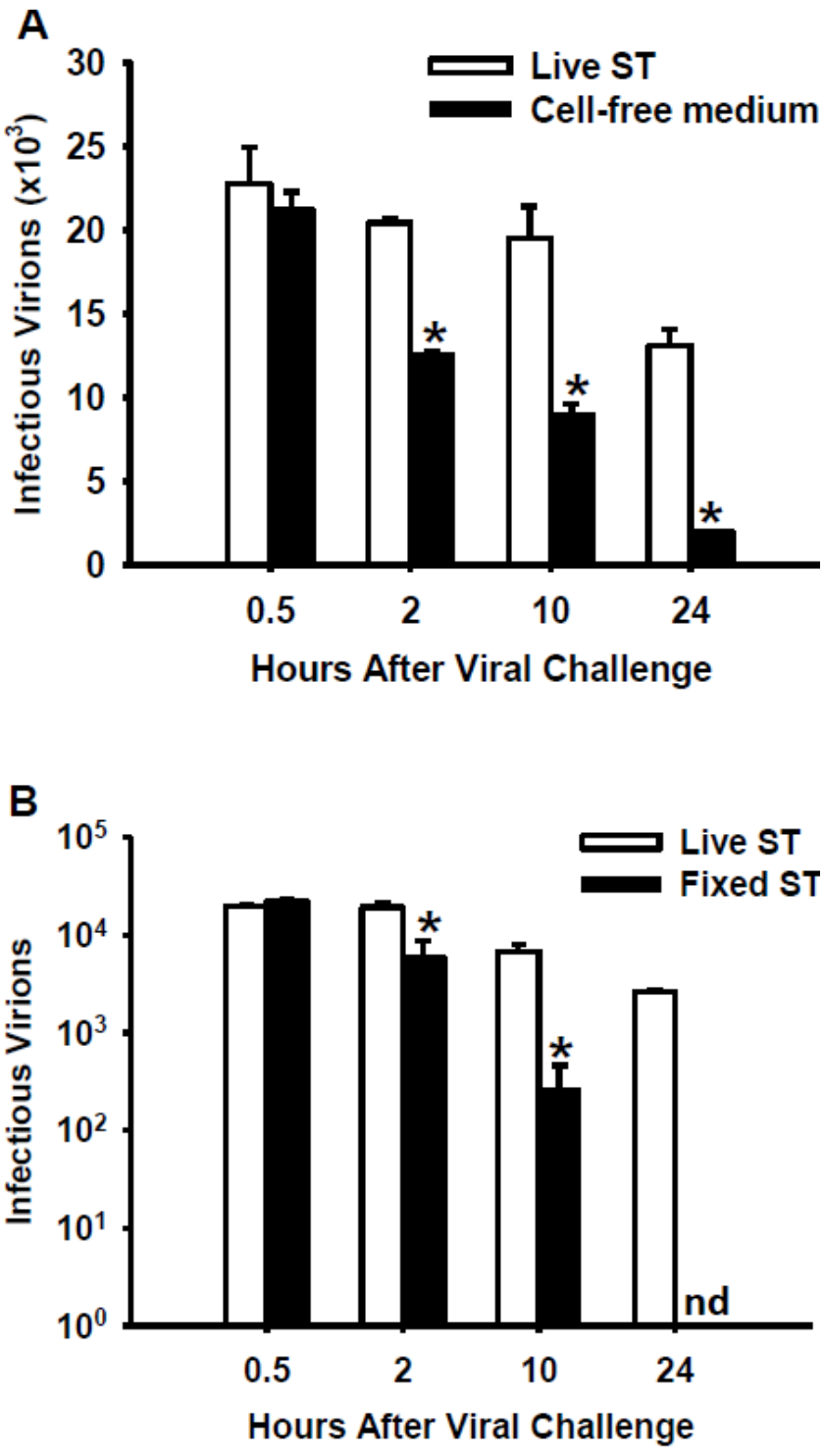

A
Live ST
Cell-free medium
Infectious Virions (x10^3)
30
25
20
15
10
5
0
*
*
*
0.5
2
10
24
Hours After Viral Challenge
B
Live ST
Fixed ST
Infectious Virions
10^5
10^4
10^3
10^2
10^1
10^0
*
*
nd
0.5
2
10
24
Hours After Viral Challenge

Figure 4.9 - HCMV is protected from inactivation by live ST cultures

(A) Kp7 was added at an MOI of 1 to ST cultures or to microwells containing medium only with no cells. Supernatants were removed from triplicate wells at each time point and assessed for infectious virus as described in the Methods. Results are presented as the mean percent ± SEM of infectious virus remaining relative to the original inoculum. **(B)** Kp7 was added (at an MOI of 10) to live ST cultures or ST cultures fixed with 4% paraformaldehyde. Infectious virus from combined cell lysates and supernatants were assessed as described in the Methods. Results are presented as the mean ± SEM from three independent experiments. * = significantly different from Live ST ($p<0.05$), nd = not detected.

4.4 Summary of Results

1. HCMV can reversibly bind to the ST as demonstrated by high levels of infectious inoculum virus found released into supernatants and remaining cell adherent over time.
2. Inoculum HCMV is protected from degradation for over 48 hrs in ST culture and requires live ST cells but is not necessarily an active process because it occurs at 4^{o} C as well.
3. Detected infectious virus was inoculum and not progeny as shown by cycloheximide treatment.
4. The amount of infectious virus available for reversible binding in fibroblasts is significantly less then ST, most likely due to CMV readily infecting fibroblasts therefore reducing the amount infectious virus available.
5. Microvilliation is likely important for reversible binding, but not ST specific as similar reversible binding was seen in CaCo2 cells.
6. ST was poorly infected even after longer exposure to infectious virus, supporting the importance of intact ST acting as a barrier in the placenta.
7. Pre-treatment of virus with heparin dramatically reduces the reversible binding of HCMV in STs.

4.5 References

Ahlfors, K., S. A. Ivarsson, et al. (1999). "Report on a long-term study of maternal and congenital cytomegalovirus infection in Sweden. Review of prospective studies available in the literature." Scand J Infect Dis **31**(5): 443-457.

Bakardjiev, A. I., J. A. Theriot, et al. (2006). "Listeria monocytogenes traffics from maternal organs to the placenta and back." PLoS Pathog **2**(6): e66.

Boppana, S. B., L. B. Rivera, et al. (2001). "Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity." N Engl J Med **344**(18): 1366-1371.

Cha, T. A., E. Tom, et al. (1996). "Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains." J Virol **70**(1): 78-83.

Compton T, N. D., Cooper NR (1993). "Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate." Virology **193**(2): 834-841.

Compton, T., D. M. Nowlin, et al. (1993). "Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate." Virology **193**(2): 834-841.

Constantin, C. M., D. Masopust, et al. (2007). "Normal establishment of virus-specific memory CD8 T cell pool following primary infection during pregnancy." J Immunol **179**(7): 4383-4389.

Demmler, G. J. (1991). "Infectious Diseases Society of America and Centers for Disease Control. Summary of a workshop on surveillance for congenital cytomegalovirus disease." Rev Infect Dis **13**(2): 315-329.

Feire, A. L., H. Koss, et al. (2004). "Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain." Proc Natl Acad Sci U S A **101**(43): 15470-15475.

Fisher, S., O. Genbacev, et al. (2000). "Human cytomegalovirus infection of placental cytotrophoblasts in vitro and in utero: implications for transmission and pathogenesis." J Virol **74**(15): 6808-6820.

Fowler, K. B., S. Stagno, et al. (1992). "The outcome of congenital cytomegalovirus infection in relation to maternal antibody status." N Engl J Med **326**(10): 663-667.

Fried, M. and P. E. Duffy (1996). "Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta." Science **272**(5267): 1502-1504.

Goff, E., B. P. Griffith, et al. (1987). "Delayed amplification of cytomegalovirus infection in the placenta and maternal tissues during late gestation." Am J Obstet Gynecol **156**(5): 1265-1270.

Griffith, B. P., S. R. McCormick, et al. (1985). "The placenta as a site of cytomegalovirus infection in guinea pigs." J Virol **55**(2): 402-409.

Guleria, I. and J. W. Pollard (2000). "The trophoblast is a component of the innate immune system during pregnancy." Nat Med **6**(5): 589-593.

Harrison, C. J. and M. G. Myers (1990). "Relation of maternal CMV viremia and antibody response to the rate of congenital infection and intrauterine growth retardation." J Med Virol **31**(3): 222-228.

Hemmings, D. G., R. Kilani, et al. (1998). "Permissive cytomegalovirus infection of primary villous term and first trimester trophoblasts." J Virol **72**(6): 4970-4979.

Hodgkin, P. D., A. A. Scalzo, et al. (1988). "Murine cytomegalovirus binds reversibly to mouse embryo fibroblasts: implications for quantitation and explanation of centrifugal enhancement." J Virol Methods **22**(2-3): 215-230.

Kumazaki, K., K. Ozono, et al. (2002). "Detection of cytomegalovirus DNA in human placenta." J Med Virol **68**(3): 363-369.

Lazzarotto, T., L. Gabrielli, et al. (2004). "Congenital cytomegalovirus infection: recent advances in the diagnosis of maternal infection." Hum Immunol **65**(5): 410-415.

Lucchi, N. W., R. Koopman, et al. (2006). "Plasmodium falciparum-infected red blood cells selected for binding to cultured syncytiotrophoblast bind to chondroitin sulfate A and induce tyrosine phosphorylation in the syncytiotrophoblast." Placenta **27**(4-5): 384-394.

McDonagh, S., E. Maidji, et al. (2006). "Patterns of human cytomegalovirus infection in term placentas: a preliminary analysis." J Clin Virol **35**(2): 210-215.

Medawar, P. (1953). "Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates." Symp Soc Exp Biol **7**: 320-338.

Morrish, D. W., J. Dakour, et al. (1997). "In vitro cultured human term cytotrophoblast: a model for normal primary epithelial cells demonstrating a spontaneous differentiation programme that requires EGF for extensive development of syncytium." Placenta **18**(7): 577-585.

Muhlemann, K., R. K. Miller, et al. (1992). "Cytomegalovirus infection of the human placenta: an immunocytochemical study." Hum Pathol **23**(11): 1234-1237.

Pereira, L., E. Maidji, et al. (2003). "Human cytomegalovirus transmission from the uterus to the placenta correlates with the presence of pathogenic bacteria and maternal immunity." J Virol **77**(24): 13301-13314.

Porath, A., R. A. McNutt, et al. (1990). "Effectiveness and cost benefit of a proposed live cytomegalovirus vaccine in the prevention of congenital disease." Rev Infect Dis **12**(1): 31-40.

Revello, M. G. and G. Gerna (2002). "Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant." Clin Microbiol Rev **15**(4): 680-715.

Revello, M. G., M. Zavattoni, et al. (1998). "Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy." J Infect Dis **177**(5): 1170-1175.

Seale, H., R. Booy, et al. (2009). "Trends in hospitalizations for diagnosed congenital cytomegalovirus in infants and children in Australia." BMC Pediatr **9**: 7.

Stagno, S. (2001). Infectious diseases of the fetus and newborn infant. K. J. Remington JS. Philadelphia, Pa, W.B. Suanders Co.**:** 389-424.

Stagno, S., R. F. Pass, et al. (1986). "Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome." Jama **256**(14): 1904-1908.

Trowsdale, J. and A. G. Betz (2006). "Mother's little helpers: mechanisms of maternal-fetal tolerance." Nat Immunol **7**(3): 241-246.

Williamson, W. D., G. J. Demmler, et al. (1992). "Progressive hearing loss in infants with asymptomatic congenital cytomegalovirus infection." Pediatrics **90**(6): 862-866.

Ziyaeyan, M., A. Alborzi, et al. (2007). "Detection of HCMV DNA in placenta, amniotic fluid and fetuses of seropositive women by nested PCR." Eur J Pediatr **166**(7): 723-726.

Chapter 5

Human cytomegalovirus viral load: A comparison of the placenta, maternal blood, maternal urine and neonatal saliva

5.1 Introduction

Human cytomegalovirus (HCMV) is the most common cause of congenital infection; however it is not routinely screened for in newborns (Demmler 1991). Occurring in approximately 1% of all births (Alford, Stagno et al. 1990; Boppana, Rivera et al. 2001), affected infants suffer a broad range of ailments. Clinical symptoms of congenital CMV-infected neonates include intrauterine growth restriction, hepatosplenomegaly, microcephaly, sensorineural hearing loss, and thrombocytopenic purpura (Paixao, Almeida et al. 2005; Ornoy and Diav-Citrin 2006). It is a primary infection in the mother that often results in these severe sequelae; subsequently, a maternal primary infection results in a 30-40% rate of viral transmission to the fetus (Raynor 1993; Kenneson and Cannon 2007). With reactivation or reinfection with HCMV, the risk of transmission to the fetus is only 2% (Raynor 1993; Kenneson and Cannon 2007); however, this is not insignificant as HCMV is endemic in the population and over 60% of neonates infected *in utero* are born to mothers with some kind of preconceptional immunity to the virus (Demmler 1991; Ornoy and Diav-Citrin 2006).

In women of childbearing age the seroprevalence of HCMV ranges between 50-85% in developed countries (Demmler 1991; Paradowska, Przepiorkiewicz et al. 2006). Seroconversion, *de novo* appearance of virus-specific IgG and/or IgM, occurs in up to 4% of all pregnancies (Hagay, Biran et al. 1996) and can be used to define a primary HCMV infection during pregnancy. A secondary HCMV infection, via reinfection with another strain or reactivation from latency can be diagnosed if there is a significant rise in IgG and or IgM antibodies in a person who was previously infected (Ornoy and Diav-Citrin 2006). However, there is a low specificity of CMV-specific IgM antibodies as a marker

of CMV infection due to their persistence in the body (Kanengisser-Pines, Hazan et al. 2009). CMV-positive IgM antibodies can be detected in both primary and secondary infections for up to18 months (Alford, Stagno et al. 1990; Ornoy 2002; Lazzarotto, Gabrielli et al. 2004). By identifying IgG post-conception and determining a seronegative IgG pre-conception valid seroconversion can be established (Revello and Gerna 1999; Yinon, Farine et al. 2010).

Mothers who are seropositive prior to pregnancy are typically asymptomatic as they exhibit a normal humoral and cell-mediated response to HCMV (Lilleri, Fornara et al. 2007; Lilleri, Zelini et al. 2009). HCMV-positive infants born to these mothers are often born asymptomatic (Ornoy and Diav-Citrin 2006); however they are at high risk for developing long-term neurological sequelae (Dollard, Grosse et al. 2007). Of these infants, 10-15% will go on to develop sensorineural hearing loss during their first 6 years of life (Fowler, McCollister et al. 1997; Grosse, Dollard et al. 2009). Therefore, previous maternal immunity does not provide complete protection against transmission to the fetus or resulting sequelae. Since most children born with congenital CMV infection have no physical abnormalities that suggest infection, neonatal screening of hearing, based on clinical identification, is often missed (Fowler, Dahle et al. 1999).

Routine serologic screening for pregnant women is not currently recommended by the public health authority in Canada (Revello and Gerna 2002; Yinon, Farine et al. 2010), largely because there is no effective prenatal treatment available (Yinon, Farine et al. 2010). However, in symptomatic congenital CMV infected infants, Kimberlin *et al* prevented hearing deterioration by treating newborn symptomatic CMV-infected infants with intravenous ganciclovir therapy (Kimberlin, Lin et al. 2003). Ganciclovir inhibits the function of DNA polymerase thereby preventing replication of viral DNA. Treatment began within the first month of life and the symptomatic infants received 6 mg/kg every 12 hrs for six weeks. No ganciclovir recipient had hearing deterioration at six months when compared with 41% of untreated patients (Kimberlin, Lin et al. 2003). A follow-

up study which administered the same dosage to another cohort of symptomatic neonates revealed fewer developmental delays at 6 and 12 months when compared to untreated patients (Oliver, Cloud et al. 2009). These studies demonstrate a possible treatment for CMV disease involving the central nervous system if detected in a newborn (Kimberlin, Lin et al. 2003; Oliver, Cloud et al. 2009). In order to identify neonates as CMV positive, efficient and effective screening must be in place.

HCMV congenital infections are the result of transplacental transmission of CMV (Yinon, Farine et al. 2010). One mechanism for HCMV to reach the fetus is through breaks in the syncytiotrophoblast (ST) layer of the placenta, which separates maternal and fetal circulations. The ST has extensive microvillae that results in a large surface area which is exposed directly to maternal blood (Chan, Hemmings et al. 2002). Our lab has shown, *in vitro,* HCMV reversibly binds to the ST thereby potentially allowing the virus to be harbored in the placenta. *In vivo,* it has been suggested that the guinea pig placenta acts as a viral reservoir for guinea pig CMV (Goff, Griffith et al. 1987; Revello and Gerna 2002). Here, the virus is protected from circulating maternal antibodies thereby maintaining the maternal infection throughout gestation (Harrison and Myers 1990). Furthermore, using a guinea pig model inoculated with CMV mid-gestation, Griffith *et al.* collected blood and placentas from dams and tested for CMV at term (Griffith, McCormick et al. 1985). None of the dams displayed viremia, however, 78% of the placentas were positive for CMV (Griffith, McCormick et al. 1985). Preferential accumulation and protection of the virus in the ST could affect the systemic distribution of CMV and increase the incidence of fetal infections.

Primary infections, reactivation or reinfection with another strain during pregnancy are all risk factors for intrauterine HCMV transmission to the fetus (Fowler, Stagno et al. 1992). All lead to an active infection; however, viral load in blood (Revello, Zavattoni et al. 1998; Lazzarotto, Gabrielli et al. 2004) or urine (Stagno 2001) does not correlate with intrauterine transmission which occurs even when virus in these compartments is low or undetectable. .We therefore

hypothesized that viral load in the placenta, but not maternal blood or urine would correlate with the incidence of CMV positive neonates. Furthermore, we sought to correlate CMV viral load at time of delivery in maternal compartments to seroconversion, maternal age, maternal weight, maternal height, birth weight, and other demographics. We obtained pilot data in which we tested maternal plasma, maternal urine and placentas for the presence of HCMV DNA via TaqMan qPCR (Appendix C). This data indicated the presence of HCMV DNA in 4/51 placentas, but not in maternal blood or urine (Appendix C). Our full study obtained data relating to seroprevalence of CMV in our pregnant cohort as well as documented seroconversion during pregnancy

5.2 Methods

All methods of this chapter are described above in 3.3.

5.3 Results

5.3.1 Demographics of Cohort

We had 175 women consented to our study for collection of maternal blood, maternal urine, placenta, and a neonatal throat swab (Table 5.1). The median age was 31 years with a gravida of 2. The median pre-pregnancy height was 163.0 cms and pre-pregnancy weight 70.0 kgs. 78% of neonates were delivered by cesarean section while the remaining 22% were normal spontaneous vaginal delivery. 51% of neonates were males and 49% females, born at a median gestational age of 38 weeks and 3 days. The median birth weight was 3430 grams and the placental wet weight median was 525.1 grams, providing an overall birth weight to placental weight ratio of 6.38. Apgar scores and cord gas results for neonates were in normal healthy clinical ranges (Table 5.1).

5.3.2 HCMV seroprevalence and seroconversion in our cohort

Serology was performed to identify HCMV IgG positive women at time of delivery. Samples were considered positive if they were above the cut-off of 0.4, which is the previously demonstrated mean OD of CMV-seronegative sera

(Figure 5.1A). Overall, 60% of our population was seropositive for HCMV IgG and 40% was seronegative (Figure 5.1B). Of the seropositive population 94/105 prenatal blood samples were available from the Provincial Public Health Laboratory (Edmonton, AB). Two of the 94 prenatal bloods were HCMV IgG negative and therefore 2.1% of our seropositive population seroconverted during pregnancy (Table 5.2). Of the two seroconversion cases, case 88 was identified as HCMV positive in urine, but not maternal blood, placenta or neonatal throat swab (Table 5.4). The second case, 161, was identified as HCMV positive in placenta and neonatal throat swab, but not maternal blood or urine (Table 5.4).

5.3.3 HCMV Seropositive versus seronegative cohort demographics

No significant differences were found between age, gravida, pre-pregnancy height/weight, mode of delivery, placental weight, sex of neonate, gestational age, arterial umbilical cord gas pH and apgar scores (Table 5.2). Venous umbilical cord gas pH was significantly different between the two groups ($p = 0.001$). The seropositive group was significantly higher (7.34) than the seronegative group (7.28); however, both were in normal clinical ranges. Similarly, the birth weight of neonates were significantly different between the groups ($p = 0.020$). The seropositive group gave birth to smaller babies (3370 g) compared to the seronegative group (3510 g), but still within the normal healthy weight range for newborns.

5.3.4 Standard curve for light cycler HCMV gB qPCR

An internal standard curve for quantification of CMV, via glycoprotein B (gB) viral load in different sample types was set up (Figure 5.2). A known copy number of plasmid DNA containing CMV DNA fragment (320 base pairs) was diluted in the 10-fold series standard curve ($2x10^2$ to $2x10^6$ genome copies) for the quantification of CMV viral load in different sample types. The standard curve is used to calculate the absolute concentration of target DNA in unknown samples. The slope, or efficiency of the standard curve is 1.962. A perfect amplification reaction would produce a curve with an efficiency of 2 because the

amount of target DNA would double with each amplification cycle. The error for the standard curve is 0.030.

5.3.5 Example of qPCR using one sample set, Case 161: maternal urine, maternal plasma, placenta and neonatal throat swab, amplification curves for HCMV gB

In an amplification reaction, the cycle at which the fluorescence of a sample rises above the background florescence is called the crossing point; in this case, the amplified product was HCMV gB. In a negative sample, no amplification is seen. After extracting DNA from samples we performed qPCR to identify HCMV gB. Unknown samples were compared against standards of known concentration ($2x10^3$ and $2x10^5$ or $2x10^6$ genome copies). Negative results, indicating the lack of detection of HCMV gB were found in maternal urine (Figure 5.3A) and maternal plasma (Figure 5.3B). Positive results, indicating the presence of HCMV gB were found in the placenta (Figure 5.3C) and neonatal throat swab (Figure 5.3D).

5.3.6 HCMV gB qPCR results

Only the HCMV seropositive population (n=105) was assessed for HCMV gB via qPCR in maternal plasma, maternal urine and placental tissue. However, neonatal throat swabs (n=175) were assessed weekly for all infants. 0/105 maternal plasma samples were positive for HCMV. 5.7% percent (6/105) of maternal urine samples were positive for HCMV (Table 5.4; Figure 5.4A). 3.8% percent (4/105) of placental samples were positive for HCMV (Table 5.3; Figure 5.4C). All positive urine and placental samples were from separate maternal-neonatal pairs. 3/175 neonates were identified as HCMV gB positive via qPCR throat swab (Table 5.4; Figure 5.4B); however, two of the neonates were confirmed negative by HCMV urine culture diagnostic testing by the Provincial Public Health Laboratory (Edmonton, AB) (Table 5.4) and follow-up qPCR urine tests. Therefore, 0.6% (1/175) of our neonatal population was confirmed positive

for HCMV congenital infection. This baby was delivered stillborn and the mother had confirmed seroconversion during pregnancy (Table 5.2).

5.3.7 Demographics of HCMV positive samples

Due to our small sample size (n=9), differences between groups could not be determined statistically. However, our HCMV-positive neonates initially identified by qPCR of throat swab were all born at an earlier gestational age and from mothers who were gravida of 2 or less and all three mothers were also 23 years or younger. Case 161, had confirmed congenital CMV infection, with maternal seroconversion. The mother was primary gravida and 19 years of age. The baby was stillborn at 26 weeks gestation at a weight of 1310 g. The placenta-positive group had a tendency for higher gravida and older age. Similarly, the urine positive group was also older in age, ranging from 27-41 years.

5.4 Summary of Results

1. 60.0% (105/175) of our population was seropositive for HCMV at the time of delivery.
2. No differences were found between seronegative or seropositive groups for maternal age, gravida, height, weight, mode of delivery, placental weight, sex of neonate, gestational age, apgar scores, arterial umbilical cord gasses or birth weight / placenta weight ratio.
3. Significant differences were found between seronegative and seropositive groups for venous cord gases and neonatal birth weight. The seropositive group had a significantly higher venous umbilical cord gas and significantly lower birth weight. However, both were still within normal healthy ranges for newborns.
4. No plasma samples were gB qPCR positive; however, 10% of the seropositive population was HCMV gB qPCR positive in maternal urine or placenta.
5. Two percent of the seropositive population had seronegative first trimester serums and were seropositive for HCMV IgG at the time of delivery.
6. The rate of congenital HCMV infection was 0.6% (1/175). The one confirmed positive neonate, case 161, was stillborn to a mother with a primary HCMV infection as confirmed by serology.
7. Case 161, displaying intrauterine transmission had virus present in the neonatal throat swab as well as placenta, but not in maternal plasma or urine.

Table 5.1 - General Demographics of Pregnant Cohort

Mothers **{n = 175}**		**Neonates** **{n=175}**	
Age **(years)**	31 (27-34) [18-50 yrs]	**Sex (M)** **(F)**	51% {n=89} 49% {n=86}
Gravida	2 (2-3) [1-10]	**Gestational Age** **(Weeks)** **(Days)**	38 (38-39) 3 (1-5)
Pre-Pregnancy Height (cms)	163 (157-168) {n = 165}	**Apgar Score** **(1 min)** **(5 min)**	9 9 [0-10]
Pre-Pregnancy Weight (kgs)	70 (61-86) {n = 169}	**Cord Gasses (pH)** **(Artery)** **(Vein)**	7.28 {n=121} 7.32 {n=124}
C/S or NSVD	78% {n=136} 22% {n=39}	**Birth Weight (g)**	3430 (3150-3710)
Placental Weight (g)	525.1 (459-616) {n = 157}	**BW/PW Ratio**	6.38 (5.67-7.12) {n = 157}

{ } = number of samples
n ( ) = Median (Inter-quartile range)
[ ] = range
C/S = caesarean section
NSVD = normal spontaneous vaginal delivery
BW/PW = Birth weight / Placental weight

Figure 5.1

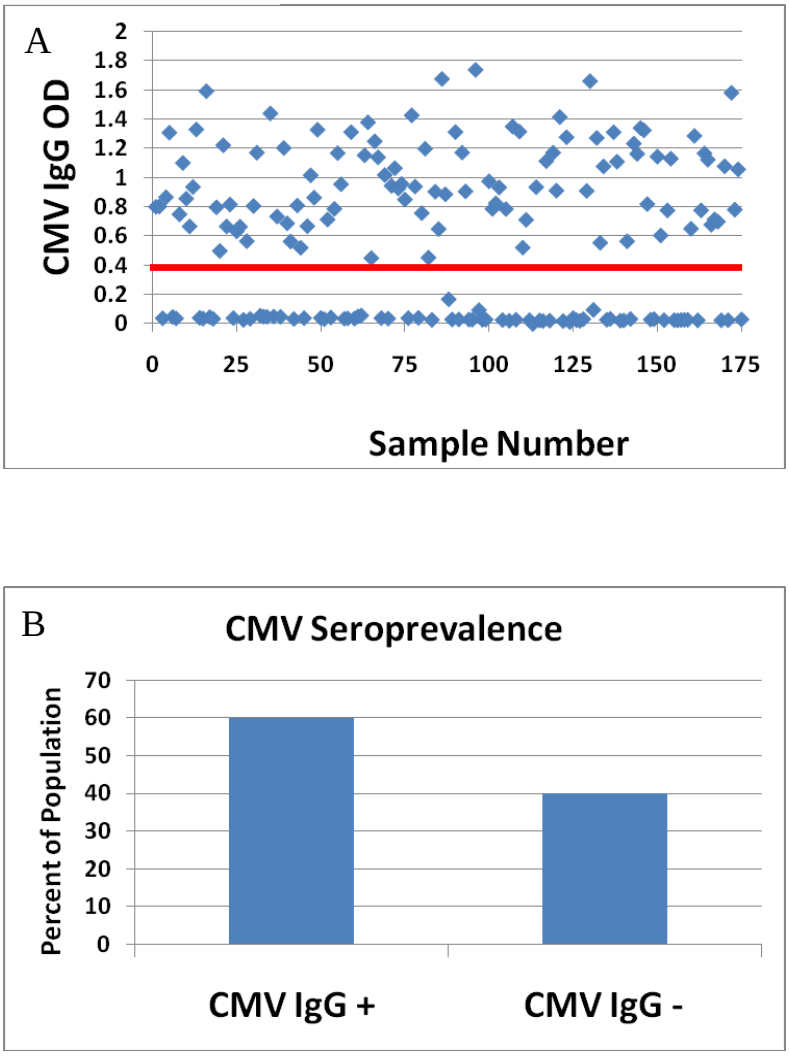


Figure 5.1 - CMV IgG OD values and seroprevalence

Table 5.2 - HCMV Seroconversion during Pregnancy

Case #	Prenatal CMV IgG	Postnatal CMV IgG
88	neg	pos
161	neg	pos

Table 5.3 - HCMV Seropositive versus Seronegative Population

Mothers {n = 175}			
	CMV IgG+ {n = 105}	**CMV IgG- {n = 70}**	***p <0.05***
Age (years)	31 (27-34)	30 (25-35)	0.151
Gravida	2 (2-3)	2 (2-3)	0.970
Pre-Pregnancy Height (cms)	163 (157-158) {n = 99}	164 (158-168) {n = 66}	0.599
Pre-Pregnancy Weight (kgs)	69.8 (60-85) {n = 102}	72 (64-87) {n = 67}	0.300
C/S or NSVD	80% {n=85} 19% {n=20}	73% {n=51} 27% {n=19}	0.749
Placental Weight (g)	519.1 (451-614) {n = 96}	555.2 (465-621) {n = 61}	0.360
Neonates {n = 175}			
	Maternal CMV IgG+ {n = 105}	**Maternal CMV IgG- {n = 70}**	***p <0.05***
Sex (M) (F)	53% {n=56} 47% {n=49}	47% {n=39} 53% {n=37}	0.151
GA (Weeks) (Days)	38 (38-39) 3 (1-5)	39 (38-39) 3 (1-4)	0.836 0.757
Apgar Score (1 min) (5 min)	9 9	9 9	na na
Cord Gasses (pH) (Artery) (Vein)	7.28 {n=72} 7.34 {n=75}	7.28 {n=49} 7.28 {n=49}	0.814 0.001*
Birth Weight (g)	3370 (3090-3640)	3510 (3250-3830)	0.020*
BW/PW Ratio	6.29 (5.6-7.1) {n = 96}	6.42 (5.8-7.1) {n = 61}	0.796

*Statistically different
{ } = number of samples
n ( ) = Median (Inter-quartile range)
C/S = caesarean section
NSVD = normal spontaneous vaginal delivery
na = no statistical analysis performed
BW/PW Ratio = Birth weight / placental weight ratio

Figure 5.2

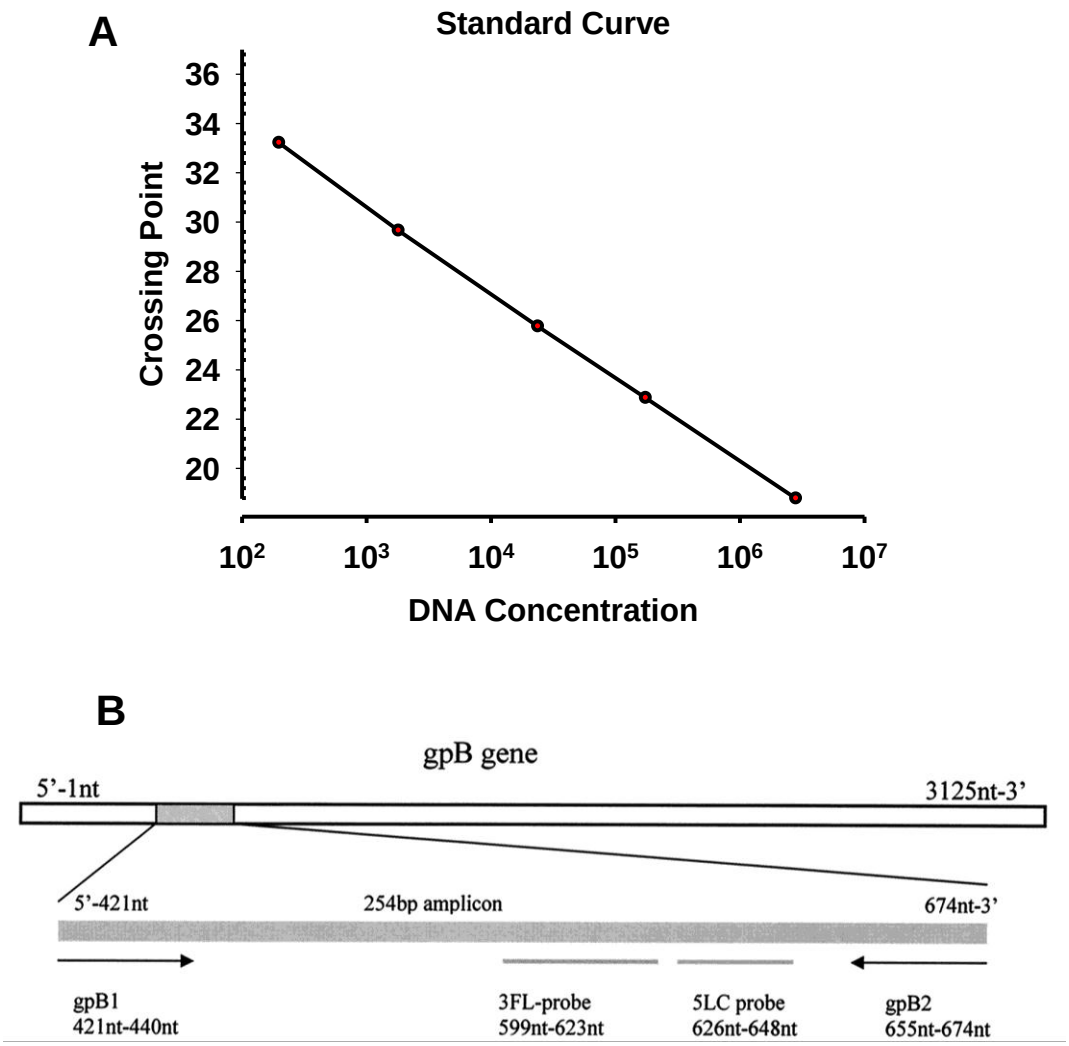


Figure 5.2 - Standard Curve for HCMV

An internal standard curve for quantification of CMV, via glycoprotein B (gpB), viral load in different sample types was set up (A). A known copy number of plasmid DNA containing CMV DNA fragment (320 base pairs) was diluted in the 10-fold series ($2x10^2$ to $2x10^6$ genome copies) standard curve for the quantification of CMV viral load in different sample types. The sequence and location of PCR primers and probes in the CMV glycoprotein B (gpB) gene (accession no. A13758) (B).

Figure 5.3

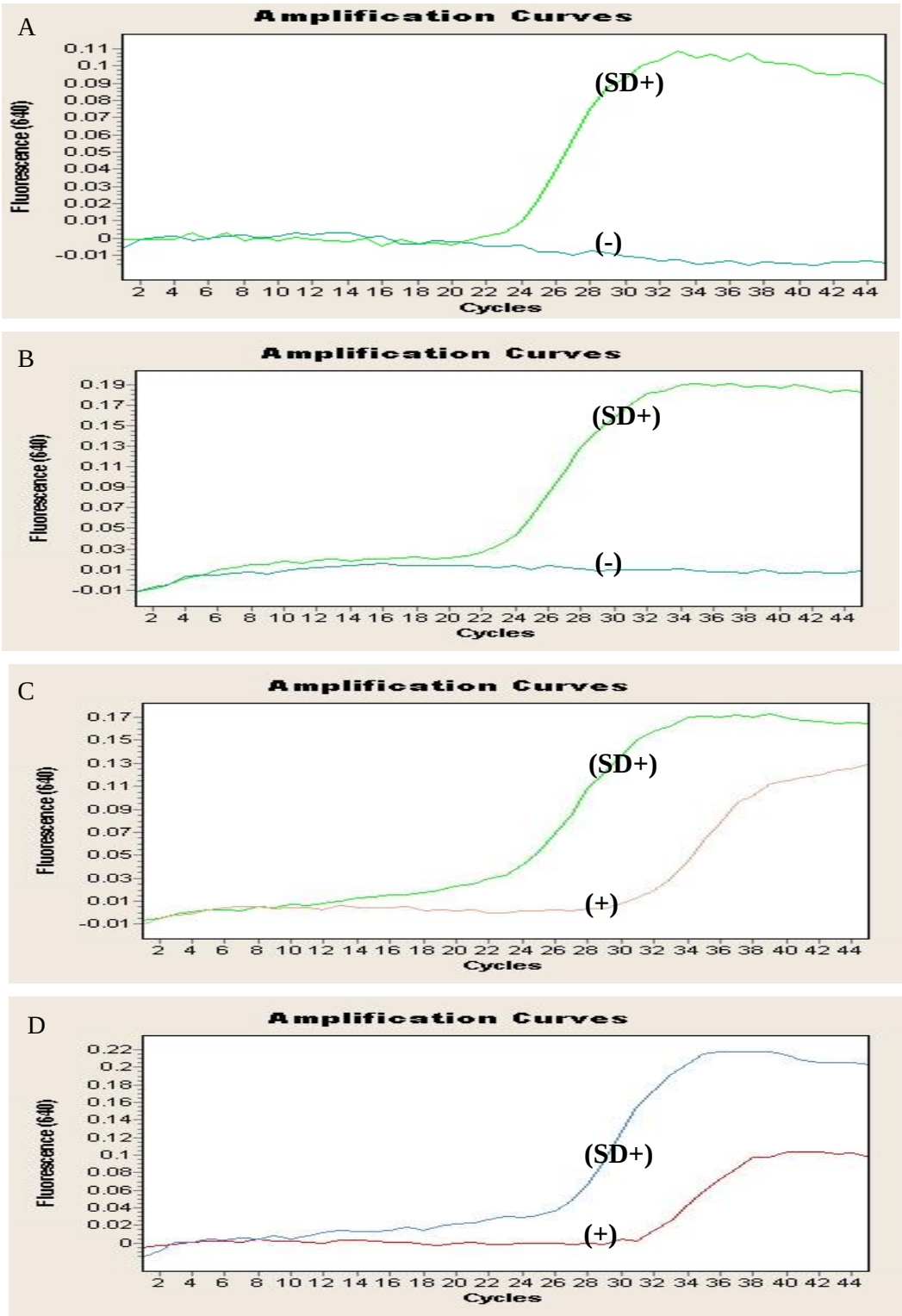

A
Amplification Curves
Fluorescence (6-FAM)
Cycles
(SD+)
(-)
B
Amplification Curves
Fluorescence (6-FAM)
Cycles
(SD+)
(-)
C
Amplification Curves
Fluorescence (6-FAM)
Cycles
(SD+)
(+)
D
Amplification Curves
Fluorescence (6-FAM)
Cycles
(SD+)
(+)

Figure 5.3 – Amplification curves for HCMV gB

Unknown samples were compared against standards (SD+) of known concentration ($2x10^5$ genome copies or $2x10^6$). The amplification curves show the fluorescence acquired each cycle and that is plotted against the cycle number. Negative results (-), indicating the lack of HCMV gB were found in maternal urine (A) and maternal plasma (B). Positive results (+), indicating the presence of HCMV gB were found in the placenta (C) and neonatal throat swab (D).

Table 5.4 - qPCR HCMV gB Positive Results

Case #	Maternal Plasma (copies/mL)	Maternal Urine (copies/mL)	Placenta (copies/µg DNA)	Neonatal Throat Swab (copies/mL)	Urine Culture
10	neg	$5.87x10^4$	neg	neg	nt
48	neg	$7.49x10^6$	neg	neg	nt
80	neg	$1.00x10^5$	neg	neg	nt
88	neg	$2.64x10^4$	neg	neg	nt
117	neg	$9.62x10^3$	neg	neg	nt
163	neg	$1.57x10^5$	neg	neg	nt
73	neg	neg	neg	$1.27x10^6$	neg
114	neg	neg	neg	$3.88x10^4$	neg
161	neg	neg	$1.04x10^5$	$2.93x10^5$	Stillborn
1	neg	neg	$1.16x10^7$	neg	nt
65	neg	neg	$4.55x10^4$	neg	nt
66	neg	neg	$1.19x10^4$	neg	nt

neg = negative result via qPCR

nt = not tested

Figure 5.4

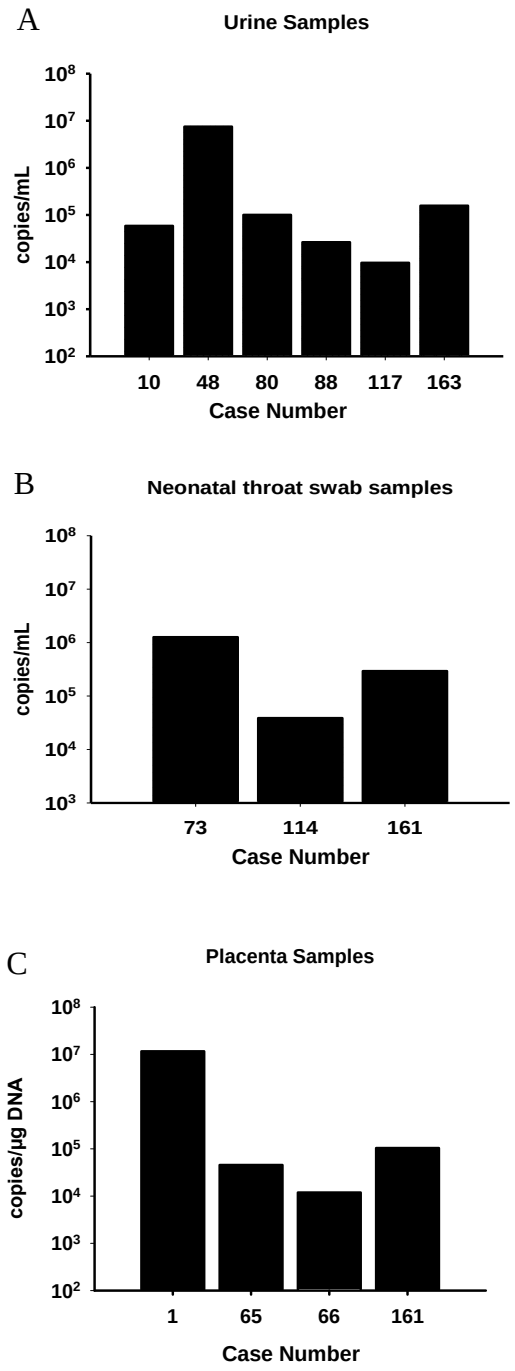


Figure 5.4 - qPCR HCMV gB Positive Results

Cases of qPCR HCMV gB positive samples for (A) maternal urine (B) neonatal throat swab samples (C) placenta samples

Table 5.5 - HCMV Positive Patient Demographics

Case #	Maternal Age (years)	Gravida	Height (cm)	Pre-pregnancy Weight (kg)	C/S OR NSVD	Sex	Gestational Age		Apgar Scores		Cord Gas		Birth Weight (g)	BW/PW Ratio
							Weeks	Days	1 min	5 min	A	V		
Urine														
10	36	3	145.00	53.00	C/S	F	38	5	9	9	7.35	7.33	3440.00	NA
48	34	2	165.10	55.60	C/S	F	39	4	9	9	7.24	7.31	2930.00	7.16
80	41	4	163.00	60.00	C/S	M	38	6	9	9	7.33	7.73	3260.00	6.58
88	36	3	157.00	55.00	NSVD	F	38	0	5	9	NA	NA	2620.00	6.37
117	30	4	158.00	43.18	C/S	M	38	2	9	10	NA	NA	3460.00	7.69
163	27	1	157.00	72.30	C/S	M	40	0	9	9	7.05	NA	4030.00	6.83
Neonatal throat swab														
73	23	2	165.00	85.00	NSVD	M	36	2	9	9	7.31	7.36	2770.00	5.93
114	23	1	NA	NA	NSVD	M	34	5	6	9	7.20	7.31	2190.00	3.55
161	19	1	170.00	65.00	NSVD	F	26	5	NA	NA	NA	NA	1310.00	2.94
Placenta														
1	43	4	165.00	69.00	C/S	M	39	0	8	9	7.29	7.36	3430.00	7.20
65	28	4	149.86	47.70	C/S	M	39	0	9	9	7.30	7.34	3300.00	5.55
66	31	6	160.00	60.00	C/S	F	38	2	7	9	7.08	7.21	3430.00	7.78
161	19	1	170.00	65.00	NSVD	F	26	5	NA	NA	NA	NA	1310.00	2.94

C/S = caesarean section
NSVD = normal spontaneous vaginal delivery
NA = not available
BW/PW Ratio = Birth weight / placental weight

5.5 References

Alford, C. A., S. Stagno, et al. (1990). "Congenital and perinatal cytomegalovirus infections." Rev Infect Dis **12 Suppl 7**: S745-753.

Boppana, S. B., L. B. Rivera, et al. (2001). "Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity." N Engl J Med **344**(18): 1366-1371.

Chan, G., D. G. Hemmings, et al. (2002). "Human cytomegalovirus-caused damage to placental trophoblasts mediated by immediate-early gene-induced tumor necrosis factor-alpha." Am J Pathol **161**(4): 1371-1381.

Demmler, G. J. (1991). "Infectious Diseases Society of America and Centers for Disease Control. Summary of a workshop on surveillance for congenital cytomegalovirus disease." Rev Infect Dis **13**(2): 315-329.

Dollard, S. C., S. D. Grosse, et al. (2007). "New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection." Rev Med Virol **17**(5): 355-363.

Fowler, K. B., A. J. Dahle, et al. (1999). "Newborn hearing screening: will children with hearing loss caused by congenital cytomegalovirus infection be missed?" J Pediatr **135**(1): 60-64.

Fowler, K. B., F. P. McCollister, et al. (1997). "Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection." J Pediatr **130**(4): 624-630.

Fowler, K. B., S. Stagno, et al. (1992). "The outcome of congenital cytomegalovirus infection in relation to maternal antibody status." N Engl J Med **326**(10): 663-667.

Goff, E., B. P. Griffith, et al. (1987). "Delayed amplification of cytomegalovirus infection in the placenta and maternal tissues during late gestation." Am J Obstet Gynecol **156**(5): 1265-1270.

Griffith, B. P., S. R. McCormick, et al. (1985). "The placenta as a site of cytomegalovirus infection in guinea pigs." J Virol **55**(2): 402-409.

Grosse, S. D., S. Dollard, et al. (2009). "Newborn screening for congenital cytomegalovirus: Options for hospital-based and public health programs." J Clin Virol **46 Suppl 4**: S32-36.

Hagay, Z. J., G. Biran, et al. (1996). "Congenital cytomegalovirus infection: a long-standing problem still seeking a solution." Am J Obstet Gynecol **174**(1 Pt 1): 241-245.

Harrison, C. J. and M. G. Myers (1990). "Relation of maternal CMV viremia and antibody response to the rate of congenital infection and intrauterine growth retardation." J Med Virol **31**(3): 222-228.

Kanengisser-Pines, B., Y. Hazan, et al. (2009). "High cytomegalovirus IgG avidity is a reliable indicator of past infection in patients with positive IgM detected during the first trimester of pregnancy." J Perinat Med **37**(1): 15-18.

Kenneson, A. and M. J. Cannon (2007). "Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection." Rev Med Virol **17**(4): 253-276.

Kimberlin, D. W., C. Y. Lin, et al. (2003). "Effect of ganciclovir therapy on hearing in symptomatic congenital cytomegalovirus disease involving the central nervous system: a randomized, controlled trial." J Pediatr **143**(1): 16-25.

Lazzarotto, T., L. Gabrielli, et al. (2004). "Congenital cytomegalovirus infection: recent advances in the diagnosis of maternal infection." Hum Immunol **65**(5): 410-415.

Lilleri, D., C. Fornara, et al. (2007). "Development of human cytomegalovirus-specific T cell immunity during primary infection of pregnant women and its correlation with virus transmission to the fetus." J Infect Dis **195**(7): 1062-1070.

Lilleri, D., P. Zelini, et al. (2009). "Human cytomegalovirus-specific CD4+ and CD8+ T cell responses in primary infection of the immunocompetent and the immunocompromised host." Clin Immunol **131**(3): 395-403.

Oliver, S. E., G. A. Cloud, et al. (2009). "Neurodevelopmental outcomes following ganciclovir therapy in symptomatic congenital cytomegalovirus infections involving the central nervous system." J Clin Virol **46 Suppl 4**: S22-26.

Ornoy, A. (2002). "[The effects of Cytomegalic virus (CMV) infection during pregnancy on the developing human fetus]." Harefuah **141**(6): 565-568, 577.

Ornoy, A. and O. Diav-Citrin (2006). "Fetal effects of primary and secondary cytomegalovirus infection in pregnancy." Reprod Toxicol **21**(4): 399-409.

Paixao, P., S. Almeida, et al. (2005). "Diagnosis of congenital cytomegalovirus infection by detection of viral DNA in urine pools." J Virol Methods **128**(1-2): 1-5.

Paradowska, E., M. Przepiorkiewicz, et al. (2006). "Detection of cytomegalovirus in human placental cells by polymerase chain reaction." APMIS **114**(11): 764-771.

Raynor, B. D. (1993). "Cytomegalovirus infection in pregnancy." Semin Perinatol **17**(6): 394-402.

Revello, M. G. and G. Gerna (1999). "Diagnosis and implications of human cytomegalovirus infection in pregnancy." Fetal and Maternal Medicine Review **11**: 117-134.

Revello, M. G. and G. Gerna (2002). "Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant." Clin Microbiol Rev **15**(4): 680-715.

Revello, M. G., M. Zavattoni, et al. (1998). "Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy." J Infect Dis **177**(5): 1170-1175.

Stagno, S. (2001). Infectious diseases of the fetus and newborn infant. K. J. Remington JS. Philadelphia, Pa, W.B. Suanders Co.**:** 389-424.

Yinon, Y., D. Farine, et al. (2010). "Cytomegalovirus infection in pregnancy." J Obstet Gynaecol Can **32**(4): 348-354.

Chapter 6
Discussion

6.1 Human cytomegalovirus is protected from inactivation by reversible binding to villous trophoblasts

Human cytomegalovirus is the most common cause of congenital infection in newborns (Demmler 1991). It was initially thought that almost all symptomatic congenital CMV infections occurred after a primary maternal infection; however, it is now known that recurrent maternal infections also lead to symptomatic congenital infections (Ornoy and Diav-Citrin 2006). These two scenarios create a danger for every pregnant woman, as developing an effective means for screening and/or prevention for HCMV remains an obstacle (Revello and Gerna 2002). In this study we examined the relationship between HCMV and cells that form a barrier between maternal and fetal circulations, the syncytiotrophoblast (ST). Using neutralizing antibodies to HCMV, we showed that HCMV can reversibly bind to the ST and be protected from degradation for over 48 hrs in culture. Importantly, we confirmed that the detected infectious virus was inoculum virus and not due to progeny virus. Although microvilliation may be important for the reversible binding process, ST were poorly infected even after longer exposure times. This demonstrated that even when virus was continuously present, the ST still functioned as a barrier to HCMV by preventing infection. However, these data support the concept that virus being sequestered within the ST would have ready access to the fetus through breaks in this layer (Schleiss 2002).

After thorough washing of ST cultures in which HCMV had been incubated, we found a pool of infectious HCMV released into culture supernatants and a pool remaining adherent to ST. This distribution of virus was maintained for at least 48 hrs. Both cell adherent and supernatant virus were significantly reduced by low pH treatment, and also by treatment with immunoglobulin containing neutralizing antibodies to HCMV. Since low pH treatment removes strongly adherent virus from the cell surface, these results indicate that strongly adherent

virus does not enter ST for the most part, but remains infectious and is released back into culture supernatants. Infectious virus that remains adherent after extensive washing binds reversibly to the ST and when released is neutralized by IVIG treatment preventing rebinding. In a clinical study conducted by Nigro *et al* (2005), pregnant women with primary CMV infections, as defined by seroconversion and CMV DNA positive amniotic fluid, were treated by IVIG at a onetime dose of 200 U per kilogram of maternal weight (Nigro, Adler et al. 2005). It was concluded that IVIG may be effective in the treatment and potential prevention of congenital CMV infection as only 3% of babies born to the IVIG-treated group were born with CMV disease compared to 50% of babies born to the untreated group (Nigro, Adler et al. 2005). It has been suggested that the function and health of the placenta is improved by IVIG treatment (Schleiss 2006). Our results support these conclusions by providing a mechanism whereby IVIG neutralizes the active virus and prevents it from re-adhering to the ST in the placenta.

Importantly, the infectious virus detected in supernatants and adherent to cells was found to be from our original inoculum and not due to progeny virus produced by infected cells. Indeed, our experiments done in the presence of cycloheximide, to inhibit viral protein synthesis, did not affect reversible binding. This is further supported by our previous study showing that progeny virus production in ST cultures does not occur until four days after exposure to HCMV (Hemmings, Kilani et al. 1998). The inoculum virus remained infectious only in the presence of live cells and not cultures fixed in formaldehyde or in cell-free medium. Interestingly, when reversible binding experiments utilizing IVIG treatment were done at 4^0C, the results were similar to those at 37^0C. While the trophoblast is the most metabolically active tissue in the placenta (Broeder, Smith et al. 1994), and it is known that HCMV entry into cells is not metabolically silent (Compton 2004), these results suggest that metabolic activity is not necessary for reversible binding to occur.

Reversible binding of HCMV to the ST in the local environment of the

placenta could lead to sequestration of the virus even in the presence of a properly established immune response. In a murine model, lymphocytic choriomeningitis virus can be effectively removed from the maternal system through an active virus-specific CD8 T-cell response, and yet still remain present in the placenta (Constantin, Masopust et al. 2007). This indicates that the lack of clearance from the murine placenta is not due to the lack of a maternal immune response (Constantin, Masopust et al. 2007). The increased and prolonged presence of HCMV within the placenta increases the probability of entry into the fetal compartment, even when viral levels are low or undetectable in maternal blood or urine (Goff, Griffith et al. 1987). Thus, the placenta has the potential to accumulate a relatively high steady-state level of virus within the intervillous space resulting from localized binding and release events at the ST. These data are consistent with previous studies, that suggest the placenta functions as a viral reservoir for guinea pig CMV which can cross the guinea pig placenta and cause an infection *in utero* (Griffith, McCormick et al. 1985; Griffith, Chen et al. 1990; Schleiss 2002). It appears as though the physical structure of the ST layer in the human placenta, which provides protection for the fetus, inadvertently also protects HCMV by allowing it to bind in and around its receptors in the ST layer. Thus, a viral reservoir is created where HCMV is maintained, although ST is not readily infectable.

Considerable reversible binding occurs in ST and CaCo2, cells which are both highly microvilliated (Collett, Walker et al. 1997; Chan, Hemmings et al. 2002). In contrast, fibroblasts with no microvilliation showed 100-fold less reversible binding while showing the highest infection levels. CTs show similar reversible binding to that of the ST, which is likely attributable to the differentiation that immediately begins as soon as CTs are plated in culture (Morrish, Dakour et al. 1997). Indeed, term CTs show some microvilliation when examined under the electron microscope, however not to the extent of that seen in STs (Hemmings and Guilbert 2002). CTs isolated using the same methodology as we have described and cultured for 24 hrs spontaneously develop microvillae

(Morrish, Dakour et al. 1997). Thus, we speculate that microvilliation may be an important cellular requirement for reversible binding of HCMV. Interestingly, the ST display the lowest infection levels, with CaCo2 cultures showing a 2.5 fold higher infection efficiency at a 2-hr time point and 3-fold higher at 24 hrs. Thus, ST is functioning as a strong protective barrier to viral infection, even in the prolonged presence of HCMV through reversible binding. The difference between infection efficiency of ST and CaCo2 could in part be attributed to the differences in expression of cell-specific receptors for HCMV tethering, docking and entry.

The initial tethering of HCMV is to HSPGs and this is followed by stronger binding to cell surface receptors that allow viral entry (Compton, Nowlin et al. 1993; Feire, Koss et al. 2004). It has previously been shown that HSPGs are a vital first step in HCMV infection; for example, HCMV does not bind to or infect fibroblast cells lacking HSPGs (Compton, Nowlin et al. 1993). We postulated that the reversible binding on ST occurs by interaction of the virus with HSPGs. We found that pre-treatment of virus with heparin dramatically reduced reversible binding in STs. Heparin binds to glycoproteins on the virus blocking the binding of HCMV to HSPGs, implicating HSPGs as the viral receptor responsible for reversible binding. HSPGs can act as either receptors or co-receptors for various ligands, stimulating signal transduction leading to gene regulation (Dreyfuss, Regatieri et al. 2009). It is therefore likely that in addition to sequestering virus in the placenta, reversible binding of HCMV to HSPGs also stimulates cell signaling. Viral attachment alone is known to stimulate the cell to produce prostaglandins, reactive oxygen species and activation of nuclear factor kappa B, leading to production of inflammatory cytokines (Speir, Shibutani et al. 1996; Fortunato, McElroy et al. 2000). This has important implications for normal placental function, particularly with respect to immune privilege.

Placental infection precedes fetal infection as shown by the presence of RNA transcripts for viral proteins in each trimester and in all cell types (Trincado, Munro et al. 2005; McDonagh, Maidji et al. 2006). We and others have previously

shown that term ST can be productively infected in culture (Hemmings, Kilani et al. 1998; Maidji, Percivalle et al. 2002). However, progeny virus is mostly retained by these cells with <30% released apically (maternal side) and <1% released basally (fetal side) (Hemmings and Guilbert 2002). Given that the ST layer *in* vivo is likely much more efficient than this cell culture model, it is therefore unlikely that an ST infection leads directly to a fetal infection. However, progeny virus released into the local placental environment on the maternal side from either intact or sloughed infected ST could interact back with the ST and accumulate through reversible binding and be protected from inactivation. Interestingly, one study showed that mouse CMV binds to fibroblasts in a stable but reversible manner prior to infecting, that leads to an equilibrium of bound and free virus (Hodgkin, Scalzo et al. 1988). However, in this case, the reversible binding only occurs over a short time frame of 5 mins *in vitro* (Hodgkin, Scalzo et al. 1988). We find a similar interaction with trophoblasts which suggests that virus generated during maternal viremia or released from infected ST could be sequestered and thereby inadvertently protected by the 12 m^2 ST layer and increase the viral load in the placenta. This occurs in the presence of an established immune response to which the placenta is not susceptible, however the ST still effectively prevents a high infection level through some cellular mechanism yet undefined. It has been shown that the interaction of HCMV with innate immune co-receptors CD14 and TLR2, on the surface of cultured ST cells, increases tumor necrosis factor alpha expression and induces apoptosis in neighboring uninfected cells, ultimately leading to damage of the villous trophoblast (Chan, Hemmings et al. 2002; Chaudhuri, Lowen et al. 2009). Thus, the continual proximity of infectious virus in or around the ST would increase the potential for breaks and also accessibility of HCMV to fetal tissues through breaks in the ST layer (Chan, Hemmings et al. 2002) or through Fc receptor uptake of virus-antibody complexes (Maidji, McDonagh et al. 2006) and ultimately increase fetal infection. Indeed, high levels of virus localized to the placenta increased the risk of *in utero* transmission in the guinea pig (Griffith,

Chen et al. 1990).

In this study, we demonstrate that the placenta can serve as a reservoir for HCMV through reversible binding of virus to HSPGs in and around the ST layer (Figure 6.1). Reversible binding protects HCMV, thereby facilitating its prolonged presence in and around the placenta. This observation and the knowledge that during an active infection, there is a lack of correlation between viral load in blood (Ahlfors, Ivarsson et al. 1999; Boppana, Rivera et al. 2001) or urine (Stagno 2001) and intrauterine transmission, has important implications for prevention and prophylactic treatment of viral infections during pregnancy. Our next step is to determine if HCMV viral load is higher in the placenta when compared to maternal blood and maternal urine during pregnancy.

6.2 Human cytomegalovirus viral load: A comparison of maternal blood, maternal urine, placenta and neonatal throat swab

Cytomegalovirus is the leading cause of congenital disease in Canada and has been estimated to affect up to 1% of the newborn population (Vaudry, Lee et al. 2007; Vaudry, Rosychuk et al. 2010). Based on our study, the rate of congenital CMV infection was 0.60% in our delivering population which is similar to that found in other urban Canadian communities. A study performed by Larke *et al*. in Hamilton, Ontario showed that 0.42% of births resulted in a congenital cytomegalovirus infection (Larke, Wheatley et al. 1980). To date, the presence of HCMV in maternal blood or urine has not been associated with intrauterine transmission of HCMV (Revello, Zavattoni et al. 1998; Stagno 2001; Lazzarotto, Gabrielli et al. 2004). Our study suggests that the presence of HCMV in the placenta, even when cleared from maternal blood or urine, may be associated with intrauterine transmission.

Sixty percent of our population was seropositive for HCMV at the time of delivery. Between our seronegative and seropositive groups, maternal age, gravida, weight, height, etc. showed no significant differences. However, birth weight was significantly decreased in the seropositive group. While still in the normal healthy range for gestational age (3290g-3877g) (Kramer, Platt et al.

2001), this is an interesting observation. A typical clinical manifestation of symptomatic congenitally infected infants is low birth weight (Muller, Eis-Hubinger et al. 2008), or small for gestational age neonates (Yinon, Farine et al. 2010). Low birth weight (<2500 g), contributes to an increased risk of neonatal mortality and morbidity; it is also associated with developmental origins of adult disease such as the development of cardiovascular complications and types 2 diabetes later in life (McIntire, Bloom et al. 1999; Barker, Eriksson et al. 2002). While our seropositive population is not classified as low birth weight, the decrease in birth weight when compared to the seronegative group indicates a possible association between HCMV seropositivity and a reduced birth weight. The venous cord gas pH was also significantly higher in the seropositive group when compared to the seronegative. As venous cord gas represents the maternal acid-base status and placental function, it is possible that the increased venous cord gas is due to the placenta trying to compensate for a lower birth weight, allowing for more oxygenated blood to reach the fetus (Thorp and Rushing 1999). However, while significantly higher, our results for both groups were still within a normal healthy range of 7.20-7.45 (Westgate, Garibaldi et al. 1994).

Within our seropositive population, 9.5% (10/105) were qPCR positive for HCMV in maternal urine or placenta, but not both. 5.7 percent (6/105) of the women were HCMV-positive in urine and not plasma. It has been demonstrated that during an active HCMV infection, viremia is usually only detected for weeks whereas the virus can be found in higher levels for months in urine (Revello, Zavattoni et al. 1998; Zanghellini, Boppana et al. 1999; Arora, Novak et al. 2010). This suggests that CMV detection in maternal urine is a better indicator for an active infection. Our data demonstrates that HCMV can be present in the placenta, even when absent from maternal blood or urine. 3.8 percent (4/105) of our seropositive population had HCMV-positive placentas. This indicates that the lack of a detectable maternal infection may not rule out the probability of having a congenital infection. Chow *et al.* tested placentas for multiple viruses at term and found that CMV was also detected in four percent of placentas during their studies

in a random pregnant population (Chow, Craig et al. 2006). Furthermore, in a cohort displaying seroconversion during pregnancy, CMV was detected in 64% of placentas (Chow, Craig et al. 2006). In our population, we were able to compare seroconversion to not only CMV detection in the placenta, but also other compartments. Of the two cases of seroconversion, we detected the presence of CMV in one of the placentas, while not present in maternal blood or urine. In the same case, I detected CMV in the neonatal throat swab, thereby associating the presence of CMV in the placenta with congenital CMV. Our lab has now shown *in vitro* that HCMV is capable of binding, releasing, and rebinding to the ST. This reversible binding is maintained *in vitro* and potentially protects the virus from maternal immune system degradation, via the placenta functioning as an immune privileged site.

Two women seroconverted during pregnancy, based on a CMV IgG negative prenatal blood and CMV IgG positive at the time of delivery. The rate of seroconversion is consistent with previously reported numbers of 1-4% (Naessens, Casteels et al. 2005; Bhatia, Narang et al. 2010). One case, 88, was positive for HCMV as detected by qPCR in urine. The baby was born in the 10^{th} percentile for weight according to gestational age (Olsen, Groveman et al. 2010), weighing 2620 grams at 38 weeks gestation. Despite being small for gestational age, the birth weight to placental weight ratio was normal. At one minute after delivery, the baby displayed a low apgar score (which is used to assess the appearance, pulse, grimace, activity and respiration of a neonate upon delivery on a scale of 0-10) of 5, but had recovered to a 10 by five minutes and the delivery was otherwise uneventful. Of interest is that this woman was a gravida 3 and 36 years old; the older the individual, the more likely it is that they have already been in contact with HCMV prior to pregnancy. The fact that this older woman seroconverted during pregnancy demonstrates the need for prenatal screening at all ages. In the other case of seroconversion, 161, we detected congenital cytomegalovirus. The infant underwent fetal demise during gestation and was stillborn. At the time of delivery, no HCMV was detected in maternal blood or

urine via qPCR; however, the placenta contained high amounts of virus, as did the neonatal throat swab. This case demonstrates the capacity of HCMV to be present in the placenta while cleared from the maternal blood or urine. The mother was 19 years of age and primigravida; young maternal age and primigravida are both risk factors for a primary HCMV infection (Larke, Wheatley et al. 1980; Kenneson and Cannon 2007). Clinically, the mother presented at week 20 with an abnormal ultrasound, indicating severe fetal non-immune hydrops. A ToRCH, serological screening for toxoplasmosis, rubella, cytomegalovirus and herpes simplex virus, was performed on the mother and a maternal primary CMV infection was confirmed. Further ultrasound showed placental thickening and oligohydraminos. An autopsy of the stillborn infant was not performed and therefore our qPCR results could not be confirmed.

Interestingly, we identified two other neonates as qPCR CMV positive by throat swab. These babies were confirmed CMV negative by urine culture, and urine qPCR. Both infants were delivered vaginally to seropositive mothers and no clinical anomalies were apparent. It is likely that the mothers were shedding CMV vaginally at the time of delivery even though they were negative in plasma and urine. This is supported by data showing that in a population of 993 pregnant women screened for CMV by vaginal swab during gestation, 7.1% were positive for HCMV with no CMV infection detected among newborns (Tanaka, Yamada et al. 2006).

Limitations of this study include the small sample size and population of only one site. Furthermore, having only one collection point for samples (term) could have impacted our detection of an active infection at another time point during pregnancy. Also, even though the placentas were sampled in multiple locations, it is still possible that upon collecting our biopsies we missed a virally infected area of the placenta. A larger cohort is necessary to further define the relationships between HCMV seropositivity and neonatal outcomes. Furthermore, confounding variables contributing to lifestyle of our women were not assessed and could also greatly contribute to the difference in birthweights. Also, our

weights were not corrected for gestational age, and while no difference was found between gestational age of our groups this is something that should be considered in a larger cohort.

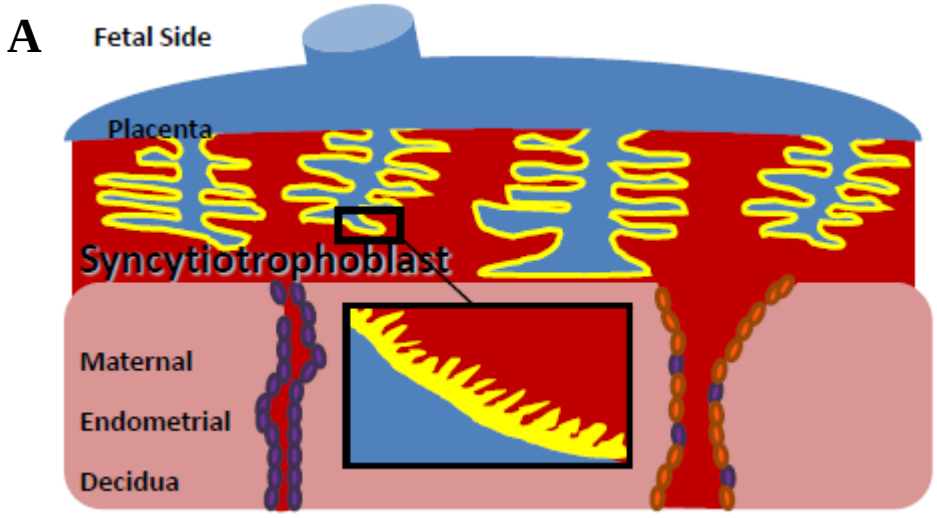
Often, HCMV-affected infants are recognized due to their severe morbidity whereas asymptomatic infants that may present later with significant neurological sequelae, often remain undiagnosed (Vaudry, Lee et al. 2007). When symptomatic at birth, neonates are routinely sent for ToRCH screening. While this offers potential for detecting CMV in symptomatic infants, asymptomatic infants would be missed. Furthermore, newborns with congenital CMV often do not produce detectable IgM, creating potential for false negative results (Vaudry, Lee et al. 2007; Kirimi, Peker et al. 2010). Another factor is that while a maternal primary infection poses a 40% risk of transmission to the fetus whereas a secondary infection has a 2% risk, more congenitally-infected babies are born to mothers with reactivation or reinfection with HCMV; this is due to the greater number of seropositive women at the time of delivery (Wang, Zhang et al. 2011). A retrospective study in the United States showed that between 1988-1994 out of approximately 40,000 babies born annually and diagnosed with congenital CMV, one quarter of them were born to mothers with primary infections while the remaining three quarters were born to mothers with pre-existing immunity to the virus (Wang, Zhang et al. 2011). This demonstrates the importance of preventive care, but also neonatal screening at the time of delivery.

A confirmed diagnosis of congenital CMV infection requires isolation of the virus from the newborn within the first three weeks of life (Vaudry, Lee et al. 2007; Vaudry, Rosychuk et al. 2010). HCMV qPCR provides an efficient and inexpensive method for initial detection of virus in multiple compartments, including the placenta (Pang, Chui et al. 2003). PCR is more sensitive than the gold standard urine culture (Revello and Gerna 2002; Gaytant, Galama et al. 2005), and throat samples have been shown to be as sensitive as urine for the diagnosis of congenital CMV infection (Warren, Balcarek et al. 1992; Balcarek, Warren et al. 1993; Vaudry, Rosychuk et al. 2010). However, as I have shown in

this study, not all HCMV-positive neonatal throat swabs are the result of a congenital infection and positive qPCR results must be confirmed by urine culture or urine qPCR of the neonate. The ease of qPCR HCMV testing as a preliminary screen for congenital CMV, should be factored into the potential development of public health measures for CMV screening during pregnancy.

It is important to focus on the correlation of viral load in the placenta to clinical diagnosis of congenital CMV infection. Further evidence demonstrating high viral load in the placenta correlated with congenital infection, even when CMV has been removed from maternal blood or urine, supports the need for neonatal screening all babies and not just ones born to mothers with a detectable maternal infection. Insights into the interaction of the placenta and virus will provide data to further understand the progression of congenital HCMV infections.

Figure 6.1



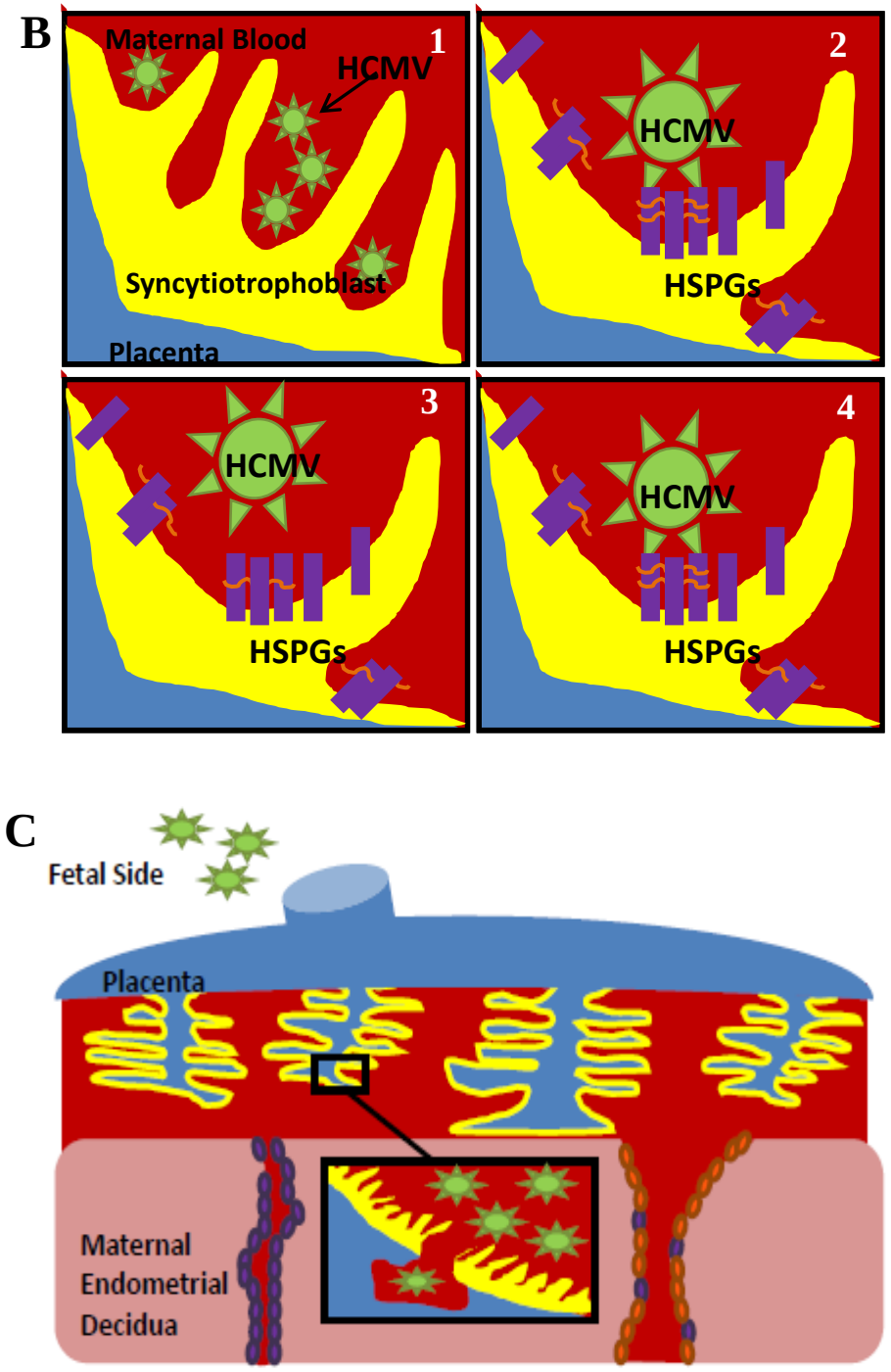


Figure 6.1 - Model for reversible binding

The ST is highly microvilliated (A) and could provide an environment that protects HCMV from the maternal immune system, thereby allowing for continual binding (B2), releasing (B3) and rebinding (B4) of the virus to the low affinity cell surface receptors, HSPGs. The accumulation of the virus in the placenta could lead to congenital infection via breaks in the ST layer (C).

6.3 Future Directions

My results demonstrate the capacity of HCMV, *in vitro*, to reversibly bind to ST. This reversible binding could in part explain my *in vivo* finding that HCMV can remain present in the placenta, even when cleared from maternal blood or urine. Collectively, these data suggest that the cellular receptor the virus is binding to for reversible binding and the cell-mediated mechanism by which the receptor is available are of importance for insight into HCMV-ST interactions.

The ST is extensively microvilliated, presents a large surface area to maternal blood and has high levels of HSPGs to which HCMV initially binds. The current model for HCMV entry into cells involves a three-step process: tethering, docking and post-attachment events. I have demonstrated that during the tethering step, reversible binding of HCMV to the ST layer occurs and protects HCMV from inactivation. This could lead to accumulation of HCMV in the placenta and increase the risk of fetal transmission by virtue of its localization.

HSPGs have been proposed as a general receptor for HCMV tethering because they are ubiquitously expressed and therefore encompass most cell types that HCMV infects (Compton, Nowlin et al. 1993). Since the placenta is an organ specific to pregnancy it is probable that its cellular receptors and response are also unique from other cell types. This tissue tropic binding is documented with RBCs that have been infected by the malaria parasite, *Plasmodium falciparum* (Alkhalil A 2000; Beeson JG 2007). The infected RBCs bind to the glycosaminoglycan, chondroitin sulfate A on the ST, but do not bind to chondroitin sulfate A on any other tissue type (Brabin BJ 2004). I have shown that when I pre-treat HCMV with heparin, there is a dramatic decrease in reversible binding of HCMV to ST. This suggests that heparin blocks the binding of HCMV to cell surface HSPGs. HCMV binding to HSPGs is a low affinity binding that could allow for release and re-binding. It has been previously noted that HSPGs are the first contact necessary for entry of the virus to the ST layer. Of interest, is the response being mediated by the ST layer upon challenge with HCMV. As EGFR and integrins represent potential receptors for docking and subsequent entry, it is important to

look at the regulation of this from the viruses' first contact to cell surface HSPGs. While my studies suggest that HSPGs are involved in tethering, the specific HSPG expressed by the ST that HCMV attaches to is uncharacterized.

There are three major types of HSPGs: syndecans, glypicans and perlecans and the placenta expresses all of them (Kirn-Safran, D'Souza et al. 2008). However, only certain syndecans and glypicans are reported to be expressed by the ST (Crescimanno, Marzioni et al. 1999). While expressed in the ST, most syndecans and glypicans are also expressed elsewhere in the utero-placental unit throughout gestation, however; Syndecan-1 (Syn-1) is the only HSPG to be constitutively expressed only in the ST throughout pregnancy (Crescimanno, Marzioni et al. 1999; Kirn-Safran, D'Souza et al. 2008).

Syn-1 is a specific HSPG expressed primarily by epithelial cells and as a transmembrane protein alters both molecular and cellular processes (Carey 1997). In most epithelia, this HSPG is expressed basolaterally and involved in cell adhesion and stabilization (Inki and Jalkanen 1996). Syn-1 also functions as a cell-surface co-receptor and activator for many growth factors and integrin molecules (Carey 1997; Lambaerts, Wilcox-Adelman et al. 2009).

The extracellular ligand of Syn-1 is what HCMV would come into contact with on the ST layer. Syn-1 has two mechanisms that function to remove the cell surface portion of its protein: endocytosis and ectodomain shedding. Similar to other HSPGs, syndecans are essential for the internalization of physiological extracellular ligands, such as epidermal growth factor receptor (EGFR) (Belting 2003), as well as certain bacteria (Smith, Gautam et al. 2006) and viruses (Gallay 2004). Upon internalization, the exact fate of syndecans is largely uncharacterized (Kirn-Safran, D'Souza et al. 2008). However, in support of removing intracellular signaling capacity, some are degraded by lysosomes (Payne, Jones et al. 2007). Another process by which the Syn-1 cellular domain is removed is ectodomain shedding which releases soluble Syn-1 into the extracellular environment (Endo, Takino et al. 2003; Hayashida, Stahl et al. 2008). The shedding is induced by heparanase and/or a variety of proteases of the matrix metalloproteinase (MMP)

family (Endo, Takino et al. 2003; Kirn-Safran, D'Souza et al. 2008). Heparanase cleaves heparan sulfate chains and while not cleaving the entire extracellular domain, it is thought to modify Syn-1 to allow for subsequent cleaving by other proteases (Mahtouk, Hose et al. 2007; Yang, Macleod et al. 2007). Syn-1 ectodomain shedding is a highly regulated process in which some kind of outside stimuli (stress, pathogen (i.e./cytokine response) outside-in signal the proteolytic cleavage of Syn-1 ectodomains at the cell surface (Hayashida, Stahl et al. 2008). The role of ectodomain shedding has been demonstrated as 1) protective in eliciting an appropriate inflammatory response (Li, Park et al. 2002; Xu, Park et al. 2005), as well as 2) pathogenic in dysregulating host defense mechanisms in a heparan sulfate-dependent manner (Park, Pier et al. 2000; Park, Pier et al. 2001).

In order to determine if Syn-1 was even a candidate for HCMV tethering, it was important to first identify the presence of Syn-1 in our primary trophoblast cultures. Previously by immunohistochemistry, Syn-1 was shown expressed in high amounts on the ST layer throughout gestation with only minimal amounts expressed on the CT (Jokimaa V 1998). In preliminary work *in vitro* on CT and ST, via immunohistochemistry for Syn-1, I have also shown high expression of Syn-1 in ST in comparison to low amounts on CTs (Appendix D; Fig 1, Fig 2). Upon determining Syn-1 was present, I then infected cells for 24 hrs and looked at Syn-1 expression (Appendix D; Fig.1, Fig.2). Assuming that Syn-1 internalization or ectodomain shedding would occur in response to an agonist as previously shown (Smith, Gautam et al. 2006; Hayashida, Stahl et al. 2008), I thought I would see a decrease in Syn-1 by immunofluorescence staining. Upon looking at the images, it appeared that uninfected ST expressed far more Syn-1 than infected ST. However, no significant difference was noted overall between uninfected and infected preparations (Appendix D; Fig.2). A possible explanation for this is that in the uninfected cultures, more cells are expressing Syn-1, whereas in the infected cultures, it appears that less cells are expressing Syn-1, but at a higher intensity leading to an equivalent overall expression in each culture. By double-

staining for IE and Syn-1, we could evaluate the cell specific expression of Syn-1 in response to an HCMV infection.

As immunofluorescence is not a strong quantitative technique, I used another method to determine Syn-1 expression in response to HCMV (Appendix D). By an in cell / on cell western assay, I determined protein expression of Syn-1 in response to increasing HCMV MOI (Appendix D). There was a trend for cell surface Syn-1 to decrease in response to increasing HCMV MOI while total Syn-1 expression remained consistent (Appendix D; Fig.3). These experiments must be replicated to ensure that the trend is a proper representation. To determine if the response to HCMV is inducing Syn-1 shedding, the supernatants from the experiments could be collected and tested for soluble levels of Syn-1. Also, looking at Syn-1 expression over a time course, with or without infection would provide information to determine if Syn-1 is shedding is accelerating in response to HCMV. Another method for looking at the role of Syn-1 on the ST in response to HCMV infection would be to use siRNA and knockdown Syn-1 expression and perform reversible binding experiments. If HCMV reversible binding was inhibited this could provide evidence for Syn-1s role as a specific tethering receptor on the ST.

Syn-1 has also been shown apically expressed in the ST indicating a role in feto-maternal communication (Jokimaa V 1998). The interaction between Syn-1 expressed on the ST and its relationship with HCMV is not yet characterized. I believe that Syn-1 represents a good potential tethering receptor for HCMV to the ST and it is the mechanism by which Syn-1 and other potential cellular receptors work together that must be further explored. As mentioned earlier, Syn-1 is a co-receptor with EGFR and several integrins. Both EGFR and integrins have been identified as potential receptors for the irreversible docking of the virus (Wang, Huong et al. 2003; Feire, Koss et al. 2004). Interestingly, microarray analyses of RNA from CMV-infected STs showed a down-regulation in EGFR as well as integrin β-chains (Schleiss, Aronow et al. 2007). If Syn-1 and EGFR are acting as co-receptors for the virus, if EGFR is downregulated, it is probable that

it is due to transmembrane signaling from Syn-1. The cell-surface domain of Syn-1 could be shed, or internalized, either leading to potential downstream signaling or lack thereof (Carey 1997; Kirn-Safran, D'Souza et al. 2008). If HCMV is only able to tether to the cell surface and not commit to cell entry by docking then the virus could be sloughed off the ST.

The regulation of Syn-1 internalization or ectodomain shedding is of interest when considering the role that it may play in intracellular signaling upon stimulation of a pathogen, such as HCMV. The cellular domain of Syn-1 has been demonstrated to bind to the small GTPase Rab5 (Hayashida, Stahl et al. 2008). Upon agonist stimulation, Rab5 dissociates from Syn-1 and could therefore be an intracellular regulator of Syn-1 shedding and another factor to investigate in trophoblasts in response to challenge with HCMV(Hayashida, Stahl et al. 2008).

While continuing to investigate the mechanisms by which HCMV interacts with the ST, I believe it is also imperative to continue work on the *in vivo* presence of HCMV in our pregnant and neonatal population. According to the Canadian Pediatric Surveillance Program, the current rate of congenital CMV infection in Canada is still unknown (Vaudry, Lee et al. 2007). Therefore, the severe morbidity and mortality associated with congenital CMV is likely underestimated. It is important to collect further epidemiological data in our population as well as screening to determine the seroprevalence and viral load in respect to neonatal outcomes. The significance of correlating viral load in women with prepregnancy immunity to neonatal outcomes supports neonatal screening at the time of delivery. This is further emphasized by our data detecting the virus in the placenta, but not maternal blood or urine. Furthermore, correlating viral load in women who seroconvert during pregnancy supports the use of potential vaccines in seronegative women prior to pregnancy. One such vaccine has completed phase II clinical trials with a positive 50% efficacy (Pass 2009). The more knowledge we can gain about the public health significance of congenital CMV, the faster research will progress towards preventative or therapeutic means for HCMV infection.

6.4 References

Ahlfors, K., S. A. Ivarsson, et al. (1999). "Report on a long-term study of maternal and congenital cytomegalovirus infection in Sweden. Review of prospective studies available in the literature." Scand J Infect Dis **31**(5): 443-457.

Alkhalil A, A. R., Valiyaveettil M, Ockenhouse CF, Gowda DC (2000). "Structural requirements for the adherence of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulphate proteoglycans of human placenta." Journal of Biological chemistry **275**(51): 40357-40364.

Arora, N., Z. Novak, et al. (2010). "Cytomegalovirus viruria and DNAemia in healthy seropositive women." J Infect Dis **202**(12): 1800-1803.

Balcarek, K. B., W. Warren, et al. (1993). "Neonatal screening for congenital cytomegalovirus infection by detection of virus in saliva." J Infect Dis **167**(6): 1433-1436.

Barker, D. J., J. G. Eriksson, et al. (2002). "Fetal origins of adult disease: strength of effects and biological basis." Int J Epidemiol **31**(6): 1235-1239.

Beeson JG, A. K., Boyle M, Duffy MF, Choong EK, Byrne TJ, Chesson JM, Lawson AM, Chai W (2007). "Structural basis for binding of *plasmodium falciparum* erythrocyte membrane protein 1 to chondroiting sulphate and placental tissue and the influence of protein polymorphisms on binding specificity." Journal of Biochemistry **262**(31): 22462-22436.

Belting, M. (2003). "Heparan sulfate proteoglycan as a plasma membrane carrier." Trends Biochem Sci **28**(3): 145-151.

Bhatia, P., A. Narang, et al. (2010). "Neonatal cytomegalovirus infection: diagnostic modalities available for early disease detection." Indian J Pediatr **77**(1): 77-79.

Boppana, S. B., L. B. Rivera, et al. (2001). "Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity." N Engl J Med **344**(18): 1366-1371.

Brabin BJ, R. C., Abedelgalil S, Mendendez C, Verhoff FH, McGready R, Fletcher KA, Owens S, d'Alessandro U, Nosten F, Fischer PR, Oridi J (2004). "The sick placenta - the role of malaria." Placenta **25**: 359-378.

Broeder, J. A., C. H. Smith, et al. (1994). "Glutamate oxidation by trophoblasts in vitro." Am J Physiol **267**(1 Pt 1): C189-194.

Carey, D. J. (1997). "Syndecans: multifunctional cell-surface co-receptors." Biochem J **327 (Pt 1)**: 1-16.

Chan, G., D. G. Hemmings, et al. (2002). "Human cytomegalovirus-caused damage to placental trophoblasts mediated by immediate-early gene-induced tumor necrosis factor-alpha." Am J Pathol **161**(4): 1371-1381.

Chaudhuri, S., B. Lowen, et al. (2009). "Human cytomegalovirus interacts with toll-like receptor 2 and CD14 on syncytiotrophoblasts to stimulate expression of TNFalpha mRNA and apoptosis." Placenta **30**(11): 994-1001.

Chow, S. S., M. E. Craig, et al. (2006). "Correlates of placental infection with cytomegalovirus, parvovirus B19 or human herpes virus 7." J Med Virol **78**(6): 747-756.

Collett, A., D. Walker, et al. (1997). "Influence of morphometric factors on quantitation of paracellular permeability of intestinal epithelia in vitro." Pharm Res **14**(6): 767-773.

Compton, T. (2004). "Receptors and immune sensors: the complex entry path of human cytomegalovirus." Trends Cell Biol **14**(1): 5-8.

Compton, T., D. M. Nowlin, et al. (1993). "Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate." Virology **193**(2): 834-841.

Constantin, C. M., D. Masopust, et al. (2007). "Normal establishment of virus-specific memory CD8 T cell pool following primary infection during pregnancy." J Immunol **179**(7): 4383-4389.

Crescimanno, C., D. Marzioni, et al. (1999). "Expression pattern alterations of syndecans and glypican-1 in normal and pathological trophoblast." J Pathol **189**(4): 600-608.

Demmler, G. J. (1991). "Infectious Diseases Society of America and Centers for Disease Control. Summary of a workshop on surveillance for congenital cytomegalovirus disease." Rev Infect Dis **13**(2): 315-329.

Dreyfuss, J. L., C. V. Regatieri, et al. (2009). "Heparan sulfate proteoglycans: structure, protein interactions and cell signaling." An Acad Bras Cienc **81**(3): 409-429.

Endo, K., T. Takino, et al. (2003). "Cleavage of syndecan-1 by membrane type matrix metalloproteinase-1 stimulates cell migration." J Biol Chem **278**(42): 40764-40770.

Feire, A. L., H. Koss, et al. (2004). "Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain." Proc Natl Acad Sci U S A **101**(43): 15470-15475.

Fortunato, E. A., A. K. McElroy, et al. (2000). "Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus." Trends Microbiol **8**(3): 111-119.

Gallay, P. (2004). "Syndecans and HIV-1 pathogenesis." Microbes Infect **6**(6): 617-622.

Gaytant, M. A., J. M. Galama, et al. (2005). "The incidence of congenital cytomegalovirus infections in The Netherlands." J Med Virol **76**(1): 71-75.

Goff, E., B. P. Griffith, et al. (1987). "Delayed amplification of cytomegalovirus infection in the placenta and maternal tissues during late gestation." Am J Obstet Gynecol **156**(5): 1265-1270.

Griffith, B. P., M. Chen, et al. (1990). "Role of primary and secondary maternal viremia in transplacental guinea pig cytomegalovirus transfer." J Virol **64**(5): 1991-1997.

Griffith, B. P., S. R. McCormick, et al. (1985). "The placenta as a site of cytomegalovirus infection in guinea pigs." J Virol **55**(2): 402-409.

Hayashida, K., P. D. Stahl, et al. (2008). "Syndecan-1 ectodomain shedding is regulated by the small GTPase Rab5." J Biol Chem **283**(51): 35435-35444.

Hemmings, D. G. and L. J. Guilbert (2002). "Polarized release of human cytomegalovirus from placental trophoblasts." J Virol **76**(13): 6710-6717.

Hemmings, D. G., R. Kilani, et al. (1998). "Permissive cytomegalovirus infection of primary villous term and first trimester trophoblasts." J Virol **72**(6): 4970-4979.

Hodgkin, P. D., A. A. Scalzo, et al. (1988). "Murine cytomegalovirus binds reversibly to mouse embryo fibroblasts: implications for quantitation and explanation of centrifugal enhancement." J Virol Methods **22**(2-3): 215-230.

Inki, P. and M. Jalkanen (1996). "The role of syndecan-1 in malignancies." Ann Med **28**(1): 63-67.

Jokimaa V, I. P., Kujari H, Hirvoren O, Ekholm E, Antila L (1998). "Expression of Syndecan-1 in human placenta and decidua." Placenta **19**(2-3): 157-163.

Kenneson, A. and M. J. Cannon (2007). "Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection." Rev Med Virol **17**(4): 253-276.

Kirimi, E., E. Peker, et al. (2010). "DNA-positive, IgM-negative symptomatic congenital cytomegalovirus infection: two case reports." J Matern Fetal Neonatal Med **23**(7): 725-727.

Kirn-Safran, C., S. S. D'Souza, et al. (2008). "Heparan Sulfate Proteoglycans and Their Binding Proteins in Embryo Implantation and Placentation." Semin Cell Dev Biol. **19**(2): 187–193.

Kramer, M. S., R. W. Platt, et al. (2001). "A new and improved population-based Canadian reference for birth weight for gestational age." Pediatrics **108**(2): E35.

Lambaerts, K., S. A. Wilcox-Adelman, et al. (2009). "The signaling mechanisms of syndecan heparan sulfate proteoglycans." Curr Opin Cell Biol **21**(5): 662-669.

Larke, R. P., E. Wheatley, et al. (1980). "Congenital cytomegalovirus infection in an urban Canadian community." J Infect Dis **142**(5): 647-653.

Lazzarotto, T., L. Gabrielli, et al. (2004). "Congenital cytomegalovirus infection: recent advances in the diagnosis of maternal infection." Hum Immunol **65**(5): 410-415.

Li, Q., P. W. Park, et al. (2002). "Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury." Cell **111**(5): 635-646.

Mahtouk, K., D. Hose, et al. (2007). "Heparanase influences expression and shedding of syndecan-1, and its expression by the bone marrow environment is a bad prognostic factor in multiple myeloma." Blood **109**(11): 4914-4923.

Maidji, E., S. McDonagh, et al. (2006). "Maternal antibodies enhance or prevent cytomegalovirus infection in the placenta by neonatal Fc receptor-mediated transcytosis." Am J Pathol **168**(4): 1210-1226.

Maidji, E., E. Percivalle, et al. (2002). "Transmission of human cytomegalovirus from infected uterine microvascular endothelial cells to differentiating/invasive placental cytotrophoblasts." Virology **304**(1): 53-69.

McDonagh, S., E. Maidji, et al. (2006). "Patterns of human cytomegalovirus infection in term placentas: a preliminary analysis." J Clin Virol **35**(2): 210-215.

McIntire, D. D., S. L. Bloom, et al. (1999). "Birth weight in relation to morbidity and mortality among newborn infants." N Engl J Med **340**(16): 1234-1238.

Morrish, D. W., J. Dakour, et al. (1997). "In vitro cultured human term cytotrophoblast: a model for normal primary epithelial cells demonstrating a spontaneous differentiation programme that requires EGF for extensive development of syncytium." Placenta **18**(7): 577-585.

Muller, A., A. M. Eis-Hubinger, et al. (2008). "Oral valganciclovir for symptomatic congenital cytomegalovirus infection in an extremely low birth weight infant." J Perinatol **28**(1): 74-76.

Naessens, A., A. Casteels, et al. (2005). "A serologic strategy for detecting neonates at risk for congenital cytomegalovirus infection." J Pediatr **146**(2): 194-197.

Nigro, G., S. P. Adler, et al. (2005). "Passive immunization during pregnancy for congenital cytomegalovirus infection." N Engl J Med **353**(13): 1350-1362.

Olsen, I. E., S. A. Groveman, et al. (2010). "New intrauterine growth curves based on United States data." Pediatrics **125**(2): e214-224.

Ornoy, A. and O. Diav-Citrin (2006). "Fetal effects of primary and secondary cytomegalovirus infection in pregnancy." Reprod Toxicol **21**(4): 399-409.

Pang, X. L., L. Chui, et al. (2003). "Comparison of LightCycler-based PCR, COBAS amplicor CMV monitor, and pp65 antigenemia assays for quantitative measurement of cytomegalovirus viral load in peripheral blood specimens from patients after solid organ transplantation." J Clin Microbiol **41**(7): 3167-3174.

Park, P. W., G. B. Pier, et al. (2001). "Exploitation of syndecan-1 shedding by Pseudomonas aeruginosa enhances virulence." Nature **411**(6833): 98-102.

Park, P. W., G. B. Pier, et al. (2000). "Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of Pseudomonas aeruginosa." J Biol Chem **275**(5): 3057-3064.

Pass, R. F. (2009). "Development and evidence for efficacy of CMV glycoprotein B vaccine with MF59 adjuvant." J Clin Virol **46 Suppl 4**: S73-76.

Payne, C. K., S. A. Jones, et al. (2007). "Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands." Traffic **8**(4): 389-401.

Revello, M. G. and G. Gerna (2002). "Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant." Clin Microbiol Rev **15**(4): 680-715.

Revello, M. G., M. Zavattoni, et al. (1998). "Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy." J Infect Dis **177**(5): 1170-1175.

Schleiss, M. R. (2002). "Animal models of congenital cytomegalovirus infection: an overview of progress in the characterization of guinea pig cytomegalovirus (GPCMV)." J Clin Virol **25 Suppl 2**: S37-49.

Schleiss, M. R. (2006). "The role of the placenta in the pathogenesis of congenital cytomegalovirus infection: is the benefit of cytomegalovirus immune globulin for the newborn mediated through improved placental health and function?" Clin Infect Dis **43**(8): 1001-1003.

Schleiss, M. R., B. J. Aronow, et al. (2007). "Cytomegalovirus infection of human syncytiotrophoblast cells strongly interferes with expression of genes involved in placental differentiation and tissue integrity." Pediatr Res **61**(5 Pt 1): 565-571.

Smith, M. F., Jr., J. K. Gautam, et al. (2006). "Microbial-induced regulation of syndecan expression: important host defense mechanism or an opportunity for pathogens?" ScientificWorldJournal **6**: 442-445.

Speir, E., T. Shibutani, et al. (1996). "Role of reactive oxygen intermediates in cytomegalovirus gene expression and in the response of human smooth muscle cells to viral infection." Circ Res **79**(6): 1143-1152.

Stagno, Ed. (2001). Infectious diseases of the fetus and newborn infant. Philadelphia, Pa, W.B. Suanders Co.

Tanaka, K., H. Yamada, et al. (2006). "Screening for vaginal shedding of cytomegalovirus in healthy pregnant women using real-time PCR: correlation of CMV in the vagina and adverse outcome of pregnancy." J Med Virol **78**(6): 757-759.

Thorp, J. A. and R. S. Rushing (1999). "Umbilical cord blood gas analysis." Obstet Gynecol Clin North Am **26**(4): 695-709.

Trincado, D. E., S. C. Munro, et al. (2005). "Highly sensitive detection and localization of maternally acquired human cytomegalovirus in placental tissue by in situ polymerase chain reaction." J Infect Dis **192**(4): 650-657.

Vaudry, W., B. Lee, et al. (2007). Congenital Cytomegalovirus Infection. Canadian Paediatric Surveillance Program Results 2007.

Vaudry, W., R. J. Rosychuk, et al. (2010). "Congenital cytomegalovirus infection in high-risk Canadian infants: Report of a pilot screening study." Can J Infect Dis Med Microbiol **21**(1): e12-19.

Wang, C., X. Zhang, et al. (2011). "Attribution of congenital cytomegalovirus infection to primary versus non-primary maternal infection." Clin Infect Dis **52**(2): e11-13.

Wang, X., S. M. Huong, et al. (2003). "Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus." Nature **424**(6947): 456-461.

Warren, W. P., K. Balcarek, et al. (1992). "Comparison of rapid methods of detection of cytomegalovirus in saliva with virus isolation in tissue culture." J Clin Microbiol **30**(4): 786-789.

Westgate, J., J. M. Garibaldi, et al. (1994). "Umbilical cord blood gas analysis at delivery: a time for quality data." Br J Obstet Gynaecol **101**(12): 1054-1063.

Xu, J., P. W. Park, et al. (2005). "Endogenous attenuation of allergic lung inflammation by syndecan-1." J Immunol **174**(9): 5758-5765.

Yang, Y., V. Macleod, et al. (2007). "Heparanase enhances syndecan-1 shedding: a novel mechanism for stimulation of tumor growth and metastasis." J Biol Chem **282**(18): 13326-13333.

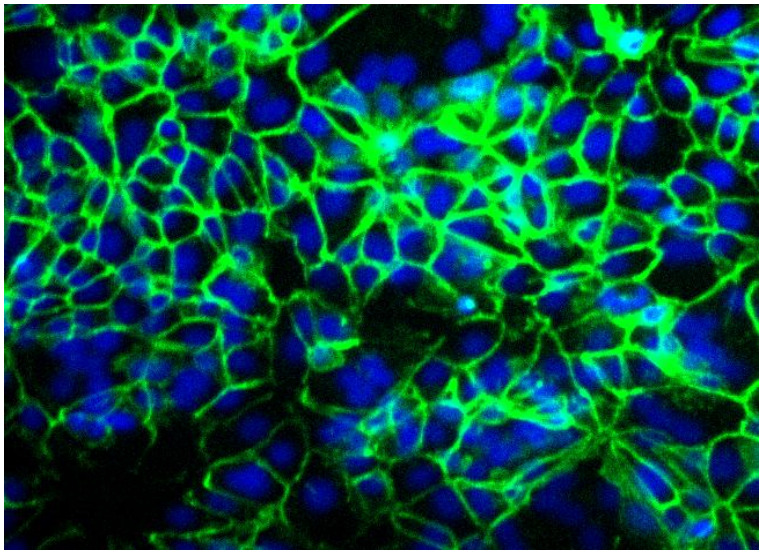
Yinon, Y., D. Farine, et al. (2010). "Cytomegalovirus infection in pregnancy." J Obstet Gynaecol Can **32**(4): 348-354.

Zanghellini, F., S. B. Boppana, et al. (1999). "Asymptomatic primary cytomegalovirus infection: virologic and immunologic features." J Infect Dis **180**(3): 702-707.

Appendix A

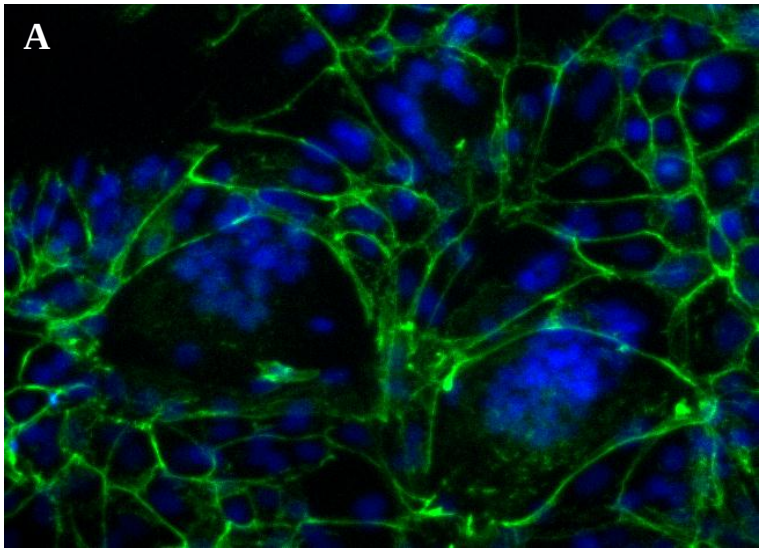
Depiction of CT and ST Cultures

Cells were plated and used as CTs or differentiated to STs by use of EGF or cAMP as described above in 3.1.1.1 and 3.1.1.2. Cells were fixed with ice-cold methanol for 10 mins at -20^{o} C and washed 3x, 2 mins each with PBS. Immunofluorescence was performed as described above, 3.1.2.2, to identify desmoplakin using FITC (green) and cellular nuclei using DAPI (blue). Digital images of each well were obtained. Multinucleation was determined by manual counting of nuclei/desmoplakin-stained cells. Mononuclear cells are defined as one nucleus per cell membrane defined by desmoplakin staining; multinuclear cells are defined as two or more nuclei per cell membrane.

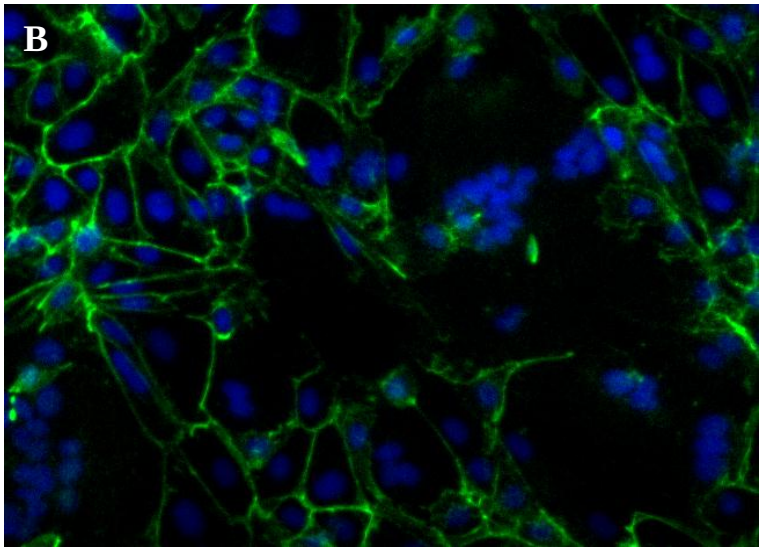


Medium Day 3

Figure 1. Immunofluorescence of Cytotrophoblast cultures at day 3.



EGF Day 5



cAMP Day 3

Figure 2. Immunofluorescence (IFC) to determine Syncytialization of trophoblast cultures (A) EGF added and IFC at day 5 (B) cAMP added and IFC at day 3

Appendix B

Forms for Clinical Study

Information Form

Title of the Research Study: **The Placenta as a Viral Reservoir**
Principal Investigator: **Denise Hemmings, Ph.D.**

Please ask for an explanation of any words or information on this form that you do not clearly understand. If you have any questions during the study, you are free to ask them at any time.

Background: Human cytomegalovirus (HCMV) is a virus that can infect an unborn baby if their mother was infected before or during pregnancy. HCMV is found in about 1 out of 100 newborns. The mother usually has few symptoms so she might not know she has an infection. Most of the time we never find out how the mother got the infection, and there is nothing she could have done to prevent it. If we find HCMV in the newborn, he or she may or may not have signs of an infection. However, even if the baby shows no signs of the infection at birth, he or she has a 1 in 10 chance of having some problems later, such as hearing loss. Therefore, close follow-up of these babies with a paediatrician and hearing specialist is very important. The placenta acts as a barrier between mother and baby during pregnancy and we believe it can hold onto a large amount of virus. The constant presence of the virus in the placenta could increase the risk of infection in the unborn baby.

Purpose of the study: We will measure the amount of HCMV in mother's blood, urine and the placenta and compare these results with whether HCMV can also be found in their newborn babies. It is important to know if the mother was infected before or during pregnancy so in mothers where HCMV is found, we will also test blood that was taken at the beginning of pregnancy for evidence of an HCMV infection. This blood is usually stored in the Provincial Laboratory of Alberta.

Procedure: If you agree to enter the study we will ask you a few questions about your pregnancy and obtain some information from your baby's chart. All of this information will be kept strictly confidential. In this study we will study samples of mom's blood and urine, baby's saliva, and the placenta. We need the following:

- ☐ About 2 tsp of blood will be taken at the same time as other blood samples
- ☐ One 5 mL container of urine from mom will be taken just before or after delivery
- ☐ The placenta
- ☐ A throat swab from your baby, within 24 hours of delivery

Please put an "X" next to the samples you will provide. Ideally we would like to have all of these samples from each participant.

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Email: denise.hemmings@ualberta.ca

Amended February 27, 2010

Possible Benefits: If HCMV is detected in your baby, paediatric specialists will carefully assess him or her for any overt symptoms and determine if any treatment is immediately necessary. The baby will be carefully followed at the neonatal follow-up clinic to watch for any long-term consequences so that early treatment can be arranged. This provides a benefit to these often asymptomatic babies since any complications can be dealt with more quickly.

Possible Risks:
Blood sample: We will collect blood at the same time as it is routinely collected so there is only a small risk of bleeding or bruising.
Baby's saliva sample: There is no risk to collection of saliva from your baby.
Placenta: None, since this tissue is normally thrown away.

If you agree to provide any or all of the samples, please read and sign the attached consent form.
Thank you for your participation.

Confidentiality: Personal records relating to this study will be kept confidential. Only the investigators listed above will have access to your records. Any report published as a result of this study will not identify you by name. By signing the consent form you give permission to the study staff to access any personally identifiable health information which is under the custody of other health care professionals as deemed necessary for the conduct of the research.

Please Note: You are free to withdraw your consent for these samples at any time, and your continuing medical care will not be affected in any way.

If you have any questions or concerns, please contact the following people:
Dr. Denise Hemmings: (780) 492-2098
Research Nurse Coordinator, RN: (780) 735-5257

If you have further concerns about any aspect of this study, you may wish to contact the Patient Relations Office of the Capital Health Authority at (780) 342-8080. This office has no affiliation with the study investigator.

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Email: denise.hemmings@ualberta.ca

Amended February 27, 2010

PART 1
Title of Project: **The Placenta as a Viral Reservoir**
Principal Investigator: **Denise G. Hemmings, Ph.D.** Phone Number: (780) 492-2098

PART 2 (to be completed by the research subject):

	Yes	No
Do you understand that you have been asked to be in a research study?	☐	☐
Have you read and received a copy of the attached Information Sheet?	☐	☐
Do you understand the benefits and risks involved in taking part in this study?	☐	☐
Have you had an opportunity to ask questions and discuss this study?	☐	☐
Do you understand that you are free to withdraw from the study at any time, without having to give a reason and without affecting your future medical care?	☐	☐
Has the issue of confidentiality been explained to you and do you understand who will have access to your medical records?	☐	☐

Who explained this study to you?_____

I agree to take part in this study: YES ☐ NO ☐

Signature of Research Subject_____

(Printed Name) _____

Date: _____

Signature of Witness _____

Signature of Investigator or Designee _____

THE INFORMATION SHEET MUST BE ATTACHED TO THIS CONSENT FORM AND A COPY GIVEN TO THE RESEARCH SUBJECT

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Email: denise.hemmings@ualberta.ca

Amended February 27, 2010

Data Sheet

Name
Hospital

Study Log Number:_____

Collection Date:_____**Normal or Preeclamptic:**_____

G:_____ **T.**_____**P.**_____**A.**_____**L.**_____

Maternal Age:_____**yrs. Race:**_____**. Pre-pregnant wt:**_____**Height**____

C/S; Y or N , NSVD; Y or N, Induced; Y or N Augment; Y or N.

If C/S any uterine activity;_____**Baby #** _____

Blood Pr (early gest)_____**Wks**_____**Cord Gasses A**_____ **V**_____

Blood Pressure at term: highest_____**Average in last 24 hrs;**_____

Epidural/Spinal : Y or N Apgars 1min___ **5min**____**Temp(fever)**__

Medications: _____

Proteinuria:_____**Urate:**_____**Hematocrit:**_____**L/L**

Hemoglobin:_____ **G/L Other lab results:**_____

Gestational Age at Delivery:___**wk**__**days Infant Birth Weight:**_____**gms.**

Sex: M or F NICU adm. Y or N Placental wt if available_____**gms.**

Smoker Y or N Other info:_____

Placenta sent to pathology Y or N, Pathologist: Dr. _____

Pertinent obstetrical history:_____

Other specimens:_____

624-3 CSC, 10240 Kingsway Ave – Edmonton, Alberta – Canada T5H 3V9 – Tel: (780) 735-5257 – Fax: (780) 735-6685 - Email: donna.dawson2@ualbertahealthservices.ca

Dear Dr._____,

This letter is written to inform you that your patient _____ (DOB) is enrolled in our study "The Placenta as a Viral Reservoir". The purpose of our study is to measure the presence and amount of Human Cytomegalovirus (HCMV) in mother's blood, urine and the placenta via Real Time PCR and compare these results with whether HCMV is also found in their newborn babies. Therefore, as part of our protocol this infant's saliva was tested for **CMV** by PCR on _____. This test has come back **POSITIVE**. This result suggests that your patient may have Congenital CMV infection. This result should be confirmed by diagnostic testing within 48 hrs. Please see attached requisition. Further diagnostic tests, management and follow up may be indicated. Dr. Wendy Vaudry (780-407-1680) is a co-investigator on this study and is also aware of the result. Please do not hesitate to contact her to discuss further. Thank you for taking the time to read this letter.

Regards,

Research Nurse Coordinator

Women's and Children's Health

Culture and Serology Requisition
Microbiology and Public Health

Provinical Laboratory of Public Health
University of Alberta Hospital,
8440 - 112 Street, Edmonton, AB T6G 2J2
Phone: (780) 407-7121 Fax: (780) 407-3864

Partners in Public Health with Capital Health

PHN / Healthcare Number | Pt. Hosp. # | Lab Accession #

Copy to Name: Dr. Wendy Vaudry
Physician Code:
Address: UAH 1D1, EDMONTON, ALBERTA T6G 2B7

☐ M ☐ F | Patient Legal Name (Last) (First) (Initial) | DOB DD MM YY

Address | City | Prov. | Postal Code

Chart # | Patient Phone # | Lab #

Ordering Physician / Practitioner | Physician Code

Ordering Address / Location | Report Location Code

Report address if different

Specimen Event Type
IA ☐ AUXILLARY
IP ☐ IN PT
OP ☐ OUT PT
AP ☐ AMBUL
HC ☐ HMCARE
ST ☐ STAFF
EN ☐ ENVIRON
WCB ☐ WORKER'S COMP

Bill Type CPL ☐ Alberta Health Care OR
CO ☐ Company OT ☐ Out of Prov
XX ☐ Pre-paid PB ☐ Patient Bill
Co. name:
Address:
Client #:

Comments Follow-up of positiv CMV result in throat swab

Date specimen collected: DD MM YY
Time (24 h)

TEST STATUS
☐ Routine
☐ Priority
☐ STAT

SPECIMEN TYPE

BODY FLUID
☐ Blood
☐ CSF
☐ Bone marrow
☒ Urine
☐ Gastric washings
☐ **Other (*specify)**

RESPIRATORY
☐ Auger suction
☐ Bronchial alveolar lavage
☐ Bronchial washing
☐ Bronchial brush
☐ ETT
☐ Nasopharynx
☐ Nose
☐ Sputum
☐ Other (*specify)

WOUND / SKIN / SURGICAL
(Specify site)
☐ Abscess
☐ Aspirate
☐ Biopsy
☐ Bone chip
☐ IV tip
☐ Skin scraping
☐ Tissue
☐ Wound Swab
☐ Other (specify)

GENITAL
☐ Cervix
☐ Urethra
☐ Vagina
☐ Other (specify)

GASTROINTESTINAL
☐ Feces
☐ Emesis
☐ Other (specify)

BACTERIOLOGY
☐ Bacterial Ag detection (specify)
☐ Bordetella pertussis
☐ Chlamydia trachomatis
☐ Clostridium difficile
☐ Culture / Sensitivity
☐ G C Screen
☐ Legionella
☐ Culture
☐ DFA
☐ Mycobacterium (TB) culture
☐ AFB smear only
☐ Mycoplasma culture
☐ Other (specify)

MOLECULAR DIAGNOSTICS*
☐ specify
*Consult laboratory

MYCOLOGY
☐ Fungus culture / exam
☐ Other (specify)

PARASITOLOGY
☐ Ova and Parasites
☐ Direct examination
☐ Other (specify)

VIROLOGY
Culture and Identification
(complete box A and E on reverse)
☐ Culture
☐ DFA
☐ Electron microscopy CMV shell
☒ Other (specify) vial cultur

SEROLOGY

VIRAL

Hepatitis
(complete box A and D on reverse)
☐ HAV IgM
☐ HAV Total (Immunity)
☐ HBV DNA
☐ HBsAG
☐ HBs Ab (Immunity)
☐ HBc Ab (Total)
☐ HBc Ab (IgM)
☐ HBeAg
☐ HBe Ab (Total)
☐ HCV Ab
☐ HCV RNA PCR*
☐ Other (specify)
*Consult Lab before submitting request.

HIV
(complete box F on reverse)
☐ HIV Ab
☐ p24 Core Ag
☐ Other (specify)

Miscellaneous
(complete box A on reverse)
☐ CMV Ab IgG
☐ CMV Ab IgM
☐ EBV Monospot
☐ EBVCA IgM
☐ EBNA IgG
☐ HHV 6 IgG (Roseola)
☐ HHV 6 IgM (Roseola)
☐ HSV IgG
☐ HSV IgM
☐ Measles IgG
☐ Measles IgM
☐ Mumps IgG
☐ Mumps IgM
☐ Parvovirus B19 IgG
☐ Parvovirus B19 IgM
☐ Ravies
☐ Rubella IgG
(complete box B on reverse)
☐ Rubella IgM
(complete box B on reverse)
☐ Varicella Zoster IgG
☐ Varicella Zoster IgM

BACTERIAL

Steptococcal
☐ Anti-Dnase B
☐ Anti-hyaluronidase
☐ ASOT

Syphilis *(complete box C on reverse)*
☐ DFA-Tp
☐ FTA-ABS
☐ MHA-Tp
☐ RPR
☐ VDRL

Other *(complete box A on reverse)*
☐ Brucella
☐ Chlamydia pneumoniae
☐ Chlamydia psittaci
☐ Diphtheria
☐ Francisella
☐ Legionella
☐ Leptospira
☐ Lyme
☐ Mycoplasma pneumoniae
☐ Pertussis
☐ Rickettsia
☐ Tetanus
☐ Yersinia
☐ Other (specify)

FUNGAL
☐ Aspergillus sp.
☐ Blastomyces sp.
☐ Candida sp.
☐ Coccidiodes sp.
☐ Farmer's lung
☐ Fusarium sp.
☐ Histoplasma sp.
☐ Paracoccidiodes sp.
☐ Penicillium Marneffei
☐ Pseudoallescheria boydii
☐ Sporothriz schenckii
☐ Zygomyctes
☐ Other (specify)

PARASITOLOGICAL
☐ Cysticercus
☐ Echinococcus
☐ Strongyloides
☐ Toxoplasma IgG
☐ Toxoplasma IgM
☐ Other (specify)

PLEASE COMPLETE PATIENT INFORMATION ON REVERSE

Culture and Serology Requisition
Microbiology and Public Health

Provincial Laboratory of Public Health
University of Alberta Hospital,
8440 - 112 Street, Edmonton, AB T6G 2J2
Phone: (780) 407-7121 Fax: (780) 407-3864

Partners in Public Health with Capital Health

PHN / Healthcare Number | Pt. Hosp. # | Lab Accession #

Copy to Name: Dr. Wendy Vaudry
Physician Code:
Address: UAH 1D1, EDMONTON, ALBERTA T6G 2B7

☐ M ☐ F | Patient Legal Name (Last) (First) (Initial) | DOB DD MM YY

Address | City | Prov. | Postal Code

Chart # | Patient Phone # | Lab #

Ordering Physician / Practitioner | Physician Code

Ordering Address / Location | Report Location Code

Report address if different

Bill Type CPL ☐ Alberta Health Care OR
CO ☐ Company OT ☐ Out of Prov
XX ☐ Pre-paid PB ☐ Patient Bill
Co. name:
Address:
Client #:

Specimen Event Type
IA ☐ AUXILLARY
IP ☐ IN PT
OP ☐ OUT PT
AP ☐ AMBUL
HC ☐ HMCARE
ST ☐ STAFF
EN ☐ ENVIRON
WCB ☐ WORKER'S COMP

Comments Follow-up of positiv CMV result in throat swab

Date specimen collected: DD MM YY
Time (24 h)

TEST STATUS
☐ Routine
☐ Priority
☐ STAT

SPECIMEN TYPE

BODY FLUID
☐ Blood
☐ CSF
☐ Bone marrow
☐ Urine
☐ Gastric washings
☐ **Other (*specify)**

RESPIRATORY
☐ Auger suction
☐ Bronchial alveolar lavage
☐ Bronchial washing
☐ Bronchial brush
☐ ETT
☐ Nasopharynx
☐ Nose
☐ Sputum
☐ Other (*specify) Throat swab in UTM (Universal Transport Media)

WOUND / SKIN / SURGICAL
(Specify site)
☐ Abscess
☐ Aspirate
☐ Biopsy
☐ Bone chip
☐ IV tip
☐ Skin scraping
☐ Tissue
☐ Wound Swab
☐ Other (specify)

GENITAL
☐ Cervix
☐ Urethra
☐ Vagina
☐ Other (specify)

GASTROINTESTINAL
☐ Feces
☐ Emesis
☐ Other (specify)

BACTERIOLOGY
☐ Bacterial Ag detection (specify)
☐ Bordetella pertussis
☐ Chlamydia trachomatis
☐ Clostridium difficile
☐ Culture / Sensitivity
☐ G C Screen
☐ Legionella
☐ Culture
☐ DFA
☐ Mycobacterium (TB) culture
☐ AFB smear only
☐ Mycoplasma culture
☐ Other (specify)

MOLECULAR DIAGNOSTICS*
☐ specify
*Consult laboratory

MYCOLOGY
☐ Fungus culture / exam
☐ Other (specify)

PARASITOLOGY
☐ Ova and Parasites
☐ Direct examination
☐ Other (specify)

VIROLOGY
Culture and Identification
(complete box A and E on reverse)
☐ Culture
☐ DFA
☐ Electron microscopy CMV shell
☒ Other (specify) vial cultur

SEROLOGY

VIRAL

Hepatitis
(complete box A and D on reverse)
☐ HAV IgM
☐ HAV Total (Immunity)
☐ HBV DNA
☐ HBsAG
☐ HBs Ab (Immunity)
☐ HBc Ab (Total)
☐ HBc Ab (IgM)
☐ HBeAg
☐ HBe Ab (Total)
☐ HCV Ab
☐ HCV RNA PCR*
☐ Other (specify)
*Consult Lab before submitting request.

HIV
(complete box F on reverse)
☐ HIV Ab
☐ p24 Core Ag
☐ Other (specify)

Miscellaneous
(complete box A on reverse)
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☐ HHV 6 IgM (Roseola)
☐ HSV IgG
☐ HSV IgM
☐ Measles IgG
☐ Measles IgM
☐ Mumps IgG
☐ Mumps IgM
☐ Parvovirus B19 IgG
☐ Parvovirus B19 IgM
☐ Ravies
☐ Rubella IgG *(complete box B on reverse)*
☐ Rubella IgM *(complete box B on reverse)*
☐ Varicella Zoster IgG
☐ Varicella Zoster IgM

BACTERIAL

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☐ VDRL

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☐ Legionella
☐ Leptospira
☐ Lyme
☐ Mycoplasma pneumoniae
☐ Pertussis
☐ Rickettsia
☐ Tetanus
☐ Yersinia
☐ Other (specify)

FUNGAL
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☐ Blastomyces sp.
☐ Candida sp.
☐ Coccidiodes sp.
☐ Farmer's lung
☐ Fusarium sp.
☐ Histoplasma sp.
☐ Paracoccidiodes sp.
☐ Penicillium Marneffei
☐ Pseudoallescheria boydii
☐ Sporothriz schenckii
☐ Zygomyctes
☐ Other (specify)

PARASITOLOGICAL
☐ Cysticercus
☐ Echinococcus
☐ Strongyloides
☐ Toxoplasma IgG
☐ Toxoplasma IgM
☐ Other (specify)

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CH-0039 Nov 2001

Appendix C

Pilot Data for HCMV Viral Load

Initially, I considered genotyping the HCMV we found in the population to see if there was a specific genotype that was more frequent in CMV infection during pregnancy. 51 samples each of maternal blood, maternal urine and placentas, were collected for DNA isolation and TaqMan real time quantitative PCR (qPCR) assessment for HCMV gB1, gB2, gB3 and gB4. The following protocol was first established by Dr. XL Pang, of the Provincial Public Health Laboratory (Edmonton, AB), and optimized for assessment of solid-organ transplant patients (Pang, Humar et al. 2008). However, this was the first time it was performed on placental tissue.

DNA isolation from maternal blood, maternal urine and placentas was performed as described above in 3.2.2. Table 1 provides the specific information of primers and probes as previously identified by the Provincial Public Health Laboratory (Edmonton, AB) (Pang, Humar et al. 2008). Each qPCR reaction contained a total volume of 25 µl: 5 µl of DNA, 12.5 µl of Universal DNA Master Mix (Applied Biosystems), 400 nM of each primer and 200 nM of each probe for gB1, gB2, gB3 and gB4 in separate tubes. Carried out in a closed tube system, ABI PRISM 7500 Sequence Detection System was used for this qPCR (Applied Biosystems). First incubated at 50^{o} C for 2 min and then at 95^{o} C for 10 min, PCR amplification occurred over 45 thermal cycles of 94^{o} C for 20 sec and 60^{o} C for 1 min. The data was collected and analyzed with Sequence Detection Software version 1.0 (Applied Biosystems).

Table 2 shows the results as positive or negative for the identification of specific genotypes (gB1-4) of HCMV in maternal blood, urine or placental tissue. Four of 51 placentas were positive for HCMV gB1, but not found in matching maternal blood or urine. While this pilot study gave a good indication to continue on with a larger sample size and include neonatal outcomes, it has to be noted that in getting the sample collection methods established some samples were not

collected. However, the last cases 39-51 are not missing any samples, demonstrating that we had an established an effective means for obtaining samples. Furthermore, serology was not performed on these samples and we could have up to 40% of the samples as seronegative. Therefore, there are gaps and more specific conclusions could not be drawn from this data. The identification of only one genotype was not unusual as gB1 was the predominant genotype identified in solid-organ transplant patients in the Edmonton area (Pang, Humar et al. 2008). Based on the finding of only HCMV gB1 positives, we decided to continue on without further genotyping.

Table 1 - Primers and Probes for TaqMan PCR system

Genotype, accession no.	Name	Sequence (5'-3')	Amplicon size (base pairs)
gB1 (HS5GLYBG)	gB1 - Forward gB1 - Reverse gB1 - Probe	CATACGACGTCTGCTGCTCACT GCTGACCGTTTGGGAAGAAG TCGATCCGGTTCAGTCTCT	72
gB2 (HS5GLXBI)	gB2 - Forward gB2 - Reverse gB2 - Probe	TCTTTGGTGGAATTGGAACGT TGTCACTCGTACTTCTTCTGGTCCTA ATCCAGTCTGAATATCA	79
gB3 (HS5GLYBM)	gB3 - Forward gB3 - Reverse gB3 - Probe	TGTTGGAACTGGAACGTTTGG TGCCCGTACTTCTCTTGGTTCT CGGTGTGAACTCCA	72
gB4 (HS5GLYBD)	gB4 - Forward gB4 - Reverse gB4 - Probe	AAACGTGTCCGTCTTCGAAACT TCCACCAGAGATTTTTGCTTGA CCGGCGGACTAGTAGT	75

Table 2 - TaqMan qPCR of Placenta, Maternal Plasma and Urine for HCMV gB1-4

Case #	Placenta				Plasma				Urine			
gB	1	2	3	4	1	2	3	4	1	2	3	4
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	n	n	n	n	n	n	n	n
3	-	-	-	-	-	-	-	-	n	n	n	n
4	-	-	-	-	-	-	-	-	n	n	n	n
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	n	n	n	n	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	n	n	n	n	n	n	n	n
14	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	n	n	n	n
16	-	-	-	-	-	-	-	-	-	-	-	-
17	+	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	n	n	n	n	n	n	n	n
19	+	-	-	-	n	n	n	n	n	n	n	n
20	-	-	-	-	n	n	n	n	n	n	n	n
21	-	-	-	-	-	-	-	-	n	n	n	n
22	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	n	n	n	n
24	-	-	-	-	n	n	n	n	-	-	-	-
25	-	-	-	-	n	n	n	n	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	n	n	n	n	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	n	n	n	n	n	n	n	n
30	-	-	-	-	n	n	n	n	n	n	n	n
31	-	-	-	-	n	n	n	n	n	n	n	n
32	-	-	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	n	n	n	n	n	n	n	n
34	-	-	-	-	-	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-	-	-	-	-	-
36	-	-	-	-	n	n	n	n	n	n	n	n
37	+	-	-	-	-	-	-	-	n	n	n	n
38	-	-	-	-	-	-	-	-	n	n	n	n
39	-	-	-	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-	-	-	-
41	-	-	-	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-	-	-	-
43	-	-	-	-	-	-	-	-	-	-	-	-
44	-	-	-	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-	-	-	-

#	Placenta				Plasma				Urine			
gB	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
46	-	-	-	-	-	-	-	-	-	-	-	-
47	-	-	-	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-	-	-	-	-	-
51	+	-	-	-	-	-	-	-	-	-	-	-

- = negative result + = positive results n = sample not collected

Pang, X., A. Humar, et al. (2008). "Concurrent genotyping and quantitation of cytomegalovirus gB genotypes in solid-organ-transplant recipients by use of a real-time PCR assay." J Clin Microbiol 46(12): 4004-4010.

Appendix D

Syndecan-1 as a Potential Receptor for Reversible Binding of HCMV to ST

I wanted to explore the role of Syndecan-1 (Syn-1) as a potential receptor for reversible binding. This appendix demonstrates some of the results to further support the rationale for further research. I first sought to identify if Syn-1 was expressed in my trophoblast populations (CT and ST). Cells were plated and used as CTs or differentiated to STs. CTs or STs were infected with clinical HCMV strain KP7 virus (MOI of 20) or left uninfected. They were centrifuged at 2500 rpm for 30 mins and incubated at 37^{o} C for 24 hrs. Cells were then fixed with ice-cold methanol for 10 mins and rinsed with PBS. Immunofluorescence was done as described in 3.1.2.2, using a primary antibody to detect Syndecan-1 (Mouse monoclonal [BC/B-B4], Abcam, Cambridge, MA; 10 ug/mL) (Figure 1). The mean intensity per field was quantified via Adobe Photoshop Elements 2.0 and divided by the number of cells in that field. The result is a measurement of mean intensity per cell (Figure 2). Immunofluorescence images appeared to show a decrease in Syn-1 expression upon infection, however once quantified, no statistical difference was noted. A possible explanation for this is that in the uninfected cultures, more cells are expressing Syn-1, whereas in the infected cultures, it appears that less cells are expressing Syn-1, but at a higher intensity leading to an equivalent overall expression in each culture.

Having identified Syn-1 expression in STs, with basal amounts being expressed in CTs, I then wanted to better assess the effect of HCMV infection on the expression of Syn-1. The following will show preliminary data, utilizing an in cell western assay, that demonstrates a trend for cell-surface Syn-1 to decrease in response to increasing HCMV MOI. In cell westerns (ICWs) are a cell-based assay that measure proteins. ICW requires signals derived from a protein of interest (POI) and a total cell count and utilizes the Odyssey® scanner to measure these outcomes by near-infrared (NIR) fluorophores (Aguilar, Zielnik et al. 2010).

In comparison with Western Blots, ICWs have been demonstrated to provide high-throughput capacity, improved precision and a reduction in sample preparation per assay (Aguilar, Zielnik et al. 2010). By permeabilizing (ICW) or non-permeabilizing (on cell western, OCW) the cellular membrane, we have the potential to identify cell surface, or total protein expression. The specific methods used are detailed below.

Trophoblasts were plated at a density of 3000 cells/mm^2 and differentiated into STs as described above. STs were then challenged with clinical HCMV isolate KP7 at MOIs of 1, 5, 10, 20, or left uninfected. Plates were centrifuged at 2500 rpm for 30 mins and incubated for 24 hrs. The cells were fixed with 3.7% formaldehyde (OCW) or 95% ice-cold methanol (ICW) and incubation at RT for 10 mins. After fixing, the plates were washed 3x with PBS, then further washed with PBS containing 0.1% Triton-x-100 (ICW), or PBS (OCW), 3x, 10 mins each on a bench top rocker and rinsed with PBS. Odyssey blocking buffer (OBB) was added at 20 ul/well for 1 hr at RT. Primary antibody detecting Syndecan-1 (Mouse monoclonal [BC/B-B4], Abcam, Cambridge, MA; 10 ug/mL) was prepared in equal amounts of PBS and OBB containing 5.0% Tween-20 and 20 ul/well, and incubated overnight at 4°C. Cells were washed with PBS, 3x, 10 mins each on a bench top rocker. Secondary antibody mixture was prepared in equal amount of PBS and OBB with 5% Tween-20 and included IRDye 800CW conjugate of goat anti-mouse IgG (Li-COR)(800-channel) used at 1:1000, 800-channel antibody signals were normalized to the 700-channel signals derived from DRAQ5 (Biostatus Ltd., Leicestershire, UK) used at 1:10,000 and Sapphire700 (LiCOR) used at 1:1000. 20 µl of secondary antibody mixture was added per well and incubated in the dark for 90 minutes. Cells were washed with PBS, 3x, 10 mins each on a bench top rocker. An Odyssey® IR scanner and Odyssey® imaging software 3.0 was used to quantify the relative expression of Syn-1 compared to controls. Note that in future experiments a negative control using a non-specific mouse monoclonal antibody should be included.

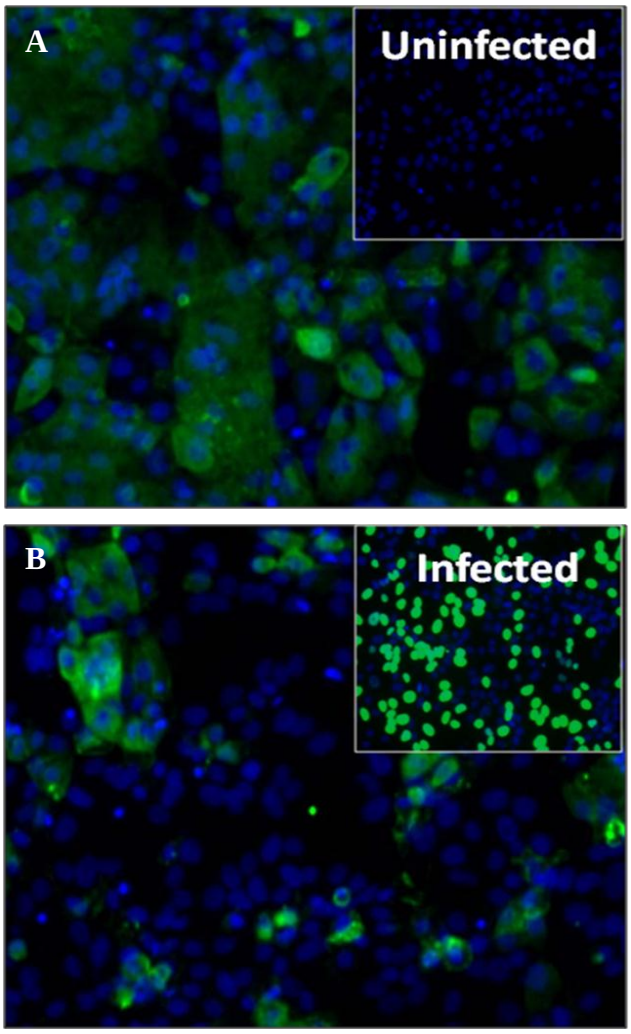

Figure 1 - Immunofluorescence for Syndcan-1 Expression in STs

Cultured ST were challenged with HCMV (MOI=20) for 24 hours and stained with primary antibodies to Syn-1 or IE, followed by a fluorescent secondary antibody. Syn-1 staining in uninfected (A) and infected (B) ST cultures. Inset in B represents expression of HCMV IE antigen in viral-challenged cultures. Syn-1 and IE were visualized with green fluorescence. Nuclei were visualized with Dapi blue.

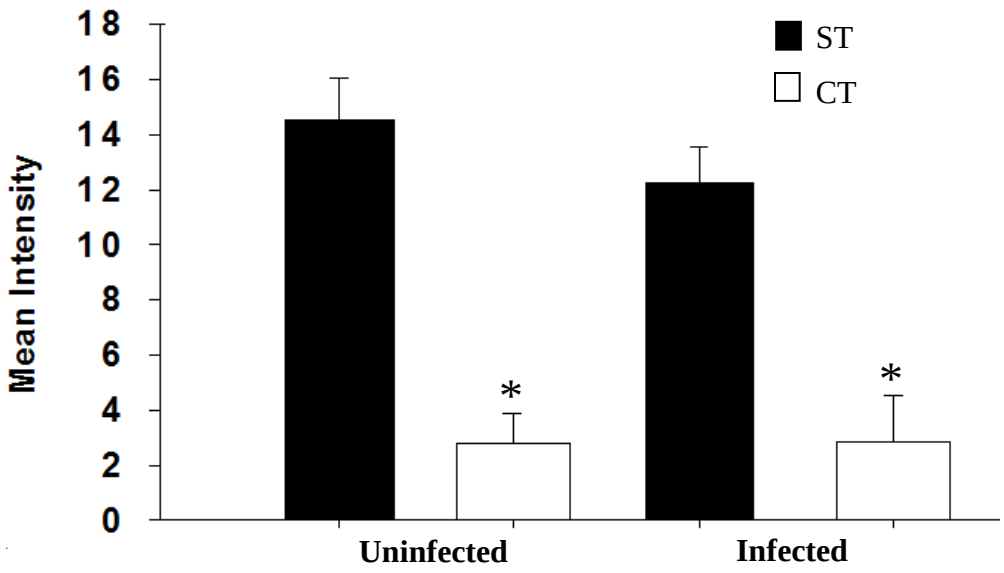

Figure 2 - Mean Intensity per cell of Syndecan-1 Expression

Cultured trophoblasts were in plain media or challenged with HCMV (MOI=20) for 24 hrs and stained with a primary antibody to Syn-1 followed by a fluorescent secondary antibody. Fluorescence was normalized to unstained controls. Mean intensity was assessed by the total intesity per field divided by the total number of cells in that field. Syn-1 expression is more abundant in ST than CT cultures. (n = 4) * = significantly different from ST ($p<0.001$).

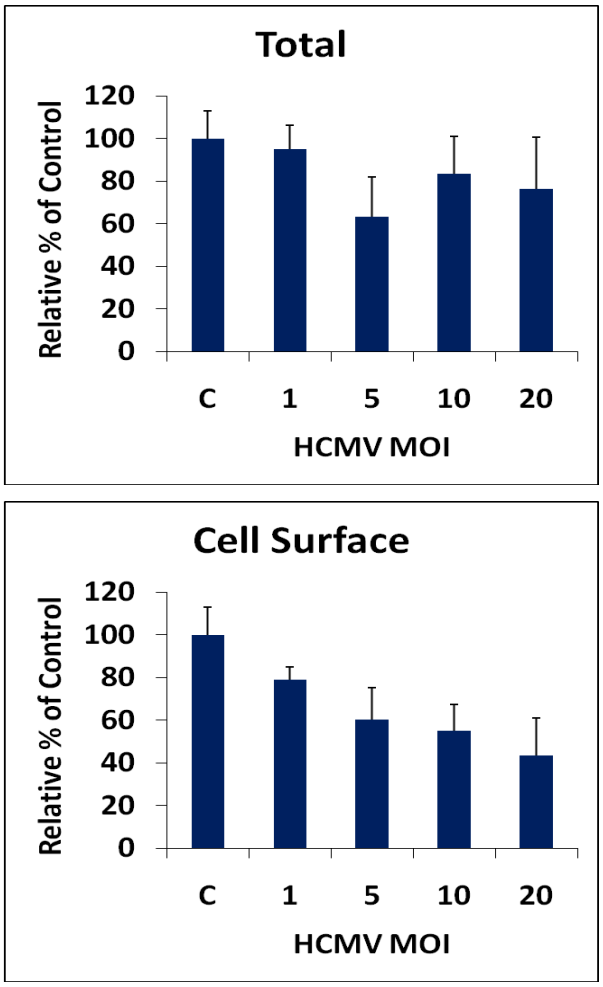

Figure 3 - In cell and On cell Western for Syndecan-1 Expression in Response to HCMV

Cultured trophoblasts were challenged with HCMV (MOI=1, 5, 10, 20) for 24 hrs and stained with a primary antibody to Syn-1, followed by a secondary antibody. Results quantified as a percent of control. Total represents in cell western and Cell surface represents on cell western. (A) Syn-1 expression stays the same as HCMV MOI increases. (B) Syn-1 expression decreases as HCMV MOI increases. 4 different placenta preps with duplicates pooled.

Aguilar, H. N., B. Zielnik, et al. (2010). "Quantification of rapid Myosin regulatory light chain phosphorylation using high-throughput in-cell Western assays: comparison to Western immunoblots." PLoS One **5**(4): e9965.