The Pennsylvania State University

The Graduate School

TARGETED MODULATION OF HOST IMMUNE PROTEINS BY HUMAN CYTOMEGALOVIRUS

A Dissertation in

Biomedical Sciences

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2021

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ABSTRACT

Human cytomegalovirus is a ubiquitous pathogen in the human population that can cause severe health consequences in immunocompromised patients and neonates. The virus modulates host immunity to facilitate viral replication within humans. This includes attenuation of innate immune activation within infected cells and dampening of the adaptive immune responses. Thus, elucidation of how the virus alters the host immune system is key to developing remedial strategies for HCMV infections.

CD4+ T lymphocytes are adaptive immune cells that are important for controlling viral infections within the host. The activation of CD4+ T cells occurs when they recognize antigenic peptides displayed on immune proteins called major histocompatibility complex class II (MHC class II). Thus, viruses hinder CD4+ T cell activation by interfering with MHC class II antigen presentation. MHC class II is constitutively expressed in specialized, antigen-presenting cells (APCs), which include cells of the myeloid lineage. Myeloid cells play an important role in the HCMV lifecycle *in vivo*. However, the regulation of endogenous MHC class II in myeloid cells during HCMV infection is not well-understood. We investigated the impact of HCMV infection on MHC class II in Kasumi-3 cells, a myeloid cell line that endogenously expresses MHC class II. We found that HCMV decreases the synthesis of MHC class II by inhibiting transcription of MHC class II and its master regulator class II transactivator (CIITA). This mechanism of MHC class II regulation was found to be independent of the immunomodulatory unique short (US) region of the HCMV genome and previously reported viral genes involved in MHC class II regulation. Importantly, the reduction in MHC class II synthesis required the expression of the immediate early proteins of the virus. Thus, we found that HCMV decreases endogenous CIITA and MHC class II expression in infected myeloid cells.

Cells encode innate immune sensors to detect presence of viral ligands. This includes sensing of viral nucleic acids within the cytoplasm, which stimulates innate immune responses. Consequently, viruses block the activation of the cytoplasmic innate sensors to prevent immune activation. We found that HCMV induces the expression of MARCH1, an E3 ubiquitin ligase that targets membrane proteins for ubiquitination and lysosomal degradation, in non-expressing fibroblasts. This induction of MARCH1 in fibroblasts is remarkable because MARCH1 expression is limited to APCs to regulate immune proteins specifically expressed within these cells. However, we observed that MARCH1 is highly expressed during the late stages of HCMV infection and localizes to the Golgi in the cytoplasmic viral assembly compartment (cVAC), the site of viral maturation. We identified stimulator of interferon genes (STING), a cytoplasmic DNA sensor, as the target of the Golgi-localized MARCH1 in HCMV-infected fibroblasts. In support of this, we saw an increase in STING expression and its associated antiviral gene transcription upon short, hairpin RNA (shRNA)-mediated MARCH1 knockdown. Consequently, there was reduced cytoplasmic viral activity and infectious virus production upon loss of MARCH1 during HCMV infections. Thus, HCMV induces MARCH1 to target the antiviral STING protein to reduce innate immune signaling and promote viral replication.

This dissertation highlights how HCMV effectively modulates the host immune response. The cessation of endogenous MHC class II synthesis upon HCMV infection reduces the expression of MHC class II, a T cell stimulating protein, and ablates the activation of the adaptive immune CD4+ T cells. Additionally, HCMV induces MARCH1 to target the immune protein STING and dampen the innate immune response in infected cells. Thus, HCMV alters the expression of cellular proteins to make the host immune environment favorable for the viral lifecycle.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BAC	Bacterial artificial chromosome
β2m	Beta 2 microglobulin
BLIMP-1	B lymphocyte-induced maturation protein 1
CCL	C-C motif chemokine ligand
cGAS	Cyclic GAMP AMP sythase
CIITA	MHC class II transactivator
CLIP	Class II-associated invariant chain peptide
cVAC	Cytoplasmic viral assembly compartment
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
EBV	Epstein Barr virus
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERp57	Endoplasmic reticulum resident protein 57
g	Glycoprotein
galK	Galactokinase K
GFP	Green fluorescent protein
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hpi	Hours post infection
HSV	Herpes simplex virus

IE	Immediate early
IFI16	Interferon gamma inducible protein 16
IFN	Interferon
IFNAR	Interferon α and β receptor
Ii	Invariant chain
IKK	IκB kinase
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
JAK	Janus kinase
kDa	Kilo Dalton
KSHV	Kaposi's sarcoma herpesvirus
LAMP	Lysosome associated membrane glycoprotein
LANA	Latency associated nuclear antigen
MARCH	Membrane associated RING CH ligases
MAVS	Mitochondrial antiviral signaling
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MIEP	Major immediate early promoter/protein
MOI	Multiplicity of infection
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NK	Natural Killer
NLS	Nuclear localization signal
ORF	Open reading frame

PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
pp	Phosphoprotein
PRR	Pattern recognition receptors
RIG-I	Retinoic acid inducible gene 1
RING	Really Interesting New Gene
RFX	Regulatory factor X
RNA	Ribonucleic acid
shRNA	Short hairpin RNA
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
STX5	Syntaxin 5
TANK	TRAF family member associated NF-κB activator
TBK1	TANK binding kinase 1
TCR	T cell receptor
TfR	Transferrin receptor
TGF-β	Transforming growth factor beta
TLR	Toll like receptor
TNF-α	Tumor necrosis factors alpha
UL	Unique long
US	Unique short
VSV-G	Vesicular stomatitis virus glycoprotein

ACKNOWLEDGEMENTS

I would like to acknowledge my mentor, Dr. Nicholas J. Buchkovich, for giving me the opportunity to work in his laboratory. He accepted me into his lab during a difficult time in my graduate career and since joining the lab, I truly enjoyed the experience of being a graduate student. He let me pursue projects of my liking and helped me overcome the challenges that I faced along the way. I was glad for the encouragement to attend scientific conferences as part of my graduate training. In addition, I have greatly benefitted from the constructive feedback I received from him for my scientific writing and presentations. Lastly, I am appreciative of the mentorship and guidance over the years, and grateful for the training I have received under his tutelage.

I am thankful to my committee members for supporting me during my graduate training and providing excellent suggestions for tackling problems that I encountered in my projects. I would also like to acknowledge Dr. John Wills and Dr. Chris Norbury for their scientific input and career advice that has been very helpful. I am also grateful to the members of the Department of Microbiology and Immunology for their encouragement during the last few years.

I have had the opportunity to interact with many wonderful people who made the lab a pleasant environment. Although I overlapped with Linda Cruz for a short period of time, I am very grateful to her for training me and helping me get started in lab. I am especially indebted to Nick Streck, who has been a great source of support and encouragement. He always lent a helping hand when needed, and shared his wealth of knowledge when it came to experiments and troubleshooting. Our fun, non-science discussions kept the lab atmosphere cheerful and I was lucky to have a friend to turn to for any advice. I have thoroughly enjoyed my conversations with Natalie, Allie and Rinki. I am very appreciative for their encouragement and friendship, and their willingness to help out in lab. I have also enjoyed interacting with Maddie, the newest member of the lab, and training her as she begins her graduate school journey.

During my stay in Hershey, I have met amazing people and their friendship has made my time in Hershey memorable. My friends, Melat, Suriya and Tanay, have shared the challenges of graduate school with me, and their company made things much easier. I will always cherish the memories of the wonderful times we have shared in Hershey. I am also thankful to other graduate students and postdocs who welcomed me into their circle of friends, lent me protocols and reagents and empathized in the struggles of science. The staff at the Graduate Education Office has been immensely helpful in answering questions and making sure I stayed on track with my graduate student obligations. All those reminders and encouraging emails made me feel I was always taken care of. Lastly, the kitchen staff in the department has been incredible and made everyday chores in the lab much easier with their help.

The journey through graduate school would have been much more arduous without the constant support of my husband Joaquin and my family. Joaquin has been there for me through thick and thin and shared in the experiences of graduate school. He always reminded me that I had a life outside the lab and I am very thankful to him for keeping me sane during my time as a graduate student. I have been very lucky to count on his support through everything and I am very appreciative of all that he does for me. My parents have been my biggest source of encouragement, always supporting me to pursue my goals and cheering me on. For their endless love and patience, I am very grateful. My brother has been a great example of resilience, motivating me never to give up. When I felt overwhelmed, he reminded to take things one day at time. I am very appreciative of his unwavering support. Lastly, I am thankful to my extended family and friends who always kept in touch to make sure I was doing well and have been rooting for me in my academic pursuits.

I would also like to acknowledge the funding source R01AI130156 for the work presented in this dissertation. The findings, conclusions and opinions in this dissertation do not necessarily reflect the view of the funding agency.

Dedicated to my late grandfather, Bhagat Singh Sandhu, for being the reason that I embarked on my scientific journey.

Chapter 1: Introduction

Human cytomegalovirus (HCMV)

HCMV history and pathology

Human cytomegalovirus (HCMV) is an opportunistic pathogen that was first isolated and described as an infectious agent by Margaret Smith. She initially isolated murine cytomegalovirus from the salivary glands of mice, and then using a similar strategy, she obtained HCMV from human salivary gland and renal tissue by culturing the virus on endometrial fibroblasts [1, 2]. Her initial observations described HCMV as a salivary gland virus causing characteristic intranuclear inclusions and enlargement of infected cells [2]. Shortly thereafter, other reports described similar morphological and growth characteristics for viruses recovered from livers of sick infants and adenoid tissues of children [3, 4]. The viruses isolated from different human tissues by Smith and others could be neutralized by multiple human donor serums [2-4]. These human viruses had distinct cytopathic effects in cell culture, produced no disease upon infection in mice and were unable to infect tissues derived from other species [2, 4]. These observations suggested that these isolated viruses were prevalent in the human population and exhibited species-specificity [2-4]. Thus, parallel studies by different research groups led to the initial characterization of HCMV. Interestingly, one of the strains isolated from the adenoid tissue of a 7 year old girl by the Rowe group, named AD169 strain, was subsequently serially passaged in cells and is one of the most commonly used lab-adapted strains of HCMV to date [3].

Evidence of disease by HCMV preceded virus isolation and characterization of its properties *in vitro*. The first report of disease occurred in 1881 where large nuclear inclusions were observed in cells from stillborn infants and in the parotid glands of young children, and thought to be protozoan in nature [5]. Years after this observation, these inclusion-bearing cells were observed by many groups with different theories regarding the nature of these cells, with a leading hypothesis that these were virus-induced morphological changes, similar to those observed with varicella and herpes simplex [6]. Several reports of disease in infants with

inclusion-bearing cells and multi-organ complications led to the term ‘cytomegalic inclusion disease (CID)’, with these enlarged cells attributed to viral infection based on evidence of similar virus-derived lesions in animals [7]. Furthermore, it was suggested to be transmitted transplacentally due to the presence of these inclusion-bearing cells in stillborn infants [7]. Finally, the isolation of HCMV from CID-afflicted infants by Smith and others led to the discovery of the cause of these cellular inclusions and disease. Thus, detailed pathological analysis of infected cells over many years paved way for the discovery that HCMV gives rise to the characteristic enlarged cells, and established its role as the etiological agent of disease and morbidity in infants.

HCMV infects humans ubiquitously. Transmission of the virus occurs through infected bodily fluids such as saliva, tears, blood, breast milk and genital secretions [8]. Within the human host, HCMV exhibits broad cell tropism and can infect multiple cell types. Commonly used cell models for experimental HCMV infections include fibroblasts, epithelial cells, endothelial cells, monocytes, hematopoietic stem cells and smooth muscle cells [9]. However, the virus has also been recovered from lymphocytes and polymorphonuclear cells from human blood samples [10, 11]. HCMV exhibits two different lifecycles in infected cells- an active, lytic lifecycle with production of infectious virus and a quiescent, latent lifecycle with limited expression of viral genes and establishment of lifelong infection. Immunocompetent individuals are usually asymptomatic for infection but in some cases these individuals may develop symptoms associated with mononucleosis [12]. However, immunocompromised people exhibit multiorgan-associated, HCMV-induced pathology commonly observed in patients undergoing solid organ transplants, human immunodeficiency virus (HIV)-infected patients and immunosuppressed individuals on chemotherapy [13]. HCMV infection in immunocompromised patients is the result of either primary infection or reactivation of the latent virus in different organs. Under such conditions, HCMV-induced pathology depends on the site of infection and usually manifests as hepatitis,

pneumonitis, colitis and retinitis, where retinitis is most commonly associated with HIV-infected patients [14]. Within infected organs, HCMV is found in several cell types such as hepatocytes in the liver and neuronal cells in the brain and eye [9]. Another significant concern associated with HCMV infections is the vertical transmission of the virus from an infected mother to the fetus [15]. This route of transmission causes severe disease in many infected newborns resulting in jaundice, microcephaly, hearing disorders and neurological abnormalities [15]. Additionally, there is increased risk of HCMV-induced disease in infants born to mothers who undergo primary infection during pregnancy than reactivation of latent infection [15, 16]. Currently, there are antiviral drugs available to target viral DNA replication and genome packaging of HCMV to treat infected, at-risk individuals. However, prolonged use of the drugs is not feasible due to toxicity issues and emergence of drug-resistant mutants [17]. Thus, HCMV infections in these risk groups is a still a major concern and a thorough understanding of the virus-host relationship is required to develop better therapeutic interventions.

Virus structure

HCMV is a member of the herpesvirus family, a large DNA virus family classified into three subgroups- *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. HCMV is the largest human herpesvirus and belongs to *Betaherpesvirinae.* The HCMV virion has a structure typical of herpesviruses with a double-stranded DNA genome enclosed by an icosahedral capsid that is surrounded by a matrix of proteins and nucleic acids called the tegument layer and a lipid envelope embedded with glycoproteins as shown in Figure 1-1 [18]. The average size of the virus particle ranges between 180-230nm making HCMV the largest and structurally most complex herpesvirus [19, 20]. The 230 kilobase pair genome of HCMV contains terminal and internal

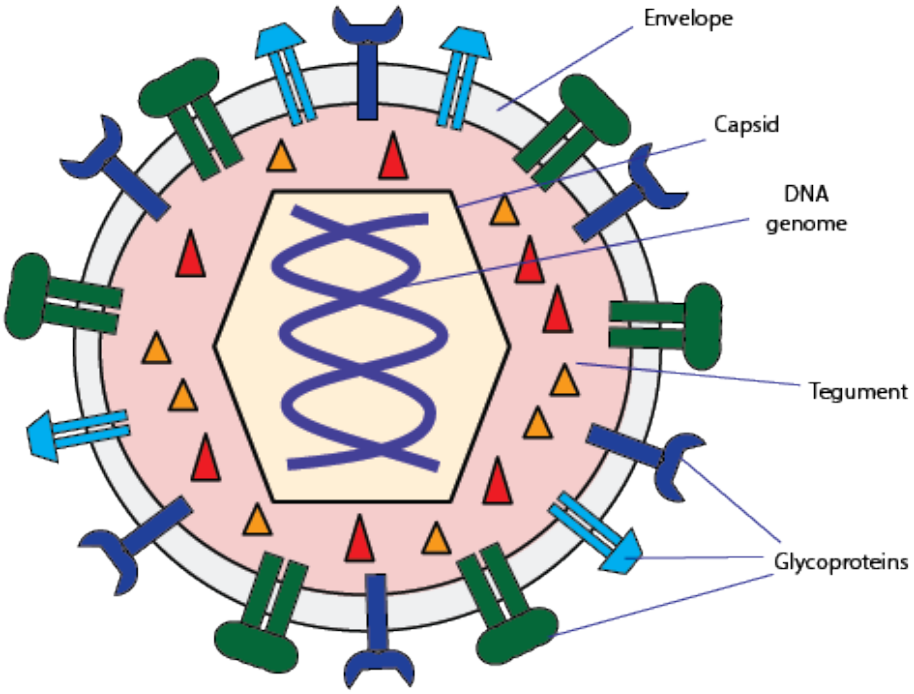

Figure 1- 1: Structure of HCMV. The DNA genome is enclosed within an icosahedral capsid, surrounded by the tegument layer. Surrounding the tegument is a lipid envelope studded with glycoproteins.

repeat sequences that separate the genome into unique short (US) and unique long (UL) genome segments [18, 21]. The different genes encoded within these regions of the genome contain the prefixes US and UL accordingly. The HCMV genome contains a single origin for replication (oriLyt) and the genome packaging and cleavage signals are found within the terminal repeat sequences [21, 22]. The genome is enclosed within a symmetrical icosahedral capsid comprised of 161 capsomeres [8].

Surrounding the capsid, the virus contains a thick layer of tegument containing multiple viral proteins. The most abundant protein is pp65 (UL83) that functions early in infection to dampen the immune response, and also is the major constituent of non-infectious dense bodies released during infection [23, 24]. Other tegument proteins packaged within the virion aid in important post-viral entry functions. These include pp71 (UL82) that acts as an important transcriptional activator for viral gene expression and pp150 (UL32) that is important for nucleocapsid stability [25-27]. HCMV can also package mRNAs and non-coding RNAs into the virus particle that aid in initiation of infection [28, 29].

The envelope of the virus contains different glycoproteins required for attachment, entry, internalization and other functions such as immunomodulation. The glycoproteins that mediate entry of HCMV are gB, gH, gL and gO. However, the glycoprotein composition in virus particles varies based on the cell type in which HCMV was propagated [30]. An example of an immunomodulatory glycoprotein packaged within the virion is US28, a viral homolog of the G protein-coupled receptor (GPCR) that binds inflammatory chemokines and modulates cell signaling and motility [31]. Thus, the HCMV virion contains the essential elements to enter and initiate efficient viral replication in a host cell. The various steps of the replication cycle, summarized in Figure 1-2, are detailed in the subsequent sections.

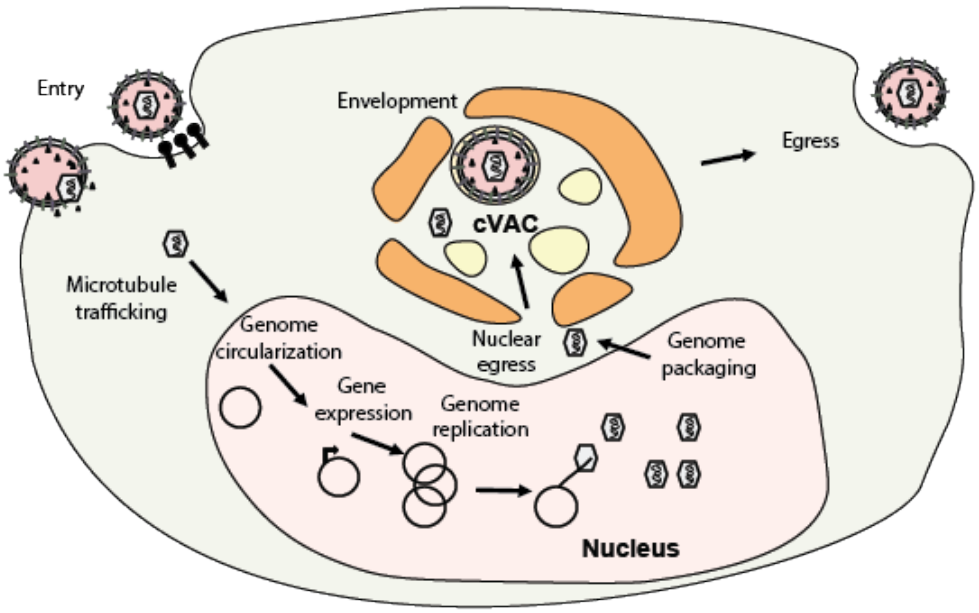

Figure 1- 2: HCMV replication cycle. The lifecycle of HCMV begins with entry of the virus, followed by trafficking of the nucleocapsid through microtubules to the nucleus. The viral genome is released in the nucleus where it initiates viral gene expression. Viral DNA replication and packaging of viral genomes into capsids also occurs in the nucleus. The packaged capsids exit from the nucleus to the cytoplasmic viral assembly compartment (cVAC) for envelopment and maturation of virions prior to egress from the cell.

Entry of HCMV

HCMV enters multiple cell types by utilizing the various viral glycoproteins that are part of the virion envelope to interact with cellular receptors. All herpesviruses use glycoproteins gB, gH and gL to enter host cells [32]. Host receptors involved in HCMV entry include integrins and heparan sulphate proteoglycans. Proteoglycans interact with viral glycoproteins gM/gN for attachment of HCMV to the cells, while integrins bind to gB to enable internalization of the virus [33-35]. Depending on the cell type, HCMV can utilize two glycoprotein entry complexes and enter through direct fusion or receptor-mediated endocytosis. The core entry complex is composed of a trimer of gH/gL/gO proteins that function along with gB as the fusion machinery [32, 36]. The trimeric gH/gL/gO complex interacts with entry receptors expressed on fibroblasts to allow the virus to enter through direct fusion [32, 37]. The cognate cellular entry receptors for the trimeric complex are platelet derived growth factor alpha (PDGFRα) and epidermal growth factor (EGFR) in fibroblasts, although the role of EGFR is controversial [38-40]. However, this mode of entry is specific to fibroblasts and the trimeric complex alone is limiting for entry into non-fibroblast cell types.

Receptor-mediated endocytosis for HCMV is important for HCMV entry into other cell types and requires low endosomal pH [41]. This mode of entry requires additional HCMV genes UL128, UL130 and UL131 to form the pentameric complex with gH/gL [41, 42]. The pentameric complex is required for efficient entry of HCMV into epithelial, endothelial and myeloid cells [41-43]. Recently, cell surface proteins like Neuropilin-2, olfactory receptor 14I1 and CD46 have been identified as receptors for the pentameric complex to allow HCMV to enter non-fibroblast cell types [44-46]. Hence, HCMV can modulate the envelope glycoprotein composition and enter various cell types via multiple receptors.

HCMV gene expression, DNA replication and capsid assembly

After entry, the viral nucleocapsids traffic to the nucleus via microtubules where the genome is released into the nucleus to initiate gene expression. The viral genome initiates a cascade of gene expression programs- immediate early (α), early (β) and late (γ) [47]. Immediate early genes are expressed within the first few hours of infection [8]. These genes work in concert with tegument proteins to suppress the host immune response, and to alter cell signaling pathways and cellular metabolism to prime the cell for infection. The earliest genes expressed are the major immediate early proteins (IE) IE1 and IE2 (also called IE72 and IE86 respectively) from the UL122-123 locus [48]. These IE proteins, aided by tegument proteins, regulate viral gene expression and initiate transcription of viral early genes while dampening the innate immune response and anti-viral gene expression [49-52].

The second wave of viral gene expression commences with early genes that are required for DNA replication and immune evasion. Early genes are further subclassified into two classes, early (β1) and delayed early (β2), based on the timing of their expression during infection. The viral machinery required for DNA replication such as DNA polymerase (UL54) and accessory factors are transcribed as early genes [47]. Early viral genes also upregulate cellular processes to increase nucleotide metabolism and regulate cyclin kinase-dependent signaling to facilitate viral replication [53]. Other early genes encoded by HCMV include an array of immunomodulatory genes to escape immune surveillance. A majority of the US region of HCMV is expressed with early kinetics to block major histocompatibility complex (MHC) class I presentation as an immune evasion strategy [54-56].

The final phase of HCMV gene expression is for the late genes that produce structural proteins for the virion. Late genes are further subdivided into leaky and true late proteins based on the requirement of viral DNA replication prior to their expression [57]. Late proteins include viral nucleocapsid, assembly and egress proteins. The late proteins accumulate in the cytoplasmic

viral assembly compartment (cVAC), a juxtanuclear region important for the late stages of the viral lifecycle, and are required for viral maturation within infected cells.

HCMV encodes its own DNA replication machinery consisting of the DNA polymerase (UL54), polymerase accessory protein (UL44), single-stranded DNA binding protein (UL57), primase (UL70), primase associated factor (UL102) and helicase (UL105). The input viral genomes are circularized prior to DNA replication [58]. HCMV expresses phosphoproteins, encoded by UL112-113, to stabilize interactions between members of the replication machinery leading to the formation of viral replication centers in the nucleus [59]. With the circularized genome as the template, DNA replication originates at the oriLyt, bound by viral proteins IE2 and UL84, through rolling amplification to generate concatemers that are substrates for genome packaging into capsids [22, 58, 60].

The virus capsid assembly occurs within the nucleus of the infected cell. However, this process is initiated by capsid and scaffold protein complex formation in the cytoplasm [61]. Both the major and minor capsid proteins, UL86 and UL85 respectively, lack a nuclear localization signal (NLS) and associate with distinct NLS-containing scaffold proteins to form complexes and translocate to the nucleus [62-64]. Once within the nucleus, these complexes associate and form procapsids with the scaffolding proteins on the inside [61]. Maturational proteolysis of scaffold proteins triggers a series of cleavage events, reducing their binding to the capsid proteins and resulting in their extrusion from the capsid interior [65, 66]. The portal complex protein, UL104, forms a portal entry at a single capsid vertex and packages DNA within the capsid, aided by the DNA cleavage activity of the viral terminase complex (UL56 and UL89), to give rise to fully assembled capsids [61, 67, 68]. The assembled capsids egress from the nucleus by phosphorylating and rearranging the nuclear lamina, via the viral kinase UL97 and the cellular kinase protein kinase C (PKC), allowing the capsids to access the nuclear membrane [69-71]. The

nucleocapsids then associate with the viral nuclear egress complex, composed of UL50 and UL54, to exit from the nucleus after transient envelopment in the perinuclear space [70].

Tegumentation, envelopment and egress of HCMV

The acquisition of the tegument layer of proteins for HCMV occurs primarily in the cytoplasm. The tegument proteins are packaged into the virion to aid in the establishment of infection and modulate the cellular environment to make it permissive for infection. HCMV encodes more than 30 tegument proteins and many of these proteins are multifunctional and essential for the viral lifecycle. Some tegument proteins have nuclear localization signals, and may associate with nucleocapsids in the nucleus, but the majority of the tegument is acquired in the cytoplasm [25, 72, 73]. Tegumentation and final envelopment are believed to occur in the cVAC, a large juxtanuclear area in the cytoplasm that serves as the final site for viral maturation [74]. The cVAC is visualized in infected cells by the accumulation of viral tegument proteins and glycoproteins and the re-organization of cellular organelles adjacent to the characteristic kidney-shaped nucleus [75, 76]. In the cVAC, concentric layers of the Golgi and trans-Golgi network are observed with the microtubule organizing center (MTOC) and endosomes aggregating in the middle [75, 76]. Tegument-coated nucleocapsids bud into vesicles to acquire the final envelope and glycoproteins [77, 78]. While the nucleocapsids obtain their tegument and envelope in the cVAC, the processes of tegumentation and membrane acquisition for final envelopment are still not fully understood. Other virus-derived structures such as dense bodies and non-infectious enveloped particles (NIEPs) are also present within the cVAC. Accumulation of pp65 tegument protein gives rise to dense bodies, and capsids that failed to undergo proper DNA packaging but egress to the cytoplasm make up the NIEPs [79]. The envelopment of both dense bodies and NIEPs also occurs in the cVAC [79]. Finally, the egress of enveloped viral particles in vesicles occurs through the exocytic pathway, where viral proteins UL103 and UL35 are thought to be required [80, 81].

Immune responses and HCMV infection

There has been a constant battle between organisms and viruses throughout evolution for co-existence. Viruses infect all living beings, and hence have evolved to overcome immune barriers in a variety of living organisms for their survival. On the other hand, organisms have measures to counteract viruses from taking over the host to promote their own survival. An example of a simple immune response in bacteria is the utilization of the clustered regularly interspaced short palindromic repeats (CRISPR)-mediated surveillance of invading viruses. However, humans have developed complex, multi-faceted immune responses to virus infections. Hence, the human immune system has multiple ways to detect and protect from invading viruses which are briefly explained in the next sections.

Innate immune responses

Upon infection of a cell with a virus, there are different components of the virus that trigger the host innate immune response. Cells can recognize specific pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) to activate downstream signaling that induces an antiviral state in the cell. The PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs) and cytosolic DNA and RNA sensors to detect foreign proteins and nucleic acids. The recognition of foreign ligands by PRRs is critical for the initiation of the immune response.

An important feature of the cellular antiviral response is the viral nucleic acid sensing during infections. For many viruses, different forms of their genome-encoding RNA and DNA activate intracellular, endosomally-localized TLRs 3, 7, 8 and 9 [82]. In addition to TLR stimulation, viral nucleic acid sensing occurs in the cytoplasm. RIG-I is a key cytosolic RNA sensing molecule that recruits adaptor protein mitochondrial antiviral signaling (MAVS) to initiate downstream antiviral signaling [83]. For DNA, sensors such as cGAS detect cytosolic DNA and stimulate STING, an endoplasmic reticulum (ER) adaptor protein that dimerizes and

translocates to the Golgi upon activation to serve as an immune signaling platform [83]. Thus, the detection of viral nucleic acids is an important trigger for the initiation of antiviral immunity.

The activation of PRRs causes a cascade of signaling that induces the interferons, the NF-κB pathway, interleukin 1(IL-1), IL-18 and other pro-inflammatory cytokines and chemokines [84]. This leads to the recruitment of other innate immune cells such as natural killer (NK) cells, neutrophils, macrophages and dendritic cells (DCs) [85]. These cells function to limit the viral infection, and receive activation signals through different pro-inflammatory cytokines. The activated macrophages and dendritic cells are then important for the initiation of the second phase of the immune response.

Adaptive immune responses

The adaptive immune system recruits T cells and B cells to combat viral infections. Broadly, the T cells function in two ways during viral infections. CD8+ T cells directly attack infected cells via perforin and granzyme B-mediated cytotoxicity after recognition of virus-associated peptides on the infected cells, while CD4+ T cells help B cells mount an antibody response and aid in optimal CD8+ T cell function [86, 87]. The antibodies produced by B cells lead to clearance of the virus and aid in the resolution of viral infection [87]. A critical step in T cell stimulation is the activation of these cells by recognizing antigenic peptides, derived from viral proteins, loaded onto the immune proteins called MHC molecules. These specialized MHC molecules are expressed on the cell surface as MHC class I and II, where MHC class I primes CD8+ T cells and MHC class II primes CD4+ T cells. The presentation of viral antigens by MHC molecules is an important step in virus clearance by the immune system.

Innate immune response modulation by HCMV

HCMV encodes more than 150 genes, many of which are dedicated to modulating the host immune response. To ensure an effective block of the immune signaling, there are multiple genes encoded by HCMV to target the immune-activating pathways at different steps.

Interferons and cytosolic sensing

The interferon pathway is the primary antiviral response initiated by infected cells and is modulated by HCMV using multiple proteins. Interferons (IFN) belong to three different classes- type I, II and III. Most infected cells initiate type I IFN (IFN α and β), while induction of type II (IFN γ) is limited to immune cells. Type III IFN (IFN λ) is the newest described IFN that can be produced by both immune and non-immune cells but the expression of IFN λ receptor is limited, restricting its activity. In contrast, type I IFN is produced by most cells upon viral infection and its receptor is ubiquitously expressed. Upon expression, type I IFNs are secreted and act through autocrine and paracrine signaling. Type I IFN binds to the type I IFN α and β receptor (IFNAR) and initiates the Janus kinase (JAK)- signal transducer and activator of transcription (STAT) signaling cascade. Upon IFNAR activation, JAK1/Tyk2 proteins phosphorylate STAT1 and STAT2. The phosphorylated STAT proteins translocate to the nucleus to induce transcription of antiviral interferon-stimulated genes (ISGs). Similarly, Type II IFN-γ binds to a widely expressed but distinct receptor, IFN-γ receptor, to trigger JAK1/2-mediated STAT1 phosphorylation to initiate a diverse set of ISGs. Upon expression, ISGs exhibit anti-viral functions and restrict viral infection at various stages. Thus, the IFNs are critical mediators of the innate immune response against viruses.

Due to the important role of type I IFN signaling, HCMV blocks the type I IFN pathway at different steps throughout the course of infection. The earliest inhibition of type I IFN during HCMV infection is mediated by the tegument proteins within the incoming virions. The tegument protein pp65 directly binds to DNA sensors IFI16 and cGAS to prevent viral DNA detection that leads to type I IFN activation [88, 89]. Similarly, the tegument protein pp71 binds to cytosolic DNA sensing adaptor STING and prevents its activation by inhibiting its trafficking and immune complex formation [90]. Hence, pp65 and pp71 block detection of viral DNA to block subsequent

type I IFN production. In addition to the tegument proteins, the early proteins US9 and UL42 disrupt localization of activated STING and MAVS while STING is degraded by IE2 [91-93]. Finally, UL94 blocks STING interaction with downstream signaling proteins while pp65 and US9 inhibit the nuclear translocation of IRF3, a STING interacting transcription factor [23, 91, 94]. Thus, HCMV inhibits viral nucleic acid sensing through multiple proteins to prevent type I IFN activation.

In addition to the inhibition of viral sensing, HCMV also blocks IFN production and signaling. The immediate early proteins IE1 and IE2 play important roles in inhibiting the IFN production and signal transduction. IE2 decreases IFN-β production in HCMV-infected fibroblasts through an unidentified mechanism [51]. To block type I IFN signaling, IE1 binds STAT2 to prevent it from initiating transcription of its target antiviral genes [50]. Additionally, the tegument protein UL23 blocks STAT1 interacting protein to prevent the signal transduction of the type II IFN receptor [95]. Hence, HCMV effectively blocks signaling of both type I and II IFNs by targeting the STAT proteins.

To prevent the antiviral function of ISGs, HCMV has mechanisms to impede ISG production and interfere with their function. Protein kinase R (PKR) is an ISG that decreases mRNA translation in cells upon activation as a global measure to limit virus replication [96]. The HCMV proteins IRS-1 and TRS-1 block the function of PKR to prevent inhibition of mRNA translation and allow viral protein synthesis in infected cells [97]. Another ISG restricting virus infection is ISG15 that exerts antiviral activities both intracellularly and as a secreted factor [96]. HCMV blocks ISG15 through the concerted action of IE1 and UL26. IE1 decreases transcription of ISG15, while UL26 directly binds to ISG15 and decreases its antiviral effect on viral proteins [98]. Thus, HCMV targets induction and signal transduction of IFN signaling and blocks ISGs to inhibit the antiviral response.

NF-κB pathway

The NF-κB pathway is activated as a consequence of viral infection, similar to type I IFN, to trigger a proinflammatory response, release chemokines and cytokines for attracting immune cells, and modulate cellular apoptosis. The activation of the NF-κB pathway results in the nuclear translocation of the IKK complex subunits RelA and B to initiate NF-κB responsive gene transcription. Like many other viruses, HCMV also modulates NF-κB activation for its own benefit by blocking the antiviral proinflammatory cytokines that signal through the NF-κB pathway, while enhancing the expression of chemokines that aid in immunomodulation and viral spread. The tegument protein UL26 blocks phosphorylation of the IKK complex and reduces the translocation of RelA, making infected cells resistant to antiviral cytokines such as TNF-α [99]. Non-coding RNAs of HCMV, miRNAs US5-1 and US112-3p, decrease IKK complex proteins to inhibit proinflammatory cytokine secretion during infection [100]. During latency, US28, a GPCR protein, suppresses NF-κB signaling to repress the major immediate early promoter (MIEP) and maintains the quiescent phase of the viral lifecycle [101]. Besides blocking proinflammatory signaling, HCMV activates NF-κB signaling to selectively upregulate immunosuppressive cytokines and chemokines that are beneficial for infection. HCMV encodes UL144 to induce NF-κB-driven CCL22 expression, a cytokine that attracts immunosuppressive T cells [102]. Additionally, UL76 increases NF-κB-mediated IL-8 secretion to attract neutrophils and enhance viral dissemination [103]. Thus, HCMV utilizes multiple proteins to block or activate the NF-κB pathway differentially during infection.

Natural Killer (NK) cells

NK cells are innate immune cells that are critical for the host defense against viral infections. These cells exhibit cytotoxicity towards virus-infected cells and are important for their clearance within the host. The significance of the NK cell function in murine cytomegalovirus

(MCMV) is highlighted by the differential susceptibility of mouse strains to MCMV infections where strains resistant to MCMV infections have enhanced activity of NK cells [104, 105]. Similar to MCMV, NK cells are required for controlling HCMV infections in humans [106]. Accordingly, HCMV utilizes multiple ways to decrease NK cell killing of HCMV-infected cells. NK cells engage with various inhibitory and activating receptors expressed on the cell surface and the integration of these interactions determines the activation state of NK cells. The absence of MHC class I on the cell surface is the primary triggering signal for NK cells. HCMV decreases expression of MHC class I to prevent T cell activation, but the loss of MHC class I can prime the NK cells to target HCMV-infected cells. To prevent NK cell-mediated cell death, HCMV modulates NK cell activity by engaging other inhibitory receptors. HCMV-encoded UL40 upregulates HLA-E to engage inhibitory receptor Natural Killer group 2 member A (NKG2A) [107, 108]. In addition, HCMV utilizes UL18 to directly bind inhibitory receptor leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB-1) [109]. Thus, the increase in NK inhibitory signals by HCMV prevents the activation of NK cells.

In addition to increasing NK cell inhibitory receptors, HCMV can directly block many NK cell activating receptors. Activating receptors for NK cells include cellular proteins expressed as part of the stress response in infected cells. Consequently, HCMV prevents the expression of the NK cell-activating cellular proteins. UL141 decreases cell surface CD155, a ligand for NK cell activating receptor DNAM-1, by retaining it intracellularly in its immature form [110]. Similarly, UL16 targets MIC-B and UL16 binding proteins (ULBP)1 and 2 for intracellular sequestration to prevent binding of these ligands to the activation receptor NKG2D on NK cells [111, 112]. In addition, MIC-A, another cellular stress protein that binds NKG2D, is targeted by multiple HCMV proteins. US18 and US20 work in concert to cause lysosomal degradation of MIC-A, while UL142 retains it intracellularly preventing expression on the cell surface [113, 114]. Thus, to balance the activation of NK cells by receptor signaling, HCMV utilizes viral

proteins to either degrade or sequester NK activating proteins and increase NK inhibitory receptor ligands.

Adaptive immune response modulation by HCMV

HCMV can modulate the adaptive immune responders, T cells, by interfering with how these cells receive signals from infected cells. This interference is accomplished by blocking the presentation of antigenic peptides derived from viral proteins for T cell recognition, ablating the co-stimulatory signals that serve as a secondary signal for optimal T cell activation and modulating chemokines and cytokines to decrease T cell trafficking and function.

MHC class I structure and presentation

The primary adaptive immune cells that clear virus-infected cells are the CD8+ T lymphocytes. The CD8+ T cells express T cell receptors (TCRs) that recognize a cognate peptide-bound MHC class I molecule displayed on the cell surface. MHC class I is a glycoprotein expressed on the cell surface of all nucleated cells and presents proteasome-processed peptides derived from intracellular proteins. The MHC class I protein is composed of the MHC class I heavy chain non-covalently associated with β2-microglobulin (β2m), and a peptide residing in the peptide-binding groove of the heavy chain [115]. The MHC class I heavy chain is co-translationally inserted into the ER to interact with chaperones BiP and calnexin to assist in its folding [116]. Binding of the MHC class I heavy chain to β2m occurs in the ER lumen, where the chaperones dissociate and allow calreticulin, ERp57, tapasin and transporter associated with antigen processing (TAP) to bind to the MHC class I complex [115, 117]. For the generation of peptides loaded onto MHC class I, processing occurs in the cytosol by the proteasome complex with subsequent translocation into the ER by TAP [118]. Once the peptide binds to the MHC class I heavy chain groove, it stabilizes the MHC class I-β2m complex, allowing it to exit the ER and traffic to the Golgi en route to the cell surface for antigen presentation [115]. Classically, the peptides generated for MHC class I are derived from intracellular proteins, such as viral proteins

within an infected cell [115]. However, extracellular proteins internalized into the cell can also be presented by MHC class I pathway through cross-presentation [115, 119]. Thus, the cytosolic protein repertoire is constantly surveilled through MHC class I antigen presentation.

Evasion of MHC class I presentation by HCMV

HCMV is a paradigm of interference with the MHC class I antigen presentation pathway to block CD8+ T cell detection of infected cells. The US region of the HCMV genome encodes four different proteins, US2, US3, US6 and US11, that block the MHC class I pathway at different steps. These genes are usually attributed as non-essential for growth in culture and are deleted as part of the viral genome editing strategies, but the existence of multiple HCMV genes to ablate the MHC class I pathway highlights the importance of regulating antigen presentation for viral persistence *in vivo*.

HCMV utilizes multiple viral proteins to target the MHC class I pathway. US11 was the first HCMV-encoded gene reported to block MHC class I in infected cells. US11 is an ER-resident, type I integral membrane protein that binds to the nascent heavy chain of MHC class I and translocates it from the ER to the cytosol to undergo proteosomal degradation [56, 120, 121]. Similarly, US2 promotes ER-associated degradation (ERAD) of the MHC class I heavy chain [55, 120]. US3, a homolog of US2, acts through a different mechanism by trapping mature MHC class I in the ER [122]. Another gene, US6, inhibits the peptide transporter TAP complex in the ER to reduce peptide import and loading onto MHC class I, resulting in decreased MHC class I stability, antigen presentation and T cell activation [123]. Additionally, there is temporal regulation of these viral proteins blocking the MHC class I pathway during infection. US3 is expressed with immediate early kinetics and has a short half-life while US2 and US11 are expressed as early genes [124]. US6 is expressed as a delayed early gene but the protein accumulates later during infection [125]. Thus, throughout the HCMV life cycle, the virus

temporally expresses different proteins with distinct functions to disrupt MHC class I processing and expression.

MHC class II structure and synthesis

MHC class II is a cell surface protein that contains two single-pass transmembrane glycoproteins, α and β, to form a heterodimer. The N terminal domain of the α-β heterodimer forms the peptide groove, which is structurally similar to MHC class I [126]. Peptides that bind to the MHC class II are longer, between 13-18 residues, than MHC class I and spread beyond the ends of the peptide binding groove in an extended conformation [127, 128]. Upon synthesis, MHC class II chains assemble in the ER but are inherently unstable without a peptide. To overcome the instability, the α and β chains associate with invariant chain (Ii or CD74) to form trimers of α-β chains associated with Ii, and this complex egresses from the ER to specialized endosomal compartments referred to as the MHC class II compartment (MIIC) [129-131]. Within this compartment, the Ii is proteolytically cleaved to a small fragment called class II-associated Ii peptide (CLIP) that remains bound to the peptide binding groove of MHC class II [132]. To acquire a high affinity peptide for the MHC class II peptide groove, the MHC class II heterodimer bound by CLIP interacts with accessory proteins HLA-DM (human leukocyte antigen DM or HLA-DM) to exchange a high affinity peptide for CLIP [133]. Other proteins like HLA-DO further regulate the MHC class II assembly process by associating with HLA-DM to finetune peptide exchange under specific conditions [134]. Once loaded, MHC class II traffics via actin-myosin1E to the plasma membrane to display peptides on the surface of the cell [119, 135]. The synthesis of MHC class II is shown in Figure 1-3. Thus, MHC class II synthesis is a multi-step process involving many cellular proteins to assemble mature MHC class II-peptide complexes.

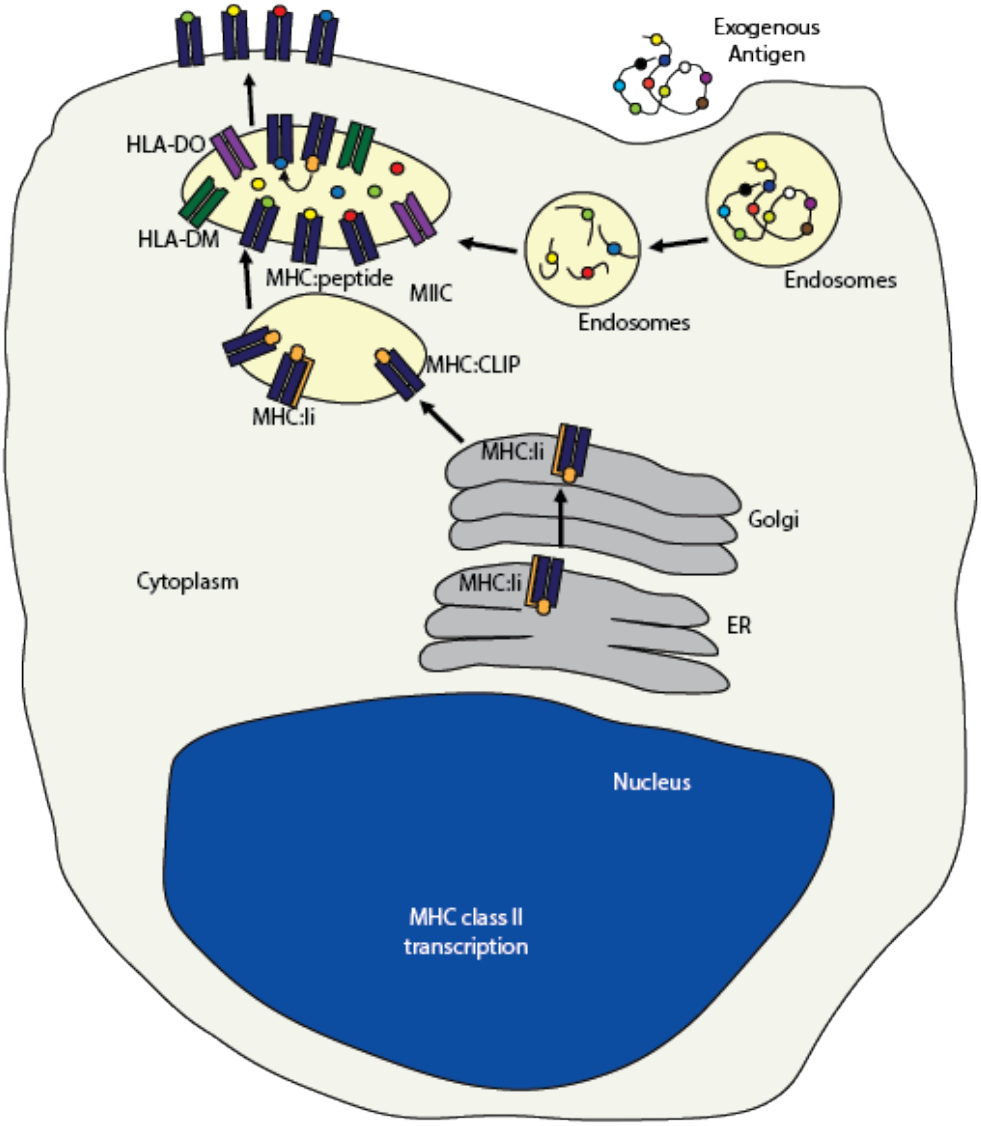

Figure 1- 3: Synthesis of MHC class II. MHC class II is synthesized with the invariant chain (Ii) and moves through the endocytic machinery where Ii is cleaved to a small peptide called CLIP. Antigens within the endosomes are proteolytically cleaved and loaded onto MHC class II in MHC class II compartments (MIIC) with the help of accessory proteins HLA-DM and -DO. Peptide-loaded MHC class II is then expressed on the cell surface.

MHC class II genes and regulation of expression

Although MHC class I and II are similar in general aspects of structure and function, the most distinct feature of MHC class II is its limited and controlled expression compared to the ubiquitous MHC class I. Unlike MHC class I which is expressed in all nucleated cells, MHC class II expression is limited to specific cell types, important in generation of immune responses, called antigen-presenting cells (APCs). Another distinct feature of MHC class II is the ability of non-APCs to induce MHC class II expression under specific conditions, such as stimulation with IFN-γ. Hence, MHC class II is expressed constitutively in APCs and inducibly in non-APCs.

In humans, both the MHC class I and II genes are located on chromosome 6. The classical MHC class II genes are highly polymorphic, like MHC class I, and are encoded by three different isotypes HLA-DP, -DQ and -DR in humans. The MHC class II genes are co-expressed resulting in different allelic combinations within an individual. Due to their function in CD4+ T cell activation, certain MHC class II alleles are implicated in increased susceptibility to autoimmune diseases due to the improper tolerance of T cells to peptides derived from self-antigens during development [136].

MHC class II is endogenously expressed in APCs such as B cells, monocytes, dendritic cells, and thymic epithelial cells that function in T cell development [119]. However, MHC class II can be expressed in other cell types upon stimulation with the cytokine IFN-γ [119, 137]. The expression of MHC class II is regulated by its transcriptional co-activator, the class II transactivator (CIITA). Cells that constitutively express CIITA express MHC class II, whereas IFN-γ stimulation can upregulate transcription of CIITA in non-MHC class II expressing cells and induce its expression [119]. Additionally, other cytokines such as IL-10, type I IFNs and

TNF-α can also regulate the MHC class II expression [138]. Thus, expression of CIITA is required for both induced and constitutive MHC class II.

The promoters of the MHC class II genes contain highly conserved sequences that allow binding of various transcription factors [138]. There are three elements within the MHC class II promoter, W/S, X, and Y, that are bound by different transcription factors [138]. The regulatory factor binding to the X (RFX) family is important for MHC class II transcription containing helix-type, DNA-binding transcription factors with three members, RFX5, RFX accessory protein (RFXAP) and RFX associated ankyrin containing protein (RFXANK), that bind to the X element MHC class II promoter [139-142]. The X element also recruits the transcriptional regulator cyclic AMP responsive element binding protein (CREB) [143]. The Y element is bound by three members of another family of transcription factors NF-Y [144, 145]. These transcription factors are ubiquitously expressed and are required for MHC class II expression. The binding of all these proteins results in a large transcriptional complex that recruits other transcriptional activators to the MHC class II gene promoters. Thus, MHC class II transcription requires an array of transcription factors and co-activators to bind to the MHC class II promoters.

CIITA, a member of the NLR family, is the master regulator of MHC class II transcription. Binding of CIITA to the transcription factor complex at the MHC class II promoters is essential for transcription [146, 147]. The transcription machinery of MHC class II is depicted in Figure 1-4. CIITA does not bind DNA itself but interacts with NF-Y, RFX and CREB to assemble the large MHC class II enhanceosome complex [148]. The CIITA protein contains an activation domain at the N terminus that binds transcription factors and chromatin modifying enzymes, and the C terminus leucine rich region (LRR) is important for its transactivation function [138, 149-151]. In addition to the transcription factors, CIITA interacts with histone modifying enzymes CREB binding protein (CBP) and p300/CBP associated factor (pCAF) that acetylate CIITA, and subsequently cause MHC class II promoter acetylation and

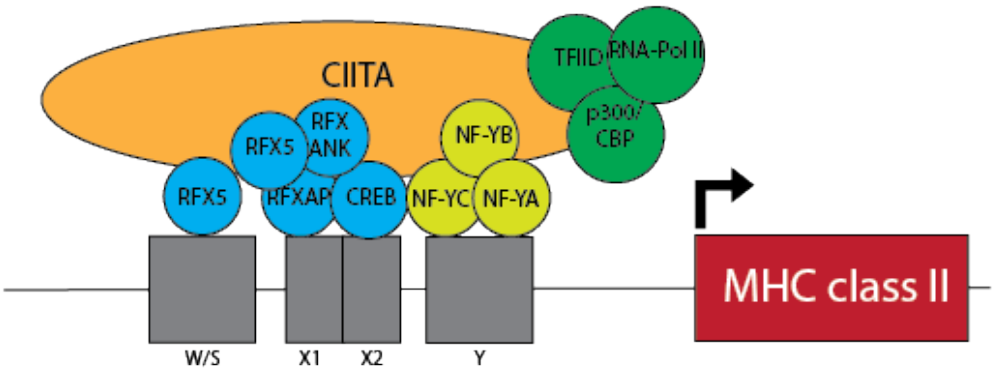

Figure 1- 4: MHC class II transcription through the MHC class II enhanceosome. The transcriptional machinery of MHC class II is bound by three elements in the promoter- W/S, X and Y. The RFX family (RFX5, RFXANK, RFXAP) binds to the X element and RFX5 also binds to the W/S element. The Y element is bound by the NF-Y family (NF-YA, B and C). These transcription factors associate with Class II transactivator (CIITA) that recruits transcription (TFIID, RNA-Pol II) and chromatin modifying machinery (p300/CBP) to form the enhanceosome and initiate MHC class II transcription. TFIID: transcription factor II D, RNA Pol II: RNA polymerase II.

transcriptional activation of MHC class II [152, 153]. Furthermore, CIITA can directly bind to the transcription initiation factor TFIID and regulate transcription elongation by recruiting cyclin-dependent kinases (CDKs) to phosphorylate RNA polymerase II [154, 155]. Hence, CIITA enlists the transcriptional and epigenetic machinery to ensure robust initiation of MHC class II transcription. The importance of the MHC class II enhanceosome for the expression of MHC class II is further illustrated in the Bare Lymphocyte Syndrome (BLS), a genetic disorder caused by deficiencies in either RFX complex genes (RFX5, RFXAP, RFXANK) or CIITA. The BLS disorder is characterized by the lack of MHC class II expression resulting in reduced T cell activity and severe immunodeficiency, leading to enhanced susceptibility to viral, bacterial and fungal infections [156]. Hence, genetic defects in the key MHC class II transcriptional proteins cause severe health problems. To summarize, CIITA, in partnership with other transcription factors, plays a pivotal role in MHC class II expression.

Regulation of CIITA by IFN-γ and promoter usage

The major transcriptional control on MHC class II is exerted by CIITA expression. Specialized APCs express MHC class II because of constitutive CIITA expression [157]. However, CIITA expression can be induced or inhibited by cytokines and is regulated by differential promoter usage in different cell types. There are three main promoters identified for CIITA expression in humans- promoter I (pI), III (pIII) and IV (pIV) [157]. The transcripts derived from these promoters differ in the first exon due to alternative splicing, leading to a unique 5' end and different translation initiation codons, while sharing other exons. Promoter I gives rise to a 132 kDa protein that is expressed in dendritic cells, promoter III is predominantly expressed in B cells yielding a 124 kDa protein, but can also be expressed in T cells and monocytes, and promoter IV is the well-characterized promoter utilized upon IFN-γ induction to result in a 121 kDa protein [158]. The three promoters use distinct transcription factor complexes

to drive CIITA transcription. Hence, the different promoters of CIITA drive its expression in various cell types.

The best-described mechanism of CIITA expression is the IFN-γ stimulated transcription of CIITA and MHC class II [147]. Upon induction with IFN-γ, JAK1/2 kinases associated with the IFN-γ receptor phosphorylate STAT1 resulting in STAT1 nuclear translocation and binding to the gamma interferon activation site (GAS) present in the pIV promoter [159, 160]. Another transcription factor, IRF-1, is also induced by IFN-γ signaling and binds to the IRF-1 binding site of pIV [161, 162]. These two induced transcription factors cooperate with constitutively expressed upstream regulatory factor 1 (USF-1), bound to the E element in pIV, to drive CIITA transcription [161]. However, the IFN-γ stimulation of CIITA transcription can be blocked or decreased by cytokines such as IL-10, TGF-β and IL-4 [160, 163]. Hence, pIV is the primary IFN-γ responsive promoter of CIITA.

The other two promoters of CIITA, pI and III, exhibit tissue-specific regulation by differential transcription factor binding to the promoters. pI is primarily active in dendritic cells but can function in other cell types as well [158]. pI is bound by transcription factors IRF8, PU.1 and RelA subunit of NFκB [164]. pIII-driven CIITA transcription has been well-characterized in B cells and is driven by binding of multiple constitutive and immune cell-specific transcription factors. Transcription elongation factor (TEF-2), activating transcription factor (ATF), CREB, E47 and IRF-4 come together with PU.1 for B cell-specific CIITA expression where PU.1 is critical for both proximal and distal regulation of pIII [165-167]. The pI and pIII transcription of CIITA is diminished with maturation of DCs and B cells. Upon maturation of B cells to plasma cells, the activation of transcription factor BLIMP-1 disassembles the MHC class II enhanceosome complex on pIII to inhibit CIITA transcription [168]. Similarly, maturation of DCs also recruits BLIMP-1 to decrease CIITA transcription, effectively reducing synthesis of new

MHC class II [161, 164]. The cessation of MHC class II production occurs in conjunction with increased transport of MHC class II to the cell surface [161]. By doing so, the mature DCs enhance the antigen presentation of pre-existing MHC class II bound to antigenic peptides and ensure that these complexes are not replaced by newly synthesized MHC class II. Hence, the different promoters of CIITA control the transcription and expression of constitutive and inducible CIITA. Figure 1-5 describes the CIITA transcription machinery for the different promoters.

Antigen presentation via MHC class II

The classical view for MHC class II antigen presentation is that the source of antigenic protein is exogenous, derived from endocytosed or internalized cargo. APCs have multiple methods of acquiring extracellular material whose proteins are then processed into peptides displayed on MHC class II for CD4+ T cell recognition. This includes viruses, bacteria and cellular proteins that enter the cell through endocytosis, phagocytosis and macropinocytosis [169]. Macropinocytosis allows for uptake of soluble exogenous antigens in macrophages and DCs [169]. For capturing other exogenous antigens, receptor-mediated endocytosis and phagocytosis occurs using lectin, complement and Fc receptors that allows for internalization of antibody or complemented-coated antigen and receptor-bound substrate [169]. From the exogenous antigens, the majority of the antigenic peptides for MHC class II are derived from the endolysosomal pathway [130]. This is well-documented for exogenous experimental antigens like ovalbumin and hen egg lysozyme that DCs, macrophages and B cells can internalize these proteins, process them and present peptides derived from these internalized proteins [170-173]. However, endogenous proteins can be presented on MHC class II to activate CD4+ T cells. The first reports of endogenous peptide generated for MHC class II came from non-secreted, intracellular immunoglobulin presentation of B cells to T cells [174, 175]. Further analysis in B

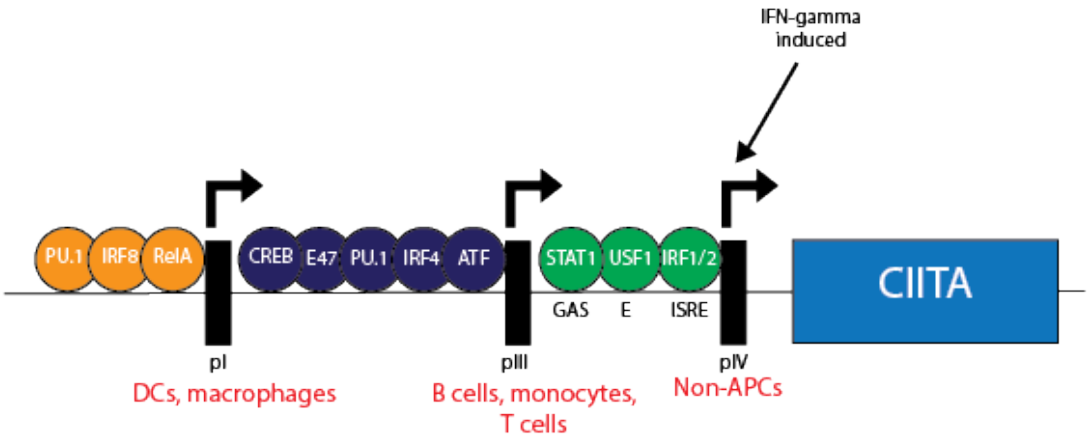

Figure 1- 5: Regulation of CIITA transcription by cell-specific promoters. Promoters I, III and IV (pI, III and IV) drive CIITA transcription in different cell types and are bound by various transcription factors. pI drives CIITA transcription in DCs and macrophages and is bound by PU.1, IRF8 and RelA. pIII is active in B cells, T cells and monocytes and is bound by E47, PU.1, IRF4, ATF and CREB. pIV is the IFN-γ inducible promoter bound by STAT1 at the GAS element, USF1 at the E element and IRF1/2 at the ISRE element within its promoter region.

cells revealed that multiple peptides were derived from self-proteins within the cell [127]. Hence, MHC class II can present peptides derived from both endogenous and exogenous antigens.

The dual antigen source for MHC class II antigen presentation has further been highlighted in viral infections. Initial observations in influenza infections suggested that MHC class II presented only exogenously-derived antigens. In influenza infected cells, MHC class I activated CD8+ T cells through peptides derived from *de novo* protein synthesis in virus-infected cells, while CD4+ T cell priming through MHC class II occurred for lysosome-processed exogenous antigen, independent of viral protein expression [176]. This suggested that viral proteins would only be presented through MHC class I in an infected cell and required internalization in APCs for MHC class II presentation. However, certain viral proteins traffic through endosomal compartments, access MIICs and be processed as endogenous antigens within an infected cell. In addition, viral infections modify cellular trafficking pathways and alter the normal antigen processing machinery. Indeed, careful analysis of other influenza viral proteins such as neuraminidase demonstrated that endogenous antigen was presented through MHC class II during influenza infection [177]. Another study demonstrated the endogenous presentation of influenza matrix protein to CD4+ T cells, using a unique antigen processing route distinct from the MHC class I pathway [178]. Furthermore, endogenous protein processing can be proteasome-dependent, and even TAP in certain instances, or occur in early endocytic compartments [179-182]. Thus, influenza virus infection results in both exogenously and endogenously derived antigens to stimulate CD4+ T cells, suggesting that the antigen repertoire is diverse during infection and viral proteins may be processed via different mechanisms to generate distinct epitopes for an optimal immune response [176, 177, 183]. These observations with influenza were soon extended to antigen presentation in other viral infections. Transformed B cell lines expressing the Epstein Barr virus (EBV) protein Epstein Barr nuclear antigen 1 (EBNA1) intracellularly stimulate EBNA-1 specific CD4+ T cells [184-186]. Endogenous glycoprotein B

(gB), a viral protein that traffics to endosomes, is presented through MHC class II to CD4+ T cells during HCMV infections [187]. Thus, endogenous proteins can give rise to peptides for presentation through MHC class II, where the processing within the cell occurs through various mechanisms to generate a diverse pool of peptides. Figure 1-6 highlights the various sources of antigen for presentation through MHC class II.

Viral evasion of MHC class II antigen processing and presentation

Since CD4+ T cells play an important role in shaping the adaptive immune system by providing the appropriate signals to cytotoxic CD8+ T cells for their optimal function, helping B cells generate antibodies and activating APCs through cytokines, viruses block the activation of CD4+ T cells by interfering with the MHC class II antigen presentation. Viruses can achieve this by either dampening the antigen presentation through cytokines or directly interfering with synthesis or trafficking of cellular proteins involved in the antigen presentation pathway. Many viruses block the IFN-γ signaling cascade, which is important for stimulating CIITA transcription in non-APCs. Poxviruses encode IFN-γ receptor decoys to bind to IFN-γ and prevent it from the interacting with the IFN-γ receptor on the cell surface [188-190]. Similarly, EBV blocks the synthesis of IFN-γ receptor via viral early gene BZLF1 [191]. Other viruses target IFN-γ pathway by inhibiting the signal transduction of IFN-γ that occurs via the JAK1-STAT1 pathway. This includes degradation of JAK1 or STAT1, de-phosphorylation of JAK1 or STAT1, inhibiting JAK1-STAT1 complex formation or STAT1 dimerization and retention of STAT1 in the cytoplasm [192]. Herpes simplex virus 1 (HSV-1) blocks phosphorylation of JAK1 and STAT1 and degrades JAK1 depending on the cell type [193, 194]. HCMV degrades JAK1 protein and decreases STAT1 phosphorylation by inducing SHP2 phosphatase [195, 196]. Furthermore, Kaposi's sarcoma herpesvirus (KSHV) increases levels of phosphatase suppressor of cytokine

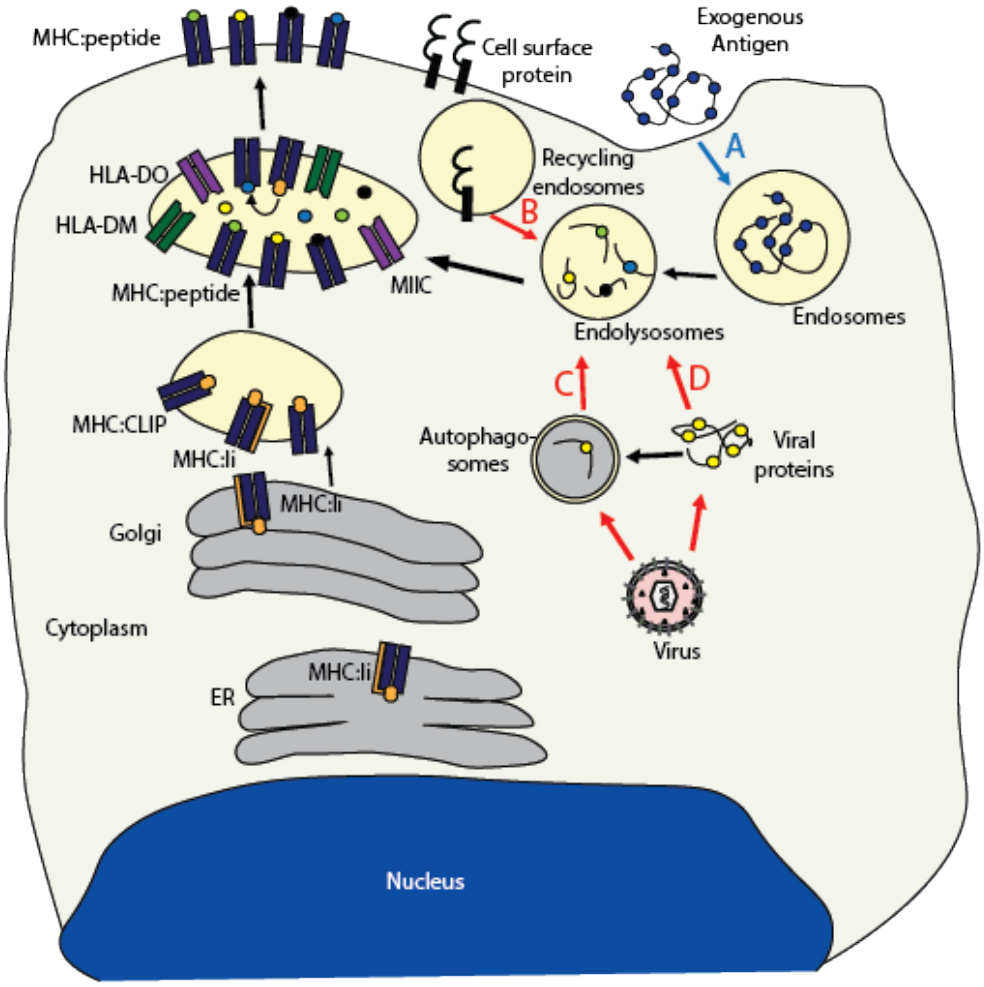

Figure 1- 6: Antigen sources for MHC class II. MHC class II can be loaded with peptides processed from exogenous proteins (A) or endogenous proteins. The endogenous antigen can be derived from cell-surface proteins that recycle through the endosomes (B), or viral proteins that are degraded through autophagy (C) or localize to the endosomes (D) during viral infections.

signaling 3 (SOCS3) to decrease STAT1 phosphorylation in infected endothelial cells [197]. In addition, the mechanisms to inhibit IFN-γ signaling include interfering with transcription of its target genes. MCMV blocks the IFN-γ pathway downstream of STAT1 nuclear translocation, preventing the induction of IFN-γ responsive genes such as MHC class II [198]. Also, KSHV utilizes viral IRF3 to decrease CIITA transcription of IFN-γ responsive pIII and pIV promoters in primary effusion lymphoma (PEL) cells [199]. Thus, viruses have many ways to inhibit the IFN-γ signaling pathway required for MHC class II induction.

Another strategy used by viruses to decrease MHC class II is through immunosuppressive cytokine IL-10. IL-10 reduces inflammatory cytokines as a general immunosuppressive effect and represses MHC class II transcription [200]. Viruses can induce secretion of IL-10 from the cell or encode a viral homolog to cellular IL-10. Many herpesviruses encode their own IL-10 homolog, and this includes HCMV and EBV from the human herpesviruses. HCMV encoded IL-10 has 30% amino acid identity to the cellular IL-10 and is expressed as two different isoforms during lytic replication and the latency cycle [201]. The lytic isoform signals through the IL-10 receptor and inhibits DC maturation while the latent isoform lacks these characteristics [202]. However, both isoforms decrease MHC class II, similar to cellular IL-10 [202, 203]. In addition to viral IL-10, HCMV also induces cellular IL-10 in monocytes, reinforcing the immunosuppressive effects of IL-10 signaling [204]. Thus, HCMV can utilize both cellular and viral IL-10 to inhibit MHC class II expression during infection. Similar to HCMV, the viral IL-10 homolog of EBV, BCRF1, exerts potent immunosuppressive activity to reduce CD4+ T cell activation and induce cellular IL-10 production in infected B cells [205, 206]. Hence, IL-10 is an important cytokine utilized by viruses to reduce MHC class II expression.

Viruses can directly block the synthesis of MHC class II. KSHV utilizes nuclear protein LANA to bind and sequester the RFX complex proteins, disrupting the assembly of MHC class II

enhanceosome required for MHC class II transcription [207]. An alternative strategy to limit the expression of the MHC class II machinery is CIITA inhibition. Another function of LANA is targeting IRF-4, a cellular transcription factor, to decrease pIII and pIV-mediated CIITA transcription in B cells [208]. EBV also targets CIITA transcription using two distinct viral proteins, BZLF1 and LMP2a. BZLF1 directly binds to the CIITA promoter and decreases its transcription while LMP2a reduces expression of transcription factors E47 and PU.1 required for pIII-mediated CIITA transcription [209, 210]. Furthermore, human immunodeficiency virus (HIV) directly interferes with CIITA activity. The Tat protein of HIV binds CIITA to prevent its interaction with positive transcription elongation factor b (p-TEFb), inhibiting CIITA-driven MHC class II transcription [211]. Thus, many viruses block CIITA to reduce MHC class II expression as a means to inhibit CD4+ T cell activation.

MHC class II presentation can also be blocked by inhibiting its maturation. Hepatitis C virus decreases cathepsin S transcription, a lysosomal protease required for Ii proteolytic cleavage, to cause improper processing of Ii resulting in reduced maturation of MHC class II [212]. Similarly, HIV-1 uses Nef protein to interfere with MHC class II maturation [213]. Additionally, vaccinia virus gene A35 alters the MHC class II maturation by increasing CLIP-MHC class II association and preventing peptide binding to MHC class II [214]. Thus, MHC class II maturation is another step in the MHC class II pathway that is targeted by viruses.

Altering MHC class II trafficking and endocytosis is another mode of interference utilized by viruses. EBV utilizes this strategy via two viral proteins, BDLF3 and gp42, to block MHC class II. BDLF3 enhances MHC class II endocytosis to reduce its surface expression [215]. gp42 directly binds to MHC class II intracellularly and traffics to the plasma membrane with the complex to hinder TCR recognition [216]. In HSV-1 infections, gB retains MHC class II in intracellular compartments, inhibiting its trafficking to the cell surface [217]. In summary, viruses have strategies to block every step of MHC class II synthesis, maturation and trafficking to

modulate the CD4+ T cell response. The various mechanisms of MHC class II inhibition by viruses are shown in Figure 1-7.

HCMV can also directly inhibit MHC class II in different cell types. HCMV utilizes US2 to degrade nascent HLA-DR and HLA-DM α chains through proteasomal degradation, while US3 binds to nascent MHC class II α and β chains, preventing their association with Ii in non-APCs [218, 219]. Additionally, pp65 blocks MHC class II expression in IFN-γ stimulated fibroblasts [220]. Most of these studies assess the impact of HCMV infection on MHC class II where the protein is forcibly expressed. Under conditions of overexpression, the regulation of MHC class II synthesis may occur via mechanisms distinct from those of the constitutively expressed protein. Thus, the regulation of endogenous MHC class II during HCMV infection is poorly understood. The experiments in this dissertation investigate the mechanism of MHC class II regulation during HCMV infection in myeloid cells that endogenously express MHC class II. Further, HCMV can decrease total CIITA transcription in mature Langerhans cells through an unidentified viral product [221]. In addition to the mechanism of constitutive MHC class II modulation by HCMV, the experiments in this dissertation determine the viral genes involved in the regulation of constitutively expressed MHC class II.

Post translational regulation of MHC class II

The presence of MHC class II on the cell surface is regulated by its trafficking within the cell. In APCs, MHC class II levels at the plasma membrane are in a constant state of flux [137, 222]. MHC class II is present in endosomes and at the plasma membrane, recycles through the endocytic machinery and is maintained at low to moderate levels on the cell surface [129, 223]. Upon receiving a maturation or activation stimulus, the constant recycling of MHC class II is halted. This, in turn, increases the density of MHC class II-peptide complexes on the cell surface for maximal antigen presentation and CD4+ T cell activation [223]. Despite the differential

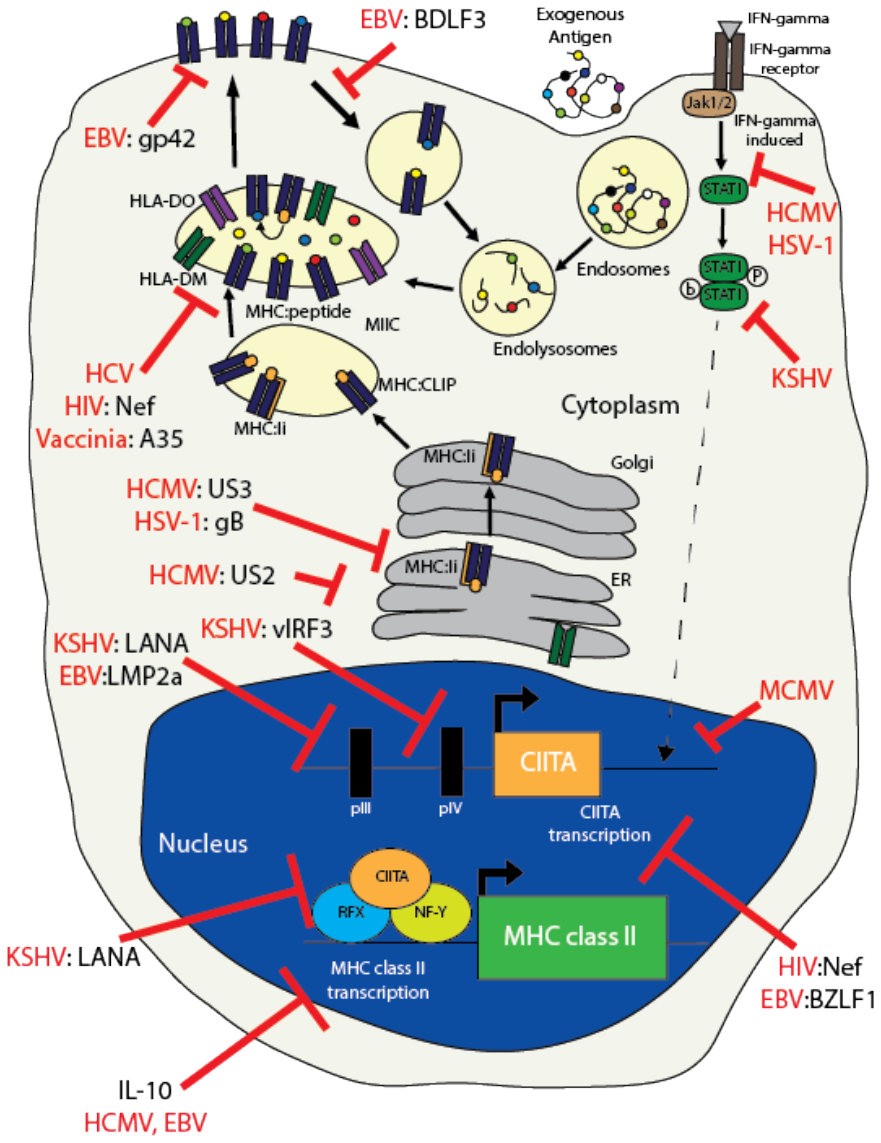

Figure 1- 7: Viral inhibition of the MHC class II pathway. Viruses can inhibit IFN-γ signaling to induce MHC class II expression, block CIITA and MHC class II transcription and synthesis, and alter trafficking and maturation of MHC class II. The various viral proteins and steps at which they function are described in detail in the text.

amounts of MHC class II expressed intracellularly in B cells and DCs, the activation of both cell types enhances MHC class II expression on the cell surface [223, 224].

The major post-translational modification for altering MHC class II trafficking is ubiquitination. The process of ubiquitination involves the addition of ubiquitin, a small protein of 7-9 kDa size, covalently attached to the β chain on the MHC class II molecule. The length of the ubiquitin chain and linkage type alter the localization of the protein or promote its degradation [225]. MHC class II ubiquitination occurs on the cytoplasmic tail of the β chain, a conserved site across many species highlighting the importance of this ubiquitin-mediated control of MHC class II protein [222]. In immature DCs, MHC class II is ubiquitinated that causes its accumulation in multivesicular bodies and subsequent degradation [226]. Upon activation, MHC class II ubiquitination is reduced resulting in less degradation and increased expression on the cell surface [226]. In B cells, the MHC class II β chain is also ubiquitinated but there is no protein degradation like immature DCs. This lack of constant turnover is due to the altered length of the ubiquitin chain on MHC class II in B cells [225]. Additionally, ubiquitination is hijacked by pathogens to decrease MHC class II. *Salmonella tymphimurium* infection induces polyubiquitination of surface MHC class II leading to enhanced endocytosis and degradation [227]. Similarly, *Franscisella tularensis*-infected macrophages induce surface MHC class II ubiquitination and degradation [228]. Hence, the regulation of MHC class II via ubiquitination of the cytoplasmic tail of β chain is important for its trafficking and degradation.

The cellular proteins mediating ubiquitination of MHC class II for endocytosis and trafficking are the E3 ubiquitin ligases MARCH1 and MARCH8 [229, 230]. MHC class II ubiquitination in B cells and immature DCs by MARCH1 and MARCH8 occurs both in humans and mice [137]. These proteins ubiquitinate MHC class II which marks it for lysosomal degradation [137].

MARCH E3 ubiquitin ligases

Overview of the MARCH proteins

The process of ubiquitination involves three stages-the activation of ubiquitin through an ATP-dependent reaction performed by a ubiquitin activation enzyme E1, the transfer of ubiquitin from E1 to an E2 ubiquitin conjugating enzyme and the final attachment of ubiquitin to target protein through an isopeptide linkage catalyzed by an E3 ubiquitin ligase enzyme [231]. The specificity for the substrate protein is determined by the E3 ubiquitin ligases, and for this reason, organisms have many genes that encode for E3 ligases to specifically target different proteins within the cell. Within the E3 ubiquitin ligases, the Really Interesting New Gene (RING) class of ligases share a catalytic domain comprised of cysteine and histidine residues coordinating two Zn^{2+} atoms [231]. MARCH (Membrane Associated RING CH) proteins are E3 ubiquitin ligases belonging to the RING class [232]. The MARCH family was discovered due to their homology to viral E3 ubiquitin ligases K3 and K5 of KSHV [233]. Since KSHV and poxviruses had similar E3 ubiquitin ligases but belonged to distinct viral families, it was hypothesized that these E3 ubiquitin ligases must have been acquired from the host [233]. A bioinformatics-based search for mammalian homologs for K3 and K5 led to the initial discovery of nine members of the MARCH family of proteins and further analysis added two more members to this family bringing the total to eleven [232, 233]. Similar to K3 and K5, these MARCH proteins have seven cysteines and one histidine positioned as cysteines(4)-histidine-cysteines(3) (C4HC3 or CH topology) in the catalytic RING domain [233]. Most of the MARCH proteins have the RING domain on their N terminus with two or more transmembrane domains, but MARCH7 and MARCH10 both lack transmembrane domains and contain the RING domain on the C terminus [234]. MARCH proteins target different substrates in the host cell, with many members localized to specific organelles. Interestingly, some MARCH proteins are homologous to another MARCH family member and function with shared substrate specificity and localization, but differential tissue

expression. Thus, the MARCH proteins are cellular E3 ubiquitin ligases with the RING domains for their catalytic activity.

MARCH1 localizes to early and late endosomes, is expressed in B cells, monocytes and DCs and has a well-understood function in the degradation and endocytosis of MHC class II and T cell co-stimulation molecule CD86 [235-237]. Recently, MARCH1 has also been shown to target STING and MAVS immune proteins [238]. MARCH8 is homologous to MARCH1, with similar localization and targets, but widely expressed in multiple cell types [229, 239]. MARCH2 and MARCH3 are another ubiquitously expressed pair of MARCH proteins that localize to endosomes, lysosomes, plasma membrane and the ER [232]. MARCH2 targets β2-adrenergic receptor G-coupled protein receptor, syntaxin6 (STX6) and cystic fibrosis conductance regulator (CFTR) through ubiquitination and degradation [240-242]. MARCH3 is about 60% identical to MARCH2 at the amino acid level and shares STX6 and CFTR as substrates with MARCH2 [243]. MARCH3 can also regulate Bap31, an ER localized protein [244]. However, Bap31 interacts with multiple members of the MARCH family, which may be due to its function as an ER chaperone. MARCH4 and MARCH9 comprise another homologous pair of MARCH proteins with approximately 60% amino acid identity. Both proteins are expressed in the Golgi, with MARCH9 localizing to lysosomes as well [233]. The shared targets of MARCH4/9 are MHC class I for lysosomal degradation as well as CD4, NK cell ligand Mult1and ALCAM glycoprotein [233, 241, 245]. However, both proteins also have independent targets, not shared amongst the pair. For MARCH4, these include Bap31, tetraspanin CD81, and syntaxin 4 (STX4) [244]. Proteins targeted solely by MARCH9 include MHC class II and accessory proteins, adhesion molecule ICAM1 and Fc receptor FCγRIIB [246-248]. However, the role of MARCH9 in MHC class II regulation is not very clear. Even with MARCH9 overexpression, the specificity and amount of degradation varies for the different MHC class II isotypes and is inconsistent across cell types. MARCH5 is a mitochondria-localized protein that interacts with mitochondrial

proteins MFN1 and MFN2 to regulate mitochondria fusion and degrade misfolded proteins within mitochondria [249, 250]. Another important function of MARCH5 is to ubiquitinate TANK, an NFκB-associated kinase, and relieve TANK inhibition of TLR7 signaling [251]. MARCH6 is an ER-associated MARCH protein that functions in ERAD, similar to its yeast homolog Doa10 [252]. Non-canonical MARCH proteins, MARCH7 and MARCH10, function in spermatogenesis along with MARCH11 that regulates the ubiquitination and degradation of SAMT1, a transmembrane protein expressed in spermatids [253-255]. MARCH7 also has a role in neuronal development, but its targets are not yet identified. The description of MARCH targets is not exhaustive and a list of all proteins targeted by the MARCH family is summarized in table 1-1. However, the elucidation of the function of endogenous MARCH proteins has been challenging due to their low expression and functional redundancy amongst MARCH family members. Most studies have relied on the overexpression of MARCH proteins which may have off-target effects and thus, the actual substrate proteins for the MARCH family *in vivo* may differ from the reported targets. In summary, the MARCH family members reside in multiple organelles and function in various tissues within the host, sharing the degradative function of protein homeostasis, but their endogenous targets are still understudied.

MARCH1 and MARCH8 proteins

MARCH1 and MARCH8 are the most studied members of the MARCH family, owing to their critical role in the host immune response. Both of these proteins have the classical MARCH topology with the N terminal RING domain, two transmembrane domains, a short lumenal domain and the C terminal tail containing motifs important for trafficking within the cell. The two proteins have greater than 70% amino acid identity with highly homologous RING and transmembrane regions, but differences in N and C terminal sequences [233]. MARCH1 and MARCH8 play an important role in immune regulation as they both target MHC class II for

Table 1- 1: Targets of MARCH proteins

Name of MARCH	Cellular Localization	TM domains	Known targets
MARCH1	Early endosomes, late endosomes, lysosomes	2	MHC class II (HLA-DR, DM, DO), CD86, CD95 (Fas), CD98, TfR, INSR, STING, MAVS
MARCH2	ER, endosomes, plasma membrane	2	CD86, TfR, DLG1, beta-2-AR, STX6, CFTR, calnexin
MARCH3	Endosomes, plasma membrane	2	Bap31, STX6, CFTR
MARCH4	Golgi	2	MHC class I, CD4, Bap31, Mult1, CD81, STX4, CD86, ALCAM
MARCH5	Mitochondria	4	MFN1, MFN2, Drp1, TANK, SOD1, LC1
MARCH6	ER	14	D2, SM
MARCH7	Cytoplasm, nucleus	0	-
MARCH8	Early endosomes, late endosomes, lysosomes	2	MHC class II (HLA-DR, DM, DO), CD86, CD95 (Fas), CD98, TfR, CD44, IL1R-AP, TRAIL-R1, STX4, Bap31, E-cadherin
MARCH9	Golgi, lysosomes	2	MHC class I, MHC class II (HLA-DR), CD4, ALCAM, ICAM-1, SLAM, Mult1, FC gamma RIIB, PTPRJ, LIR-1, CD81
MARCH10	Cytoplasm	0	-
MARCH11	Golgi, multivesicular bodies	2	SAMT1

TfR: transferrin receptor, INSR: Insulin receptor, DLG-1: Discs large homolog 1, STX6: syntaxin 6, STX4, syntaxin 4, CFTR: cystic fibrosis transmembrane conductance regulator ,ALCAM: activated leukocyte cell adhesion molecule, MFN:mitofusin, DNM1L: dynamin-1 like protein TANK: TRAF family member associated NFkB activator, SOD1: superoxide dismutase , LC1: dynein light chain, ICAM1: intercellular adhesion molecule, SLAM: signaling leukocyte activation molecule , PTPRJ: protein tyrosine phosphatase receptor type J, LIR-1: leukocyte immunogobulin-like receptor subfamily B member 1, D2: dopamine receptor, SM: Squalene monoxygenase, SAMT1: spermatogenesis associated multipass transmembrane protein 1

degradation. MARCH8 was first described to decrease CD86 and MHC class II in a transgenic mouse overexpressing MARCH8, with decreased expression of MHC class II in thymic epithelial cells, APCs and B cells, resulting in impaired CD4+ T cell development, altered APC function and resistance to CD4+ T cell-mediated autoimmune diseases [229, 256]. This decrease in MHC class II was due to the ubiquitination of a single lysine residue on the β chain [256]. In addition to the β chain ubiquitination *in vivo*, the α chain can also be ubiquitinated in cell lines both by MARCH1 and MARCH8 [257]. The ubiquitin-mediated control of MHC class II is counterbalanced by glycoprotein CD83, which antagonizes the functions of MARCH1 and MARCH8 to stabilize expression of MHC class II [239, 258]. Similar to MARCH8, MARCH1 functions *in vivo* to regulate MHC class II. MARCH1 deficient mice exhibited increased surface MHC class II in B cells [230]. Conversely, MARCH1 overexpression decreased MHC class II in human DCs [259]. Interestingly, there is an indirect effect on MHC class I expression due to the increase of MHC class II in MARCH1 knockout mice, with some cell types expressing lower surface MHC class I [260]. Thus, MHC class II is a bonafide target of MARCH1 and MARCH8 *in vivo*.

A distinct difference between the role of MARCH1 and MARCH8 in MHC class II regulation is the relationship between MARCH1 and activation state of cells. The expression of MARCH1 is tightly linked to the maturation state of DCs. MARCH1 is highly expressed in immature DCs to ubiquitinate and degrade MHC class II to regulate its levels. Upon DC maturation, MARCH1 expression is diminished to prevent MHC class II turnover and increase MHC class II on the cell surface for antigen presentation [259]. There is some ambiguity regarding the contribution of ubiquitination to enhanced MHC class II endocytosis in B cells and DCs [226, 261, 262]. However, it is clear that MARCH1 is required for the constant turnover of MHC class II under resting conditions, and that the cessation of MARCH1 activity occurs upon activation to increase MHC class II-peptide complexes on the plasma membrane. But there is no

evidence of MARCH8 expression being diminished upon maturation of cells. Thus, MARCH1 expression is modulated to alter the levels of MHC class II and regulate antigen presentation during activation of immune cells.

Within the MHC class II family of proteins, MARCH1 and MARCH8 target all three isotypes, HLA-DR, -DP and -DQ and downregulate MHC class II accessory proteins HLA-DO and DM [263, 264]. In addition to the ubiquitination of the β chain, both MARCH proteins also ubiquitinate a conserved lysine residue on the α chain of HLA-DM [263]. Interestingly, MARCH8 internalized and degraded HLA-DM, but mutation of the ubiquitination sites on HLA-DM did not inhibit MARCH8 activity [263]. Instead, MARCH8 required a tyrosine-based trafficking motif on the HLA-DM β chain for efficient endocytosis of HLA-DM [263]. This suggests that MARCH8 has a role in enhancing endocytosis of HLA-DM independent of its ubiquitination activity. Thus, the mechanism of action for MARCH1 on HLA-DM is divergent from MARCH8. Hence, MARCH1 and MARCH8 can target both canonical and non-canonical MHC class II proteins.

MARCH1 and MARCH8 can target other membrane proteins for degradation in addition to MHC class II and CD86. The common list of proteins targeted by both include Fas (CD95), transferrin receptor (TfR) and the glycoprotein CD98. Cell-surface and intracellular TfR levels decrease upon MARCH1 and MARCH8 overexpression, and this decrease requires the RING domain of MARCH proteins for activity [233, 265]. CD98 is a cell-surface, heterodimeric glycoprotein important for lymphocyte function. The heavy chain of CD98 can bind to integrins and signal for cell proliferation and survival, while the light chain acts as an amino acid transporter for leucine, isoleucine and valine [266]. MARCH1 and MARCH8 ubiquitinate CD98 to re-route CD98 from recycling endosomes to lysosomes for degradation [236]. In T cells, the introduction of ubiquitination-resistant CD98 caused increased proliferation and clonal expansion compared to wild type CD98 [267]. In addition to CD98, MARCH1 and MARCH8 can target cell

death receptors. Cell surface levels of Fas, a programmed cell death receptor, are decreased by MARCH1 and MARCH8, while another death receptor TRAIL-R1 was targeted by MARCH8 alone [233, 268]. MARCH1 can also function in metabolism by regulating insulin receptor. MARCH1 ubiquitinates insulin receptor to regulate plasma membrane levels and modulate insulin sensitivity [269]. In addition, MARCH1 can target STING and MAVS proteins in innate immune signaling [238]. Thus, MARCH1 and MARCH8 can target multiple membrane proteins and regulate their turnover.

Since MARCH8 is more ubiquitous in expression, the list of MARCH8 target proteins is greater than MARCH1. MARCH8 functions in innate immune activation by controlling the activation of inflammatory IL-1 signaling. Overexpression of MARCH8 degrades IL-1 receptor accessory protein (IL1RAP) via lysine 48 (K48)-mediated polyubiquitination and inhibits IL-1β signaling pathway [270]. Proteomic screening for additional MARCH8 targets further identified CD44, CD81 and Bap31 as interacting partners. However, the functional consequence of such interactions is still unclear. Lastly, MARCH8 also decreases E-cadherin, a cell adhesion molecule, in developing zebrafish embryos [271]. Thus, MARCH8 degrades multiple membrane proteins independent of MARCH1.

MARCH1 domains for function and localization

The analysis of MARCH1 protein has identified key domains and residues that are required for the interaction and targeting of substrates. The most important domain for the E3 ubiquitin ligase activity is the RING domain. Mutation of the MARCH1 RING domain disrupts its ability to decrease MHC class II, due to the lack of E3 ubiquitin ligase catalytic activity [230, 246, 272]. Substrate recognition for MARCH1 is mediated by transmembrane domains. With MHC class II as a substrate, domain swapping experiments with MARCH9, a protein from the same family that targets MHC class I, demonstrated the requirement of both the N and C terminal transmembrane regions of MARCH1 to interact with MHC class II and target it for degradation.

In addition to the transmembrane regions, both murine and human MARCH1 require a certain region within the C terminal tail for functional activity against target proteins [272, 273]. Thus, the transmembrane and the RING domains of MARCH1 are critical for substrate targeting and ubiquitination respectively.

Another important observation regarding MARCH1 is the low abundance of the MARCH1 protein even after overexpression [246]. This may occur due to the relative instability and short half-life of the protein. Interestingly, the half-life of murine MARCH1 is less than that of human MARCH1 [246, 272]. For many E3 ubiquitin ligases, the short half-life occurs due to their ability to self-ubiquitinate and regulate their own protein levels. MARCH1 has a similar self-ubiquitinating activity mediated by K48 ubiquitin linkage [246]. Additionally, MARCH1 ubiquitination decreases upon expression of inactive RING domain-containing MARCH1 mutant, suggesting that the protein can induce its own ubiquitination [246]. However, the inactive MARCH1 mutant is still ubiquitinated, albeit to a lower level, suggesting the involvement of other cellular E3 ubiquitin ligases to regulate MARCH1 protein levels [246]. Importantly, MARCH1 does not require ubiquitination for its function. Deletion or alteration of lysine residues on MARCH1 reduced its ubiquitination but still decreased surface MHC class II [246]. Thus, the expression of MARCH1 is tightly regulated within cells by ubiquitination.

MARCH1 localizes to early endosomes, late endosomes and lysosomes. In addition, murine MARCH1 also co-localizes with trans-Golgi markers, not observed for human MARCH1 [272]. Due to the endosomal localization, MARCH1 can also be incorporated into extracellular vesicles (EVs). This EV incorporation has been attributed to tyrosine-based sorting motifs present in the N and C terminus that could target MARCH1 protein to these vesicles [273]. Interestingly, mutation of the tyrosine residues in the C terminus tail did not alter the localization of human MARCH1 in late endosomal compartments, but reduced its incorporation into EVs [273]. However, murine MARCH1 requires the C terminal tyrosine motifs for its localization [272]. The

important domains for MARCH1 function are depicted in Figure 1-8. Although majority of the MARCH1 protein domains have conserved functions in both humans and mice, there are some differences in localization and structure-function relationships between human and murine MARCH1.

MARCH1 protein can also exist as a dimer when over-expressed in cells. MARCH1 self-dimerizes or forms heterodimers with MARCH8 and MARCH9 [246]. These interactions were independent of RING domains and required the transmembrane domains for dimerization [246]. While it is speculated that dimerization of MARCH1 may increase its stability, whether these dimers are functional or exist under endogenous conditions is still unknown.

Regulation of MARCH1 expression

The induction of MARCH1 expression has been observed under immunosuppressive conditions. In monocytes, MARCH1 mRNA expression is induced by treatment with anti-inflammatory cytokine IL-10 in monocytes [274]. The increase in MARCH1 due to IL-10 results in the ubiquitination of MHC class II [274]. A similar effect of IL-10-mediated MARCH1 induction to decrease MHC class II and CD86 is observed in human macrophages [275]. However, the effect of IL-10 on MARCH1 induction is dependent on cell types. In DCs, IL-10 does not directly induce MARCH1 expression but suppresses DC activation [275]. In specialized B cells, IL-10 induced an increase in MHC class II with a concomitant decrease in MARCH1 expression, resembling a B cell activation state [276]. This is in contrast to the usual immunosuppressive function of IL-10. Additionally, pathogens have usurped the IL-10-driven MARCH1 induction to decrease MHC class II. The reduction of MHC class II in macrophages infected with *Francisella tularensis* occurs by cellular IL-10 activation to increase MARCH1 expression for MHC class II targeting [277]. Hence, IL-10 regulates MARCH1 but this regulation is distinct in different cell types depending on the activation state of cells.

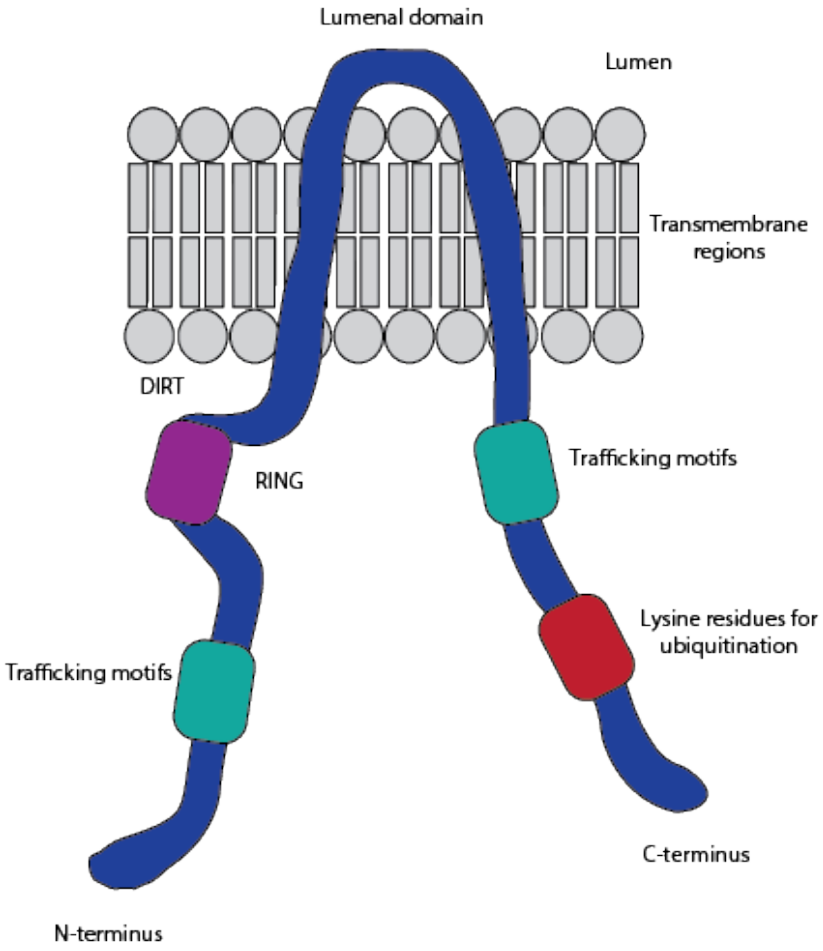

Figure 1- 8: Key features of the MARCH1 protein. MARCH1 is an E3 ubiquitin ligase that contains the RING domain for its catalytic activity and the DIRT (domain between RING and transmembrane regions) region for its activity on the N terminus. It has two transmembrane domains, important for targeting substrate proteins, and a short lumenal domain. The C terminus contains lysine residues that are ubiquitinated and regulate MARCH1 protein expression. Both the N and C terminus have tyrosine-based trafficking motifs.

MARCH1 expression is influenced by other inflammatory and immunosuppressive cytokines. Lipopolysaccharide (LPS) treatment of DCs decrease MARCH1 both at the transcriptional and translational level [259]. Both DCs and B cells decrease MARCH1 expression upon receiving maturation signals in order to stop MHC class II turnover [230, 259]. Other cytokines such as TGF-β, TNF-α and IL-1β can increase MARCH1 expression in ovarian cancer cell lines [278]. In insulin-dependent regulation, MARCH1 expression is controlled by FOXO1 transcription factor where insulin stimulation displaces FOXO1 bound to MARCH1 promoter and decreases its expression [269]. Furthermore, type I interferons can also stimulate MARCH1 expression in monocyte-derived macrophages [279]. Hence, MARCH1 expression can be modulated by a variety of cytokines and stimuli altering the activation of cells.

Role of MARCH proteins in viral infections

The role of the MARCH proteins during viral infections has only recently been studied. MARCH8 interacts with specific viral glycoproteins and increases their intracellular retention. HIV-1 glycoprotein Env and vesicular stomatitis virus glycoprotein G (VSV-G) both are targets of MARCH8 and have decreased cell surface levels upon MARCH8 expression [280]. Env is intracellularly sequestered by MARCH8 while VSV-G is retained within the cell and degraded via lysosomes [280]. This interaction with viral glycoproteins is not limited to MARCH8 only. MARCH1, 2 and 9 also reduce lentivirus infectivity upon overexpression, and exert similar effects on Env and VSV-G by reducing incorporation of viral glycoproteins into virus particles [279]. The interaction of both MARCH proteins with viral glycoproteins requires the RING domain, although none of the viral proteins were tested for ubiquitination [279, 280]. Additionally, MARCH2 is also induced upon HIV-1 infection in various cell types, suggesting that MARCH2 activity could be utilized by the cell to inhibit viral infection [281]. This suggests that the MARCH proteins could serve as antiviral factors in HIV-1 infections. Conversely, MARCH8 acts as a proviral factor required for hepatitis C virus envelopment and assembly

[282]. MARCH8 ubiquitinates non-structural protein 2 (NS2) through lysine 63 (K63) linkage and this ubiquitination is important for hepatitis C virus envelopment [282]. The role of MARCH proteins during virus infections is depicted in Figure 1-9. Thus, MARCH proteins can play divergent roles during virus infections where two scenarios exist: 1) viruses can hijack MARCH proteins to facilitate infection or 2) the antiviral response of the cell induces MARCH proteins to block viral infection. The experiments described in this dissertation demonstrate a role for MARCH1 during HCMV infection with the induction of MARCH1 late in infection. They investigate the impact of this induction during productive viral replication, and determine the localization and target of MARCH1 during HCMV infection. Interestingly, the expression of MARCH1 too early in infection is unfavorable, illustrating the importance of temporal regulation for its optimal function.

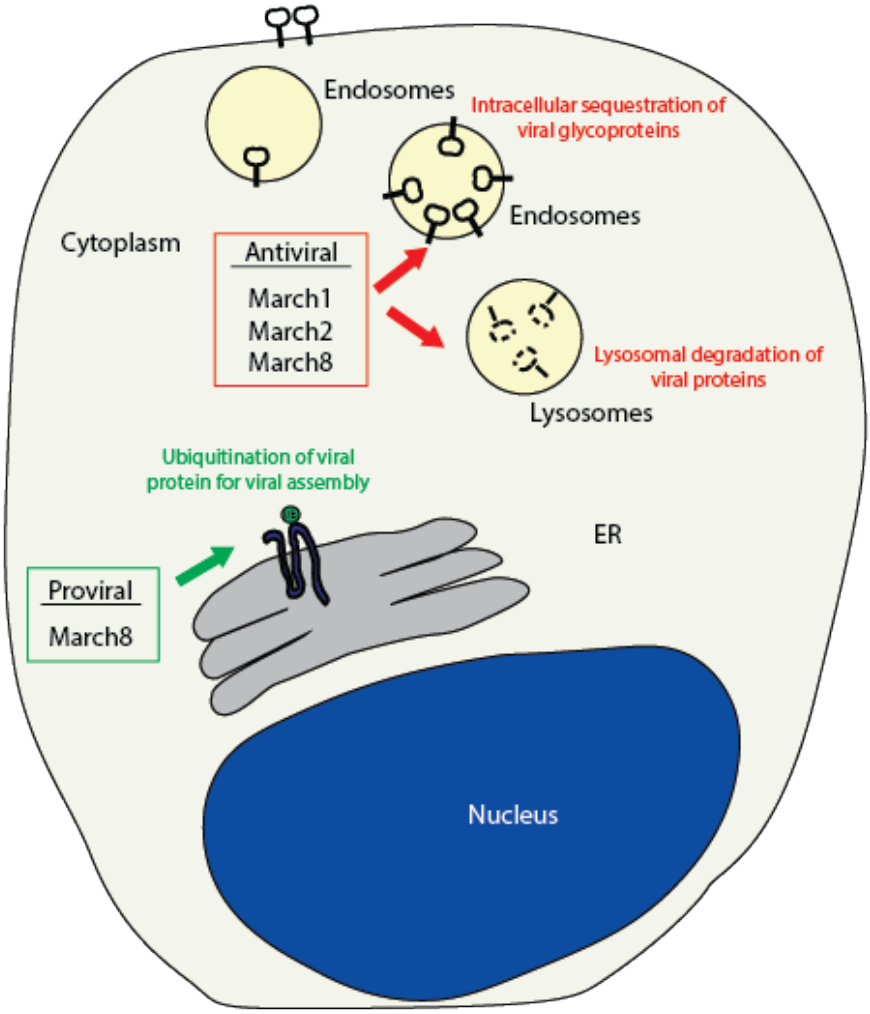

Figure 1- 9: MARCH proteins in viral infections. MARCH1, 2 and 8 sequester viral glycoproteins VSV-G and Env and induce the degradation of VSV-G. MARCH8 ubiquitinates viral protein NS2, important for its function in viral assembly of HCV.

Chapter 2: HCMV decreases MHC class II by regulating CIITA transcript levels in a myeloid cell line

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Published in *Journal of Virology* April 2020 Volume 94 Issue 7

Abstract

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that encodes many proteins to modulate the host immune response. Extensive efforts have led to the elucidation of multiple strategies employed by HCMV to effectively block NK cell targeting of virus infected cells and the major histocompatibility complex (MHC) class I primed CD8+ T cell response. However, viral regulation of the MHC class II-mediated CD4+ T cell response is understudied in endogenous MHC class II-expressing cells, largely because popular cell culture systems utilized for studying HCMV do not endogenously express MHC class II. Of the many cell types infected by HCMV in the host, myeloid cells, such as monocytes, are of particular importance due to their role in latency and subsequent dissemination throughout the host. We investigated the impact of HCMV infection on MHC class II in Kasumi-3 cells, a myeloid-progenitor cell line that endogenously expresses the MHC class II gene, HLA-DR. We observed a significant reduction in expression of surface and total HLA-DR at 72 hpi and 120 hpi in infected cells. The decrease in HLA-DR is independent of previously described viral genes that regulate the MHC class II complex or the unique short (US) region of HCMV, a region expressing many immunomodulatory genes. The altered surface level of HLA-DR was not a result of increased endocytosis and degradation, but a reduction in HLA-DR transcripts due to a decrease in expression of the class II transactivator (CIITA).

Importance

Human cytomegalovirus (HCMV) is an opportunistic herpesvirus that is asymptomatic for healthy individuals but can lead to severe pathology in congenital infections and immunosuppressed patients. Thus, it is important to understand the modulation of the immune response by HCMV, which is understudied in the context of endogenous MHC class II regulation. Using Kasumi-3 cells as a myeloid progenitor cell model endogenously expressing MHC class II (HLA-DR), this study shows that HCMV decreases expression of HLA-DR in infected cells by reducing transcription of HLA-DR transcripts early during infection, independent of previously implicated genes. This is an important finding as it highlights a mechanism of immune evasion utilized by HCMV to decrease expression of MHC class II in a relevant cell system that endogenously expresses the MHC class II complex.

Introduction

Human cytomegalovirus (HCMV) is a clinically significant herpesvirus that drastically alters the host cell during its protracted replication cycle. Among the well-characterized alterations is a striking change to the cellular proteome and a remodeling of the protein composition of the plasma membrane [283]. Plasma membrane composition can be altered by multiple mechanisms, which include modifying the kinetics of endocytosis, recycling and lysosomal degradation of target proteins, altering their trafficking and localization away from the plasma membrane, and changing the rate of synthesis at the transcriptional and translational levels.

Immune proteins constitute a significant portion of the plasma membrane proteins regulated by HCMV. Examples of a few of the many immune proteins whose surface expression is regulated by this virus include MICA (MHC class I polypeptide-related sequence A), MICB, tumor necrosis factor α (TNFα), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), TRAIL-R2, and the UL16 binding proteins 1, 2, 3 & 6 [112, 113, 284-286]. Perhaps the best-studied example is major histocompatibility complex (MHC) class I, as HCMV utilizes several well-described strategies to ensure reduced MHC class I antigen presentation [55, 56, 122, 287]. Modulating presentation of MHC class II, which presents both exogenous and endogenous antigens, would also be beneficial for HCMV infection. The pool of MHC class II presented antigens could include antigens derived from viral proteins expressed within infected cells [288]. CD4+ T cell response to endogenous antigen has been demonstrated for many different viruses [289]. HCMV-infected cells can present endogenous antigen to prime the CD4+ T cell response [219]. CD4+ T cells specific for both MCMV and HCMV express granzyme B and possess cytolytic activity to control infection [290-292]. Therefore, controlling the action of CD4+ T cells would be advantageous for HCMV infection, specifically to block endogenous presentation of viral proteins.

The MHC class II pathway involves synthesis of MHC class II proteins, their association with invariant chain (Ii) within the endoplasmic reticulum, trafficking through the Golgi apparatus to the MHC class II loading compartment (MIIC) and loading of peptide with help from accessory factors in endosomal MIICs [293]. These mature MHC class II molecules move to the plasma membrane to present peptide to CD4+ T cells. Pathogens employ various mechanisms to block MHC class II presentation at different steps of the pathway. These strategies targeting MHC class II and accessory factors can be broadly divided into three categories- 1) synthesis, assembly and loading, 2) altered trafficking and localization and 3) targeted degradation. Blocking assembly of MHC class II components, for example interfering with the interaction between the invariant chain and the MHC class II alpha and beta chains, is an attractive strategy. In fact, US3 mediated inhibition of Ii-MHC class II complex formation has been reported for HCMV [218]. Altered localization of MHC class II can be achieved by rerouting MHC class II to endosomes or lysosomes where the complex is degraded or retained in these vesicles away from the plasma membrane. HCMV infected cells have been shown to contain MHC class II retained within perinuclear vesicles in the cell and this aberrant localization has been observed in fibroblasts, myeloid cells and endothelial cells [195, 220, 294]. Additionally, US2 directly targets MHC class II for degradation by binding and degrading the alpha chain of HLA-DR and HLA-DM within HCMV-infected glioblastoma U373 cells [219]. For MCMV, M78 binds to MHC class II and degrades it in the lysosomes within infected myeloid cells [295].

In addition to these mechanisms, viruses can use cytokine-mediated effects to regulate MHC class II. MHC class II is induced by interferon gamma (IFN-γ), and blocking IFN-γ signal transduction is an effective approach for preventing induced MHC class II production. Accordingly, HCMV blocks IFN-γ induced surface MHC class II in infected endothelial and glioblastoma cells by degrading Jak1 to prevent IFN-γ signal transduction [195, 296]. MCMV

exerts a similar effect on IFN-γ driven MHC class II expression [198]. While IFN-γ induces MHC class II synthesis, IL-10, an immunosuppressive cytokine, decreases MHC class II via multiple mechanisms. HCMV encodes an IL10 homolog, UL111A, that has immunomodulatory function on responder cells and decreases MHC class II during latent infections [202, 203]. Additionally, HCMV can induce cellular IL-10 to achieve a similar function and MCMV utilizes IL-10 signaling for viral persistence in salivary gland [297, 298]. Thus, blocking antigen presentation by MHC class II molecules is a strategy employed by cytomegaloviruses to promote infection.

HCMV can infect many different cell types such as fibroblasts, epithelial cells and myeloid cells. HCMV infections in vitro have classically been studied in fibroblasts because of the robust lytic replication of the virus in this cell type. However, the dissemination of the virus in vivo occurs primarily through cells of the myeloid lineage. The virus infects myeloid progenitor cells and undergoes latency. Upon reactivation of the virus, myeloid cells are integral for viral spread throughout the host. Given their importance for viral spread and their ability to act as antigen-presenting cells, it is critical to understand how HCMV manipulates myeloid cells to its advantage during infection. Using primary cells as an HCMV infection model has been challenging due to the fact that these cells obtained ex vivo have considerable donor variability in terms of infectivity and are not amenable for techniques that require teasing out specific viral proteins and mechanisms. However, HCMV can infect several cell lines of the myeloid lineage that mimic primary cells with regards to many aspects of HCMV infection, and thus have great potential as experimental models for studying HCMV infection in vitro [299-302]. One of these cell lines, Kasumi-3, expresses appreciable levels of MHC class II HLA-DR and we took advantage of this line to investigate how HCMV controls endogenously expressed MHC class II complexes. These cells are a clonal myeloid progenitor cell line derived from a myeloperoxidase-negative leukemia patient, express surface CD34 and MHC class II and serve as a model of latency and re-activation [299, 300, 303]. Previous work implicating viral genes for MHC class II

regulation has involved IFN-γ stimulation to drive MHC class II expression in non-myeloid cells. The impact of HCMV infection in myeloid progenitor cells for MHC class II has been understudied. Using Kasumi-3 cells enables us to understand the regulation of endogenous MHC class II in infected myeloid cells without differentiation or activation of cells. We found that HCMV does downregulate surface levels of MHC class II, but that this decrease was independent of the mechanisms reported for regulating MHC class II under either induced or overexpressed conditions. We observed that surface MHC class II molecules are endocytosed and degraded at the same rate in uninfected and HCMV-infected cells, indicating that HCMV does not promote degradation of surface MHC class II molecules. Rather, our results show that HCMV repression of MHC class II primarily occurs at the transcriptional level as a result of downregulation CIITA expression.

Results

HCMV reduces surface and total levels of MHC class II

To determine a suitable model for addressing the mechanism of MHC class II downregulation in an endogenously expressing system, we checked for surface levels of the MHC class II human leukocyte antigen DR isotype (HLA-DR) in three different myeloid cell lines, Kasumi-3, KG1 and THP-1. While both KG1 and Kasumi-3 cells were positive for surface HLA-DR, very little was detected in undifferentiated THP-1 cells (data not shown). Since we found the Kasumi-3 cells to be more amenable to infection and could attain a higher percentage of infected cells, we chose them as our model for addressing the mechanism of MHC class II regulation by HCMV. We next investigated the surface levels of other MHC class II HLAs on Kasumi-3 cells. We detected no HLA-DQ, as the cell population was indistinguishable from unstained cells (Figure 2-1A). While there was very little staining for HLA-DP, the predominant HLA subtype detected on Kasumi-3 cells was HLA-DR (Figure 2-1A). Thus, we used HLA-DR for our subsequent experiments to investigate how HCMV regulates MHC class II surface levels.

Since reduced surface expression of MHC class II complexes would benefit a viral infection, we hypothesized that surface HLA-DR would decrease following infection by HCMV. To test this hypothesis, we infected Kasumi-3 cells with a virus expressing mCherry from its genome to allow for identification of infected cells [304]. Although only a minority of the population was infected, these cells could be identified as mCherry positive (Figure 2-1B) and surface HLA-DR levels could be assessed on both the mCherry positive (infected) and negative (uninfected) populations. Gating the cells on mCherry allowed us to separate infected and uninfected cells, however we did not characterize the infection as lytic or latent. Following HCMV infection, surface HLA-DR levels were reduced when compared to uninfected samples at both 72 and 120 hpi (Figure 2-1C). Quantification using the geometric mean fluorescence showed that levels were reduced to ~60% at 72 hours, further decreasing to ~20% by 120 hpi (Figure 2-1D). Thus, MHC class II surface expression steadily declines throughout HCMV infection of Kasumi-3 cells. To determine whether this surface downregulation also occurred in primary cells, we next infected CD14+ human peripheral blood monocytes and monitored surface HLA-DR at 72 hpi. We observed decreased surface HLA-DR (Figure 2-1E), similar to what was observed in the Kasumi-3 cells.

We next wondered whether the decrease in surface MHC class II correlated with a decrease in total MHC class II. Reduced surface levels could be due to intracellular sequestration of the protein, in which total MHC class II levels would remain steady. Total HLA-DR levels were measured in permeabilized Kasumi-3 cells that were uninfected or infected for 72 or 120 hpi. Similar to the surface levels, total HLA-DR protein was reduced at 72 hpi and further declined by 120 hpi (Figure 2-2A). The decrease in total HLA-DR protein in infected cells was confirmed by western blot analysis (Figure 2-2B). Immunofluorescence analysis of uninfected

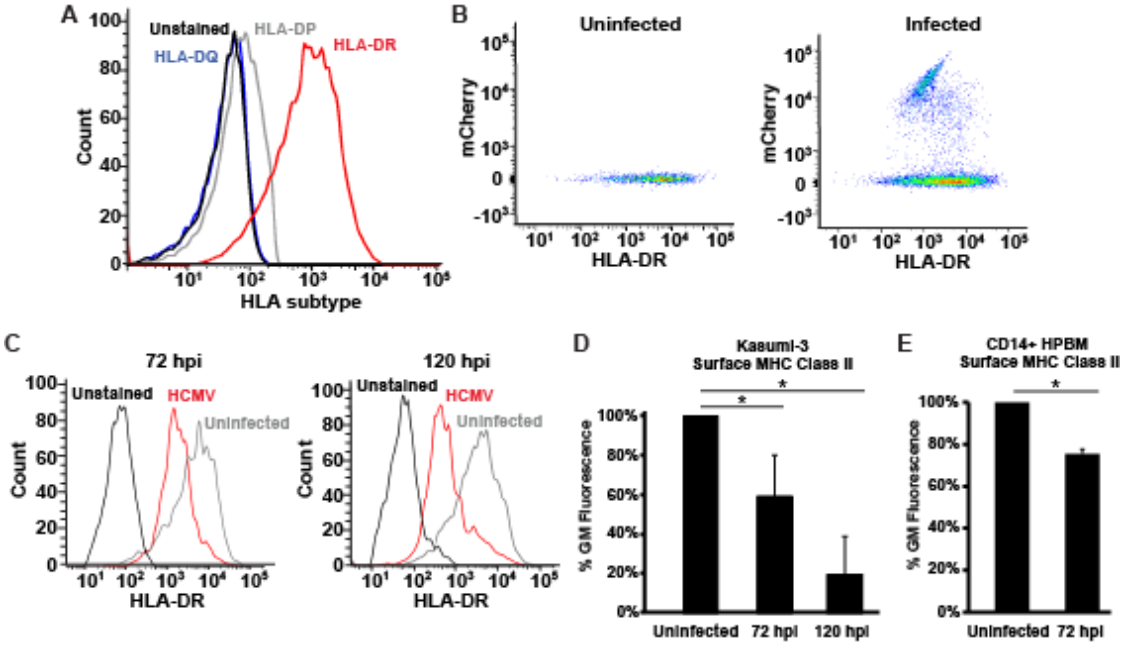

Figure 2- 1: HCMV downregulates surface expression of MHC class II in a myeloid progenitor cell line. (A) Flow cytometry analysis of unstained Kasumi-3 cells or cells stained for HLA-DR, HLA-DQ and HLA-DP. (B) Flow cytometry scatter plot of HCMV-infected (72 hpi) and uninfected cells showing relationship between mCherry (marker of HCMV infection) and HLA-DR. (C) Histograms of HCMV-infected (mCherry) and uninfected Kasumi-3 cells stained for surface HLA-DR at 72 and 120 hours post infection. (D) Bar graph of the geometric mean fluorescence values from samples in (C) displayed as a percentage of the uninfected sample. (E) Geometric mean fluorescence values from uninfected or HCMV-infected CD14+ human peripheral blood monocytes (HPBM) at 72 hpi. Values are averages from a minimum of three independent experiments. * indicates $p<.05$.

Kasumi-3 cells showed HLA-DR localized to both the cell surface and intracellular puncta (Figure 2-2C). At 72 hours post infection some surface staining remained, but much of the HLA-DR protein localized to internal puncta. Any HLA-DR detected at 120 hpi was entirely localized to intracellular puncta and very little HLA-DR was detected in some cells (Figure 2-2C). At 72 hpi, most of the HLA-DR puncta either co-localized with or were adjacent to LAMP1 (Figure 2-2D); however, some HLA-DR puncta with no correlation to LAMP1 were present. At 120 hpi, almost all HLA-DR puncta co-localized with LAMP1. These LAMP1/HLA-DR positive puncta could represent MHC class II loading compartments or degradative lysosomes, which would be consistent with the decrease observed for both surface and total HLA-DR molecules during infection

The HCMV-dependent reduction in MHC class II is independent of viral proteins previously reported to downregulate MHC class II

Several mechanisms for how HCMV regulates MHC class II have been reported in cell lines in which the complex is not natively expressed [202, 218-220, 295, 305]. We next sought to determine which of these mechanisms might apply to the regulation of endogenously expressed MHC class II in cells of the myeloid lineage. Two HCMV proteins expressed from the unique short region of the genome, US2 and US3, alter the stability, loading, and trafficking of MHC class II molecules [218]. However, the expression of these genes was disrupted during the generation of the BAC used to propagate the TB40/E strain [306]. As confirmation, both US2 and US3 were detected in cDNA harvested from cells infected with the AD169 strain, but not the TB40/E strain utilized for the above experiments (Figure 2-3A). Thus, the MHC class II-regulation we observed is not dependent on US2 or US3.

MHC class II regulation has been reported for two other viral proteins, the tegument protein pp65 and the viral IL-10 homologue UL111A [202, 220, 305]. In IFN-γ stimulated fibroblasts, transfection of pp65 alone was sufficient to reduce surface HLA-DR levels [220]. To

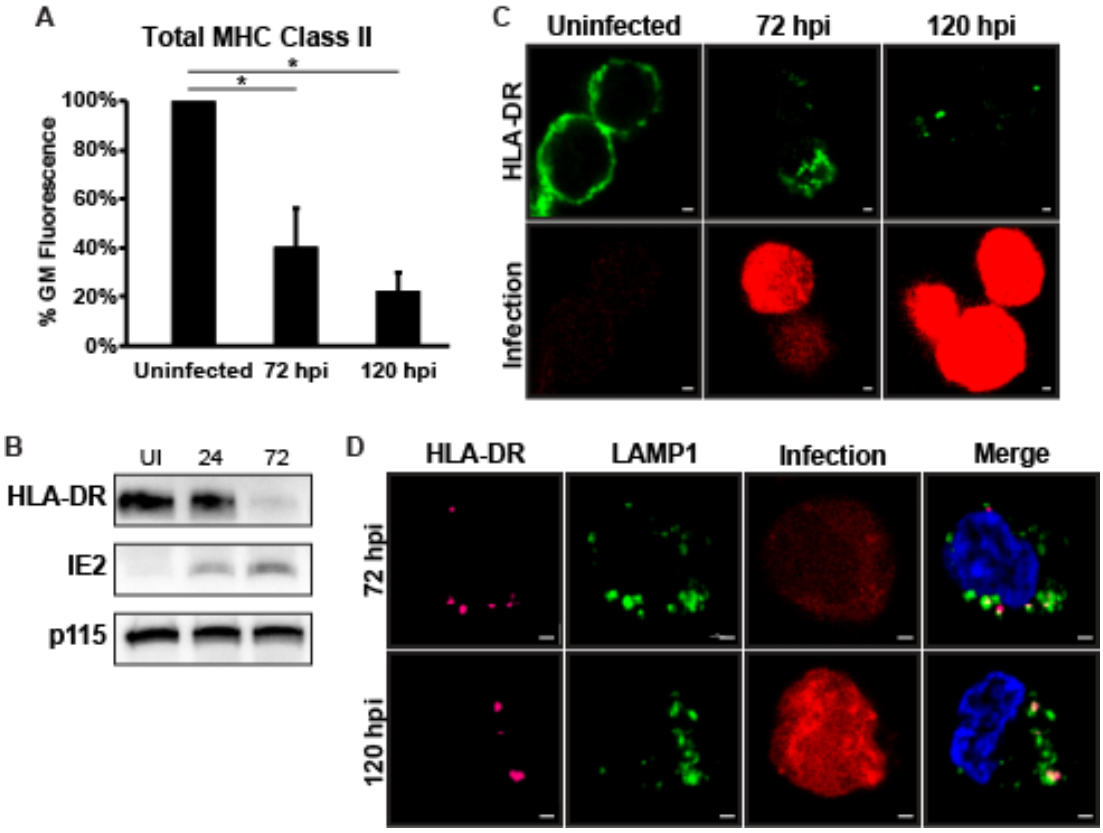

Figure 2- 2: Total MHC class II is reduced during HCMV infection. (A) Bar graph of the geometric mean fluorescence values of total HLA-DR protein in uninfected and infected samples (72 & 120 hpi). Values are a percentage of uninfected sample and are averages from at least three independent experiments. * indicates $p<.05$. (B) Western blot analysis of uninfected and HCMV-infected Kasumi-3 cells at 24 and 72 hpi to detect HLA-DR, IE2 and p115 (loading control) protein levels. (C) Immunofluorescence of total HLA-DR (green) protein in uninfected and HCMV-infected Kasumi-3 cells at 72 and 120 hpi. Infected cells expressed mCherry (red) from genome as marker for infection. (D) Immunofluorescence of total HLA-DR (pink), LAMP1 (green) in HCMV-infected (red) Kasumi-3 cells at 72 and 120 hpi. Nuclei stained with DAPI. Scale bars in (C) & (D) equal 1 μm.

test whether endogenously expressed MHC class II was reduced in the presence of pp65, we expressed either GFP or GFP-pp65 in Kasumi-3 cells and compared the expression of surface HLA-DR in the GFP positive populations. Expression of pp65 did not reduce MHC class II surface levels (Figure 2-3B). Thus, pp65 alone is not responsible for the downregulation of MHC class II during infection of Kasumi-3 cells. To test the contribution of the viral IL-10, or UL111A protein, we generated a virus in which the start codon of UL111A was replaced with a stop codon. The UL111A-STOP virus produced infectious virions at wildtype levels (Figure 2-3C) and western blot analysis confirmed that UL111A was not expressed (Figure 2-3D). MHC class II surface levels were still decreased despite the absence of UL111A (Figure 2-3D). Thus, the HCMV downregulation of MHC class II is independent of the viral IL-10 protein.

A recent study using mouse cytomegalovirus demonstrated a role for the MCMV M78 protein in degrading MHC class II [295]. To test whether the HCMV homologue may be playing a similar role, we generated a virus deficient for UL78 expression by replacing the entire UL78 ORF with *galK*. The UL78-*galK* virus grew as well as wildtype virus (Figure 2-3C), perhaps even slightly better, and was able to downregulate MHC class II with similar kinetics as the wildtype virus (Figure 2-3E). To confirm that UL78 was not responsible for the observed MHC class II surface downregulation, we next transfected Kasumi-3 cells with a vector control or plasmid expressing UL78-FLAG and confirmed expression of the UL78 construct by western blot analysis. We observed no decrease in surface MHC class II in the UL78-transfected samples (Figure 2-3F). Collectively these data show that UL78 is not responsible for MHC class II downregulation in Kasumi-3 cells.

Class II molecule downregulation is independent of the unique short region of the HMCV genome

HCMV downregulates MHC class II in the myeloid lineage Kasumi-3 cell line; however, this downregulation appears to be independent of previously published mechanisms. We sought

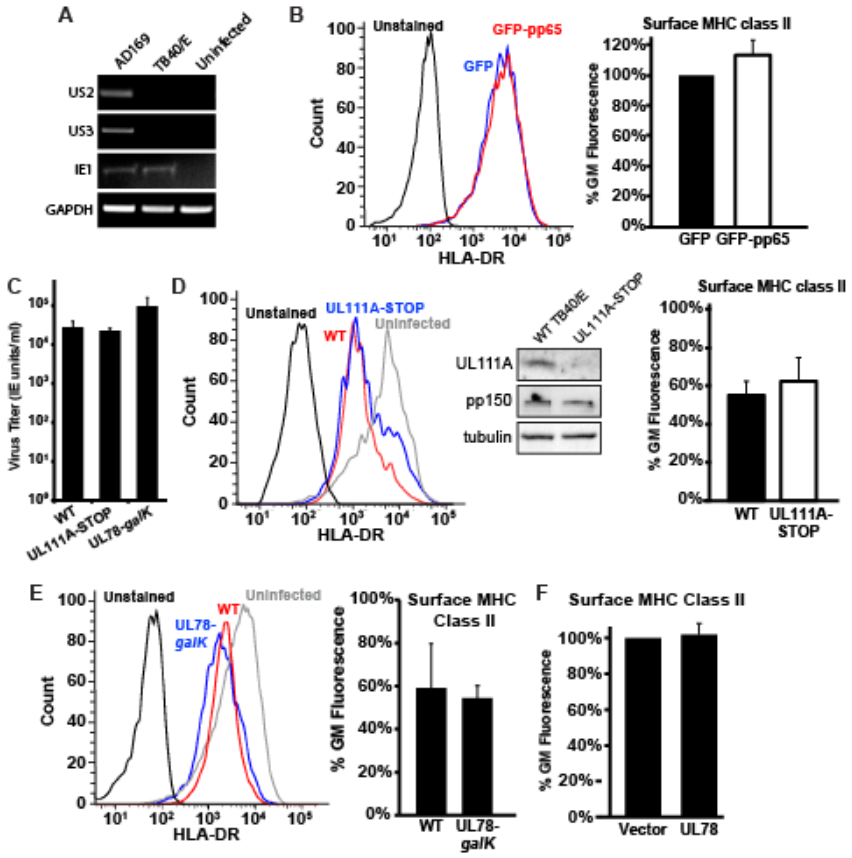

Figure 2- 3: Kasumi-3 cells do not regulate MHC class II using viral proteins previously reported to decrease MHC class II expression and localization. (A) RT-PCR analysis of US2, US3, IE1 and GAPDH transcripts from uninfected fibroblasts or fibroblasts infected with the AD169 or TB40/E strains of HCMV (96 hpi). (B) Histograms of surface HLA-DR protein in Kasumi-3 cells 48 hours after electroporation with either GFP control or GFP-pp65. Quantification of the geometric mean fluorescence is shown on right as a percentage of the GFP control sample. (C) Infectious titers at 120 hpi of wildtype TB40/E (WT), and the UL111A-STOP and UL78-*galK* viruses following MOI=3 infection of fibroblasts. (D) Histograms of surface HLA-DR protein in Kasumi-3 cells infected with mCherry expressing wildtype TB40/E or a virus lacking UL111A (UL111A-STOP) expression at 72 hpi. Western blot analysis confirms absence of UL111A protein. Quantification of the geometric mean fluorescence is shown on right as a percentage of the uninfected sample. (E) Histograms of surface HLA-DR protein in Kasumi-3 cells infected with mCherry expressing wildtype TB40/E or a virus lacking UL78 expression (UL78-*galK*) at 72 hpi. Quantification of the geometric mean fluorescence of TB40/E or a virus lacking UL78 (UL78-*galK*) is shown on right as a percentage of the uninfected sample. (F) Graph representing geometric mean fluorescence of surface HLA-DR protein in Kasumi-3 cells 48 hours post electroporation with either vector control or plasmid expressing UL78. Histograms in (B), (D) and (E) are representative images from one of at least three independent experiments. Values from bar graphs in (B), (C), (D), (E) and (F) are percentages of the geometric mean fluorescence from uninfected or control transfected cells and are averages from at least three independent experiments.

to determine the viral factors responsible for downregulating MHC class II and hypothesized that the factor(s) responsible may be expressed from the unique short region of the genome, a region that has been shown to encode several factors important for immune modulation and regulation of MHC class I and NK ligands. To screen this region for class II regulating factors, we generated five viruses with five to six contiguous genes replaced with *galK* (ΔUS7-US12, ΔUS13-US18, ΔUS19-US24, ΔUS26-US30, ΔUS31-US34A) as depicted in the schematic in Figure 2-4A. Successful replacement of the US gene segments with *galk* was confirmed by PCR (Figure 2-4B).

We were able propagate all of the US deletion viruses and importantly, none of the viruses exhibited a growth phenotype on fibroblasts (Figure 2-4C). Analysis of surface MHC class II levels following infection with US deletion viruses showed that all five viruses were competent for downregulating MHC class II surface expression (Figures 2-4D & 2-4E). In cells infected with the ΔUS13-US18 virus, a slight increase was observed in HLA-DR surface levels when compared to levels in a wildtype infection, however this increase was not statistically significant and the virus still largely retained the ability to downregulate surface MHC class II. This may indicate that a factor in this region could be a minor participant in regulating surface HLA-DR, however this factor is clearly not solely responsible for reducing surface MHC class II. Thus, MHC class II surface levels were still reduced in the absence of each the HCMV US proteins. This suggests that none of these proteins alone are responsible for the MHC class II downregulation, although we cannot rule out redundancy amongst other US genes that were still expressed in each of the deletion viruses. Attempts to replace the entire US region of the genome were unsuccessful, as the virus was unable to spread following electroporation of the US-deficient BAC.

HCMV utilizes an immediate or early protein to regulate surface MHC class II levels

To this point, none of the viral factors investigated were responsible for the decrease in MHC class II surface protein during infection of the myeloid progenitor Kasumi-3 cell line. It is

unclear which, if any, of the above gene products are even expressed during an infection of Kasumi-3 cells. To investigate the expression of viral genes in our Kasumi-3 infections, we examined the protein levels of two immediate early (IE1 & IE2), two delayed early (UL71 and pp150) and two late proteins (pp28 and pp71) at 24, 72 and 120 hours post infection. We found that both the immediate early and early proteins were expressed and peaked at 72 hpi, with markedly reduced levels at 120 hpi (Figure 2-5A). Both late proteins, which are present in the virion tegument layer, were present at 24 hpi, likely a result of incoming protein from the high MOI infections. However, very little late protein was detected at 72 and 120 hpi, indicating suppressed expression of late proteins in our Kasumi-3 infections. This may indicate that the infected cells were shutting down viral protein expression, although we have not adequately characterized the infection as lytic or latent. At least some cells were undergoing a productive infection as low amounts of infectious virions could be recovered (data not shown). Thus, we hypothesized that MHC class II downregulation was a result of immediate or early gene expression. To test this, we investigated whether acyclovir could prevent the decrease in surface MHC class II levels. Expression of viral late proteins is dependent upon DNA replication, which can be blocked by the addition of a replication inhibitor such as acyclovir. Addition of acyclovir did not prevent the decrease in HLA-DR surface levels (Figure 2-5B). Thus, regulation of MHC class II is not dependent on viral DNA replication, and therefore appears to be dependent on a viral factor expressed with immediate early or early kinetics.

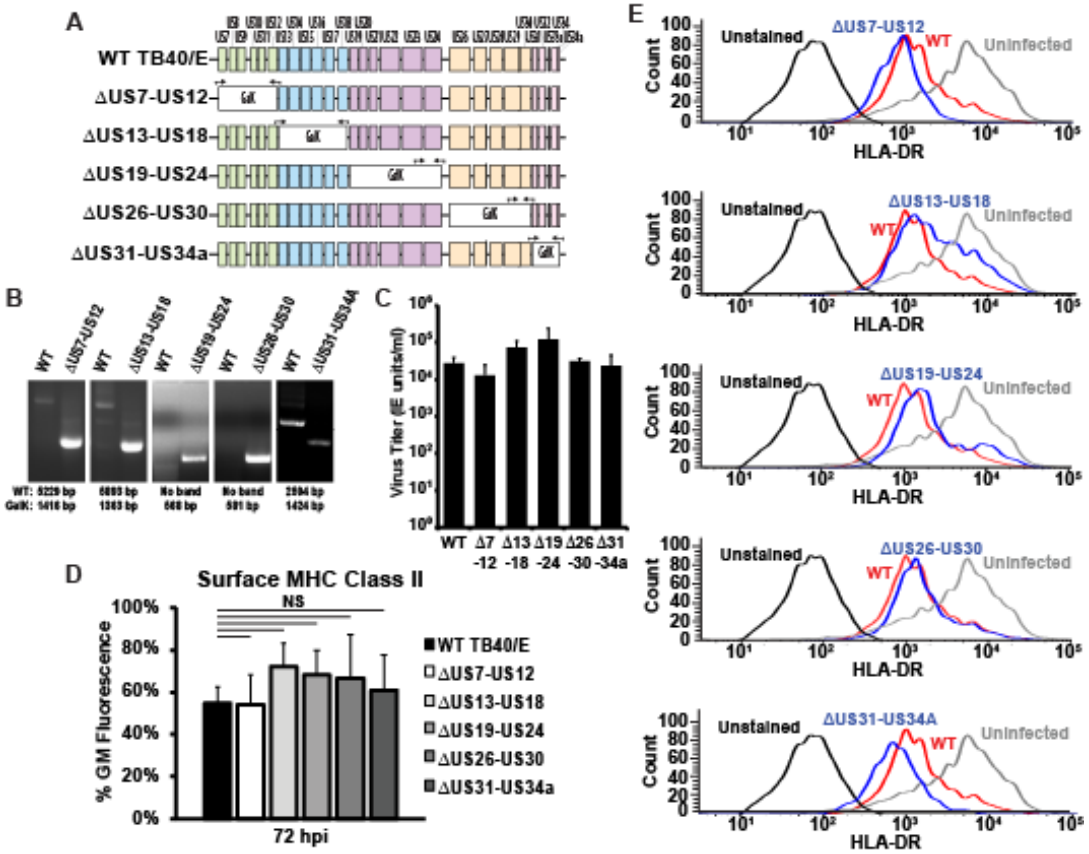

Figure 2- 4: The unique short region is not required for downregulation of surface MHC class II molecules in Kasumi-3 cells. (A) Schematic of the US region of the HCMV genome showing strategy for segmentally knocking out proteins expressed from the US region. Black arrows indicate primer sets either flanking *galK* insertion region or binding within *galk* and a neighboring flanking region. (B) PCR analysis showing replacement of the indicated segment of the US region with *galK*. Expected values of WT and *galK*-containing bands are indicated below image. (C) Infectious titers at 120 hpi of wildtype TB40/E (WT), and the US deletion mutants (Δ7-12, Δ13-18, Δ19-24, Δ26-30, Δ31-34A) following MOI=3 infection of fibroblasts. (D) Bar graph generated from geometric mean fluorescence values of surface HLA-DR staining of Kasumi-3 cells infected with wildtype TB40/E or viruses lacking segments of the US-gene region (ΔUS7-US12, ΔUS13-US18, ΔUS19-US24, ΔUS26-US30, ΔUS31-US34A) at 72 hpi. ns = not significant. (E) Histograms of one representative experiment for the samples in (D). Values in (C) & (D) are the averages of three independent experiments.

Our protein expression data detected the presence of tegument proteins at 24 hpi. While the levels of these proteins were nearly undetectable at 72 and 120 hpi, when the greatest reduction in surface MHC class II is observed, it is nonetheless important to rule out a contribution of incoming virion proteins in downregulating MHC class II. This is particularly important due to the high number of virions added to the Kasumi-3 cells to initiate infection. To test whether incoming virion proteins were responsible for the MHC class II downregulation, we added UV-inactivated virus to Kasumi-3 cells and measured surface MHC class II levels. Levels of surface HLA-DR were indistinguishable from levels on uninfected cells (Figure 2-5C), indicating that incoming virion proteins were not responsible for the MHC class II downregulation. Thus, downregulation of MHC class II appears to be mediated by a gene whose expression is not dependent on DNA replication.

One factor of interest expressed with immediate early kinetics is UL20. UL20 is a putative T-cell receptor homologue that is immediately trafficked to the lysosome for degradation following expression and is not required for infection in fibroblasts [307]. We hypothesized that in Kasumi-3 cells, UL20 may be binding to newly synthesized MHC class II molecules and delivering them to lysosomes for degradation, thus resulting in decreased surface localization. To test this hypothesis, we generated a UL20-null virus that expresses GFP in place of UL20. UL20 has previously been reported to be dispensable for growth on fibroblasts, and we similarly found that infectious virion production was equivalent between the ΔUL20 and wild-type viruses (Figure 2-5D) [308]. No difference in the reduction of MHC class II molecules was observed in the absence of UL20 (Figure 2-5E). Thus, UL20 is not the factor responsible for reducing surface MHC class II levels.

HCMV does not alter the internalization rate of class II molecules

Surface expression of MHC class II is reduced by HCMV and understanding the mechanism of this downregulation is important for identifying the viral factor mediating this

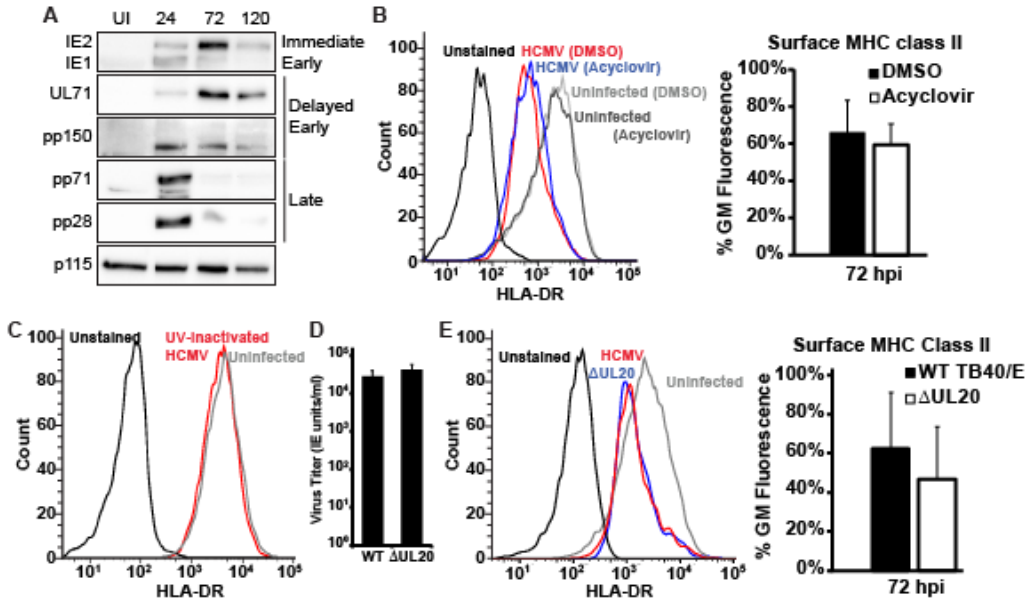

Figure 2- 5: Reduced MHC class II protein requires early viral gene synthesis. (A) Western blot analysis of uninfected (UI) or HCMV-infected Kasumi-3 cells at 24, 72 and 120 hpi showing two immediate early genes (IE1 & IE2), two delayed early genes (UL71 & pp150), two late genes (pp71 & pp28) and a loading control (p115). (B) Histograms of one representative experiment of surface HLA-DR staining of uninfected Kasumi-3 cells or cells infected with wildtype TB40/E and treated with DMSO or acyclovir. Bar graph at right shows geometric mean fluorescence values of infected samples at 72 hpi. (C) Histograms of one representative experiment of surface HLA-DR staining at 72 hpi of Kasumi-3 cells infected with UV-inactivated virus. (D) Infectious titers at 120 hpi of wildtype TB40/E (WT), and the ΔUL20 viruses following MOI=3 infection of fibroblasts. (E) Histograms of one representative experiment of surface HLA-DR staining at 72 hpi of Kasumi-3 cells infected with wildtype TB40/E or a virus expressing GFP in place of UL20 (ΔUL20). (Right) Bar graph generated from geometric mean fluorescence values. Values graphed in (A, D, & E) are averages of a minimum of three independent experiments.

effect. Potential mechanisms of downregulation include actively targeting the MHC class II molecules for internalization and degradation, preventing the proper assembly and loading of the MHC class II complex, or modulating MHC class II expression at the transcriptional or translational level. We first tested whether HCMV increases the endocytosis rate of surface MHC class II molecules (Figure 2-6A). HCMV infected Kasumi-3 cells were labeled with antibody against HLA-DR at 24 hours post infection. Secondary antibody conjugated to fluorescent probe was then added at 0, 4, 8, 12, 24 and 36 hours post addition of primary antibody and immunofluorescence was measured using flow cytometry. The internalization of MHC class II as measured by the rate of fluorescence loss was indistinguishable between uninfected and HCMV-infected cells (Figure 2-6B). Thus, the difference in surface MHC class II is not likely due to an increase in internalization and degradation of the complex.

HCMV reduces class II transcription by preventing CIITA expression

We next tested whether MHC class II was regulated at the transcriptional level. At 24 hpi, levels of the MHC class II HLA-DRα transcript were unchanged. However, at 72 hpi transcript levels were significantly reduced (Figure 2-7A). Thus, HCMV either reduces expression or enhances degradation of HLA-DRα transcripts. The HLA class II promoter is dependent upon the MHC class II transactivator (CIITA). We next investigated whether HCMV also transcriptionally downregulates CIITA. We detected a small but not significant decrease in CIITA transcripts at 24 hpi, with a much greater and significant reduction in transcript levels observed at 72 hpi (Figure 2-7B). Thus, the reduction in MHC class II can be explained by the downregulation of its essential transcriptional transactivator.

CIITA contains four promoters, and to define how HCMV downregulates CIITA transcription it is first important to understand the contribution of each of these promoters. Transcriptional activity has largely been assigned to promoters I, III and IV. Promoter I is active in monocyte-derived dendritic cells, promoter III in B cells, T cells and monocytes, and promoter

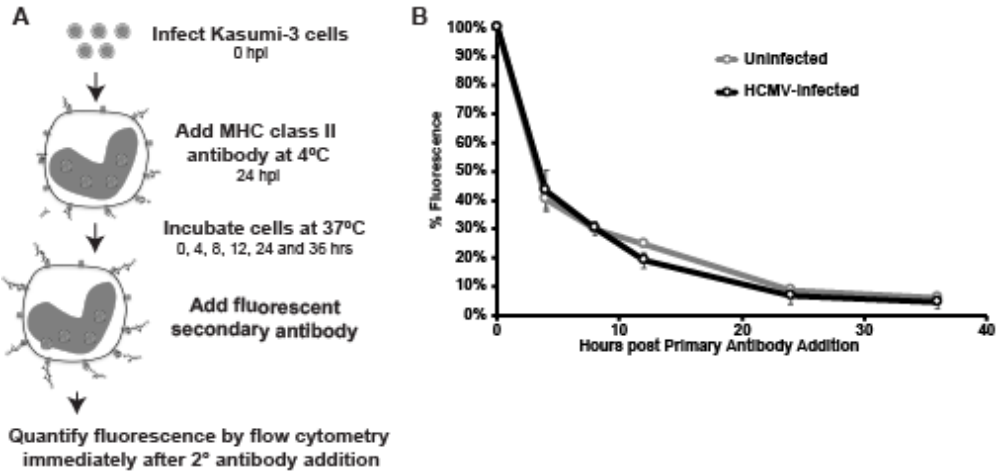

Figure 2- 6: HCMV does not alter the rate of MHC class II internalization in Kasumi-3 cells. (A) Schematic showing strategy for measuring internalization rate of MHC class II. (B) Graph plotting remaining surface HLA-DR levels in uninfected or infected samples at 0, 4, 8, 12, 24 and 36 hours post addition of primary antibody. Values are the mean of three independent experiments.

IV in response to IFN-γ induction [158, 161, 167]. We investigated which promoter(s) were active in uninfected Kasumi-3 cells and the effect of infection on promoter usage. The predominant transcripts in uninfected Kasumi-3 cells were a product of promoter III (Figure 2-7C). A very small population of transcripts derived from promoter IV were also present but no transcripts corresponding to promoter I could be detected. Thus, Kasumi-3 cells primarily utilize promoter III to endogenously express CIITA. As expected, transcripts generated from promoter III were decreased at 24 and 72 hpi, similar to what was observed for total CIITA transcript levels (Figure 2-7C). The minor pool of transcripts derived from promoter IV remained relatively constant throughout infection. Thus, the decrease in CIITA transcript levels can be attributed to either repression of promoter III transcription or a decrease in the stability of transcripts derived from promoter III.

The major immediate early (MIE) proteins are multifunctional proteins essential for HCMV infection that regulate the transcription of a diverse set of genes. We hypothesized that these genes transcriptionally repress the CIITA promoter III. This would be consistent with our observation that the factor responsible for downregulation is an immediate early or early gene, since addition of acyclovir did not block the downregulation of surface HLA-DR. We first transfected Kasumi-3 cells with a plasmid that contains the entire major immediate early gene region and can express both IE1 and IE2 as well as the other gene products produced from this region [309]. We observed reduced surface HLA-DR levels in these samples 48 hours post transfection (Figure 2-7D). The reduced surface HLA-DR correlated with a decrease in CIITA transcripts (Figure 2-7E). We next wanted to determine which of the major immediate proteins was responsible for this downregulation. The major immediate protein 2 (IE2) was the more abundantly expressed MIE protein in our infections (Figure 2-5A) and can act as a transcriptional repressor, which was first identified by its ability to directly bind a target sequence in its own promoter termed the cis repression signal [310, 311]. IE2 has since been shown to repress the

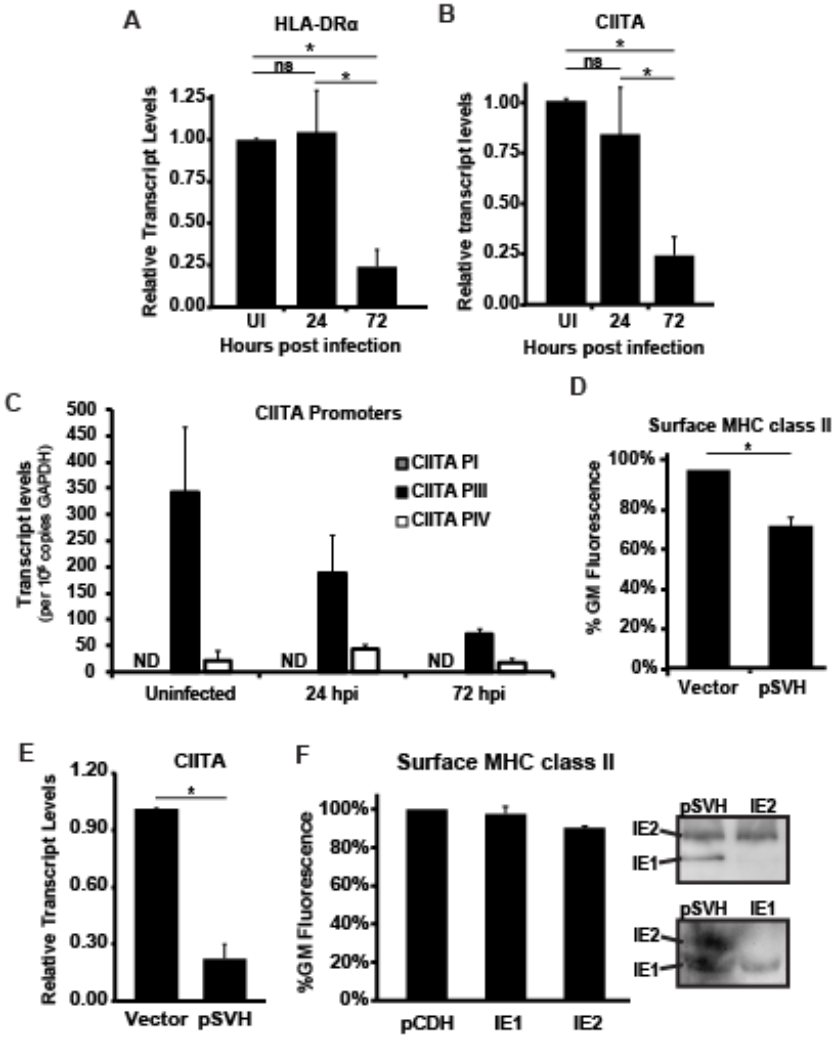

Figure 2- 7: HCMV reduces MHC class II in Kasumi-3 cells by downregulating expression of CIITA. (A & B) Quantitative PCR analysis of (A) HLA-DRα or (B) CIITA transcript levels in uninfected (UI) and HCMV-infected samples at 24 and 72 hpi. Values shown are relative to uninfected samples after normalization to GAPDH. (C) Quantitative PCR analysis of CIITA transcripts derived from Promoter I, III or IV in uninfected and HCMV-infected samples at 72 hpi. Values are absolute numbers of transcripts per 10^5 copies of GAPDH. ND = not detected. (D) Quantification of the geometric mean fluorescence for surface HLA-DR in Kasumi-3 cells electroporated with a DNA control (Vector) or pSVH. (E) qPCR analysis of CIITA transcripts following electroporation of DNA control (Vector) or pSVH into Kasumi-3 cells. (F) Quantification of the geometric mean fluorescence for surface HLA-DR in Kasumi-3 cells electroporated with pCDH vector, pCDH-IE1 or pCDH-IE2. Western blots from samples in (D & F) showing expression of IE1 and IE2 after transfection of pSVH, pCDH-IE1 and pCDH-IE2 plasmids. All values in (A-F) are averages from at least three independent experiments. * indicates $p<.05$, ns = not statistically significant.

transcription of other genes, and we hypothesized that IE2 was the MIE protein responsible for the transcriptional repression of CIITA [312-315]. We next transfected Kasumi-3 cells with a plasmid that would express IE2 alone. Interestingly, surface HLA-DR was not reduced in the presence of IE2 alone, suggesting that IE2 was insufficient by itself to mediate the transcriptional repression of CIITA and hence MHC class II transcription and the subsequent decrease in surface protein (Figure 2-7F). IE1 was also insufficient by itself to mediate the downregulation of surface MHC class II (Figure 2-7F). Western blot analysis confirmed protein expression of the transfected plasmids. Therefore, while HCMV decreases MHC class II surface expression by using the MIE region, IE1 and IE2 are alone insufficient. Thus, IE1 and IE2 either work in concert or one of the other gene products expressed from the pSVH plasmid is responsible for repressing CIITA transcript levels.

Chapter 3: HCMV induces E3 ubiquitin ligase MARCH1 to decrease STING-induced antiviral response

Abstract

Human cytomegalovirus alters the host immune environment for successful viral replication. This can include a combination of downregulating inhibitory immune proteins and the induction of immunosuppressive proteins. We investigated the role of the E3 ubiquitin ligase MARCH1, a post-translational regulator of immune membrane proteins, during HCMV infection. We found that MARCH1 was induced by HCMV infection in fibroblasts, a cell type not known to express MARCH1 under physiological conditions. MARCH1 increased late during infection and localized to the viral assembly compartment, suggesting a function during the late stages of infection. Importantly, we show that MARCH1 is required for productive viral replication in fibroblasts as MARCH1 knockdown using short, hairpin RNA (shRNA) reduced infectious virus production. We found that this decrease in viral activity upon MARCH1 knockdown was due to increased STING levels resulting in increased transcription of interferon-stimulated genes. Thus, HCMV induces the immunosuppressive MARCH1 ligase to target STING and inhibit the antiviral response in infected cells.

Importance

Human cytomegalovirus is an opportunistic herpesvirus that can cause serious health complications in immune-suppressed patients and congenitally-infected neonates. Understanding how the virus modulates immune responses will help develop better therapies to combat virus infections in these risk groups. In this study, we report a novel regulation of cellular E3 ubiquitin ligase MARCH1 by HCMV. We show that MARCH1 is specifically induced during HCMV infection and is required for efficient viral replication. Importantly, we find that HCMV induces MARCH1 late in infection to decrease STING and downstream interferon-stimulated genes. This is an important finding since it highlights a new strategy of immune evasion by HCMV by usurping MARCH1 to decrease an immune effector during HCMV infection.

Introduction

Human cytomegalovirus (HCMV) is an opportunistic herpesvirus that causes disease in immunocompromised individuals and neonates. HCMV has adverse consequences in congenital infections, transplant patients, HIV-infected individuals, and cancer patients on an immunosuppressive regimen [14, 16]. There is a need to fully understand the interplay between the virus and the host to develop better therapeutic strategies for these risk groups. Throughout the course of infection, HCMV usurps many cellular pathways to facilitate its own replication in infected cells. A common strategy used by the virus for modulating the cellular environment is to modify the protein composition of the cell to aid viral infection in cells. For instance, HCMV increases cellular metabolic proteins while downregulating immune effector proteins, which is beneficial for the virus [283, 316]. Identifying cellular proteins that are essential for the viral lifecycle will reveal new targets for treatment of HCMV infections.

Viruses utilize post-translational modifications to alter protein localization or to promote their degradation. Ubiquitination is a post-translational modification where the small ubiquitin protein is covalently attached to the target protein [231]. This ubiquitin tag serves as a signal for altered trafficking or degradation. Ubiquitination is a three-step process with the cell expressing enzymes specific for performing ubiquitin activation (E1), conjugation (E2) and ligation (E3) [231]. E3 ubiquitin ligases provide specificity for the substrate, and hence many E3 ubiquitin ligases are modulated by viruses to specifically target cellular and viral proteins [317, 318]. One family of E3 ligases are the Really New Interesting Gene (RING) proteins, which consist of a catalytic RING domain containing cysteine and histidine residues to balance two zinc atoms [231]. Viruses utilize RING ligases to degrade undesirable cellular proteins and even encode RING ligase homologous proteins to facilitate viral infection.

Among the RING ligases are the MARCH (Membrane-associated RING CH) proteins, which were discovered as mammalian homologs to K3 and K5 proteins of Kaposi's sarcoma

herpesvirus (KSHV) [233]. MARCH proteins function similar to K3 and K5 in the ubiquitination and degradation of membrane proteins. Members of the MARCH family target immune proteins like major histocompatibility complex (MHC) class I, MHC class II, T cell co-stimulatory molecule CD86 and natural killer cell ligands [229, 230, 233, 237, 241, 256]. MARCH1 and MARCH8 are two well-characterized MARCH family members that promote degradation of MHC class II [230, 232, 237, 256, 259]. MARCH1 and MARCH8 ubiquitinate lysine residues on the cytoplasmic tails of MHC class II, targeting the protein for lysosomal degradation [256, 259]. In addition to MHC class II, both MARCH1 and MARCH8 target other cell-surface receptors like transferrin receptor (TfR), Fas (CD95) and CD98 [233, 267]. While MARCH8 is ubiquitously expressed in different tissues, MARCH1 expression is limited to immune cells such as dendritic cells, monocytes and B cells [230, 233, 259, 274]. Within these immune cells, MARCH1 is regulated by immunosuppressive cytokine IL-10 [274, 276]. Due to their roles in MHC class II modulation, MARCH1 and MARCH8 are important for antigen presentation and immune responses in vivo [229, 230, 256, 259].

The functions of MARCH proteins remain understudied due to their low expression in many different tissues and redundancy in function within members of the MARCH family. Recently, the role of MARCH proteins has been described in viral infections. MARCH1, MARCH2 and MARCH8 ubiquitinate viral glycoproteins Env (envelope protein of HIV) and VSV-G (G protein of vesicular stomatitis virus) to prevent their expression on the plasma membrane and retain them intracellularly, reducing glycoprotein incorporation into virions [279, 280]. Furthermore, MARCH2 is induced in HIV-1 infected cells as a cellular response to inhibit viral infection [279, 281]. Thus, these three MARCH proteins are antiviral in function during HIV-1 infection. Conversely, MARCH8 ubiquitinates NS2 protein of Hepatitis C virus to facilitate viral envelopment, acting in a proviral manner [282]. However, whether any MARCH proteins play a role, proviral or antiviral, during HCMV infections remains yet to be determined.

Recently, we have reported that MHC class II is decreased upon infection in HCMV-infected Kasumi-3 cells [319]. Due to its role in MHC class II regulation in immune cells, we looked at MARCH1 and the effect of HCMV infection on MARCH1 expression. Although we found no role for MARCH1 in regulation of MHC class II, we now describe a previously uncharacterized role of MARCH1 during HCMV infection. We observed that MARCH1 is induced upon HCMV infection in fibroblasts, a striking finding since fibroblasts do not normally express MARCH1. This induction requires viral late gene expression. Further, MARCH1 is localized to the viral assembly compartment during the late stages of HCMV infection and is required for efficient viral replication. Herein, we identify STING, a cytosolic DNA sensor, as a target of MARCH1 during HCMV infection. Our results demonstrate a positive role for MARCH1 in HCMV infection and highlight how the virus uses a cellular protein to its own advantage. Thus, MARCH1-dependent STING regulation during HCMV infection is required for effective production of infectious virus.

Results

MARCH1 is induced during HCMV infection

While we found that MARCH1 was induced at the transcript levels during HCMV infection in cells of the myeloid lineage, we previously showed that MARCH1 is not involved in MHC class II degradation (Figure C-1) [319]. This led us to hypothesize that MARCH1 plays an alternative role during HCMV infection. We observed a steady increase in MARCH1 transcripts in infected fibroblasts over the course of HCMV infection (Figure 3-1A). Interestingly, we found MARCH8 transcripts to remain relatively constant throughout the course of HCMV infection, indicating that the MARCH1 induction was specific (Figure 3-1B).

We next determined if the increase in MARCH1 transcription caused a corresponding increase in MARCH1 protein. Similar to MARCH1 transcripts, we saw a steady increase in MARCH1 protein over the course of infection (Figure 3-1C). As expected, MARCH1 protein was

not expressed in uninfected fibroblasts but was detected faintly at 48 hpi and considerably at 72 hpi, 96 hpi and 120 hpi (Figure 3-1C). To ensure that this was not a strain-specific effect limited to the TB40/E strain of HCMV used for infections, we utilized another strain of HCMV, AD169, and found that MARCH1 protein levels still increased during infection with robust expression of the protein 72 hpi onwards (Figure 3-1D). For both AD169 and TB40/E infections, MARCH1 protein was expressed temporally similar to the late viral protein pp28 (Figures 3-1C-D). Thus, HCMV increases MARCH1 protein expression at late time points during infection.

MARCH1 requires late viral gene expression

We next wanted to understand how HCMV was inducing MARCH1 expression. Previous studies of MARCH1 reported that immunosuppressive cytokine interleukin 10 (IL-10) induces MARCH1 in B cells and monocytes [274, 276]. Furthermore, HCMV infection induces cellular IL-10 in monocytes [297]. Even though IL-10 production is limited to specialized immune cells, it is possible that HCMV infection could induce IL-10 expression in infected fibroblasts. To test whether cellular IL-10 was induced, we looked at IL-10 transcription in infected fibroblasts and used infected Kasumi-3 cells as a positive control for IL-10 induction. We saw an increase in cellular IL-10 transcription in infected Kasumi-3 cells, but there was no induction of cellular IL-10 in infected fibroblasts (Figure 3-2A). Thus, cellular IL-10 is not responsible for MARCH1 induction during HCMV infection. However, HCMV encodes a viral homolog of IL-10 [201, 202]. Hence, we hypothesized that viral IL-10 was inducing MARCH1 expression in infected fibroblasts. To determine if viral IL-10 was responsible for MARCH1 induction, we utilized a viral mutant lacking UL111A, the gene encoding viral IL-10 (Figure 2-3D) [319]. We found that MARCH1 was still induced both transcriptionally and at the protein level using the virus lacking

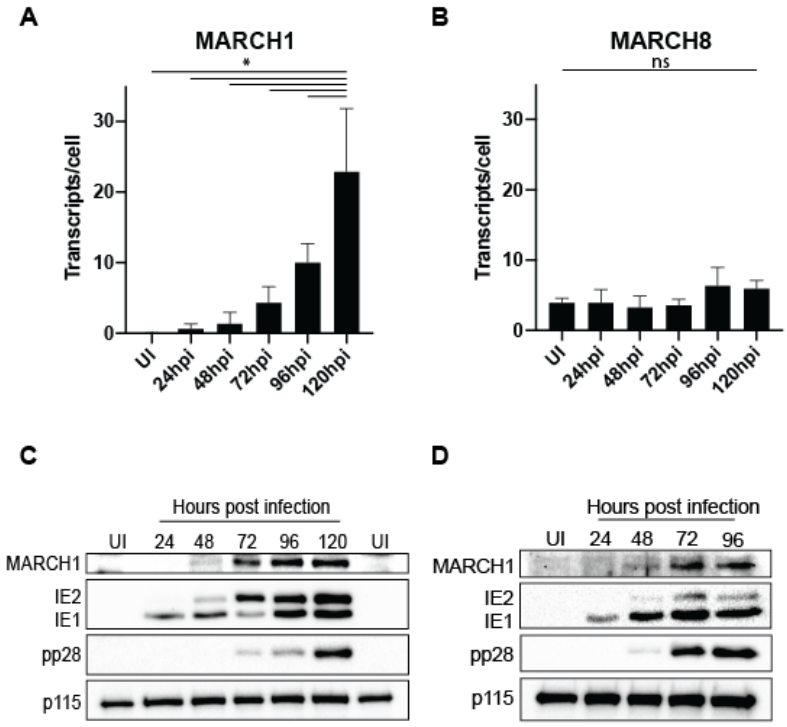

Figure 3- 1: MARCH1 is induced during HCMV infection in fibroblasts. Quantitative PCR for (A) MARCH1 transcripts and (B) MARCH8 transcripts in uninfected cells and cells infected with HCMV (TB40/E strain, MOI=3) at 24, 48, 72, 96 and 120 hpi in primary fibroblasts. Values for qPCR experiments (A-B) are absolute copies of transcript relative to GAPDH as loading control and are averaged from minimum of three independent experiments. (C and D) Western blot analysis for MARCH1, immediate early viral proteins (IE1, IE2), late viral protein (pp28) and loading control (p115) in uninfected and cells infected with HCMV at MOI=3 in primary fibroblasts where (C) is infection with HCMV TB40/E strain at 24, 48, 72, 96 and 120 hpi and (D) is infection with AD169 strain at 24, 48, 72 and 96 hpi. * indicates $p<0.05$, one-way ANOVA with Tukey's post-hoc analysis.

UL111A (Figures 3-2B and 3-2D). Thus, MARCH1 is induced independent of both the viral IL-10 homolog and cellular IL-10 during HCMV infection.

Late viral gene expression is dependent upon viral DNA replication. Thus, late gene synthesis is susceptible to viral DNA replication inhibition by the use of drugs such as acyclovir. Upon acyclovir addition to our fibroblast infections, MARCH1 transcription was highly reduced and MARCH1 protein was not expressed (Figures 3-2C,D). This inhibition of MARCH1 induction with acyclovir was also independent of UL111A (Figures 3-2C,D). Although we did not define the exact mechanism of MARCH1 induction, we find that it is dependent on viral DNA replication and late gene synthesis.

MARCH1 is required for viral replication

MARCH1 induction could be the result of a global effect on protein expression due to infection or specifically induced by the virus to promote infection. To determine whether MARCH1 is required by HCMV, we utilized shRNA-expressing lentivirus transduction of fibroblasts to knockdown MARCH1 transcripts (Figure 3-3A-B). Using this shRNA approach to knockdown MARCH1 during HCMV infection, we saw about a log reduction in viral titers in the MARCH1 shRNA knockdown cells relative to control cells transduced with only the lentivirus vector (Figure 3-3C). This decrease in infectious virus upon MARCH1 knockdown occurred at 96 hpi and 120 hpi, when MARCH1 is highly expressed in infected fibroblasts (Figures 3-3B and 3-1C). Hence, MARCH1 is required for productive HCMV infection in fibroblasts.

Many MARCH1 targets are expressed in immune cells specifically. The known MARCH1 targets expressed in fibroblasts include Fas, transferrin receptor (TfR) and insulin receptor (INSR). We assessed whether the increase in MARCH1 protein caused a concomitant decrease in the known MARCH1 targets expressed in fibroblasts. While the levels of both Fas and TfR are higher at 24 hpi and 48 hpi, these proteins are expressed at levels similar to or higher

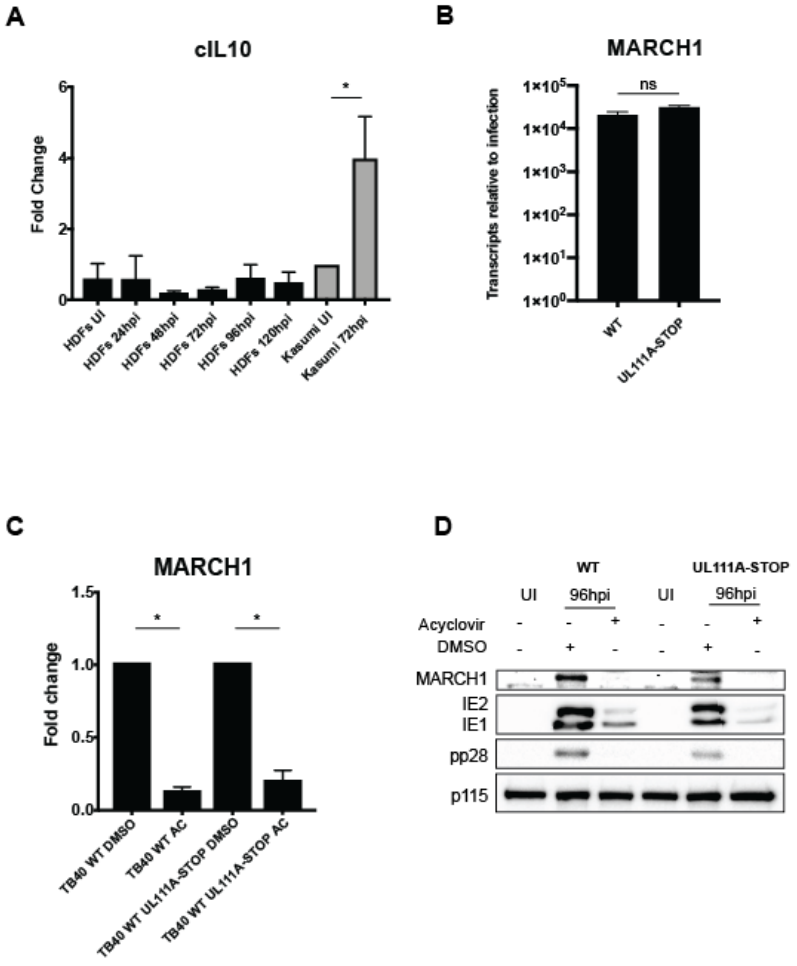

Figure 3- 2: MARCH1 is induced by late viral gene expression. (A) Quantitative PCR for human IL-10 transcripts in uninfected and TB40/E -infected fibroblasts (MOI=3 at 24, 48, 72, 96 and 120 hpi), uninfected Kasumi-3 cells and infected Kasumi-3 cells (MOI>3) at 72hpi. Fold change is relative to uninfected Kasumi-3 cells with GAPDH as a loading control. (B) Quantitative PCR for MARCH1 absolute transcripts normalized to pp28 for cells infected with HCMV TB40/E wild-type and UL111A-STOP viruses at MOI=3 in MRC-5 fibroblasts (C) Quantitative PCR for MARCH1 transcripts at 96hpi in uninfected cells and cells infected with HCMV TB40/E wild-type and UL111A-STOP viruses at MOI=3 in MRC-5 fibroblasts treated with DMSO or acyclovir. Fold change is calculated with absolute transcripts normalized to WT or UL111A-STOP DMSO with GAPDH as loading control. (D) Western blot analysis for MARCH1, immediate early viral proteins (IE1, IE2), late viral protein (pp28) and loading control (p115) in uninfected cells or cells infected as described in (B) in primary fibroblasts. Values for qPCR are derived from at least three independent experiments. * indicates $p<0.05$, one-way ANOVA with Tukey's post-hoc analysis.

than uninfected fibroblasts at the late time points when MARCH1 is most expressed, indicating that MARCH1 induction during HCMV infection does not cause a proportionate decrease in Fas and CD95 (Figure 3-3D). Next, we determined if the MARCH1 knockdown affected Fas and TfR that could contribute to the decrease in virus titers. Importantly, both Fas and TfR were expressed at similar levels between control and MARCH1 knockdown cells, suggesting that reduction in viral titers was not due to a change in Fas or TfR protein levels (Figure 3-3D). Previously published proteomic data indicates that INSR expression increases during HCMV infection, suggesting that INSR is not the target of MARCH1 [283]. Thus, HCMV specifically increases MARCH1 upon infection in fibroblasts, and does not affect levels of Fas, TfR and potentially INSR.

MARCH1 knockdown decreases viral proteins and cytoplasmic viral activity

We next sought to determine at what stage viral replication was blocked in the MARCH1 knockdown samples. We first analyzed the expression of different viral proteins in control and MARCH1 shRNA knockdown cells. We found that immediate early protein IE1 was slightly reduced while IE2 and delayed early protein UL44 were relatively unchanged between control and MARCH1 shRNA cells (Figure 3-4A). However, a subset of delayed early proteins like pp150, UL71, gB, and the late protein pp28 were reduced upon MARCH1 knockdown at 96 hpi (Figure 3-4A). To ensure that infection initiated equally in both control and knockdown cells, we also looked at an earlier timepoint of 24 hpi to probe for expression of IE proteins. IE1 and IE2 were expressed similarly at 24 hpi, suggesting that infection initiated comparably between control and MARCH1 shRNA cells (Figure 3-4B). This was not unexpected since MARCH1 is not expressed in infected cells at 24 hpi (Figure 3-1C). However, at 96 hpi, there was still a decrease in late viral proteins such as pp28 similar to what was shown previously (Figure 3-4B). We also assessed if other cytoplasmic cellular proteins important for viral replication were affected by MARCH1 knockdown. Previously, we have shown that syntaxin 5 (STX5), a Golgi-localized

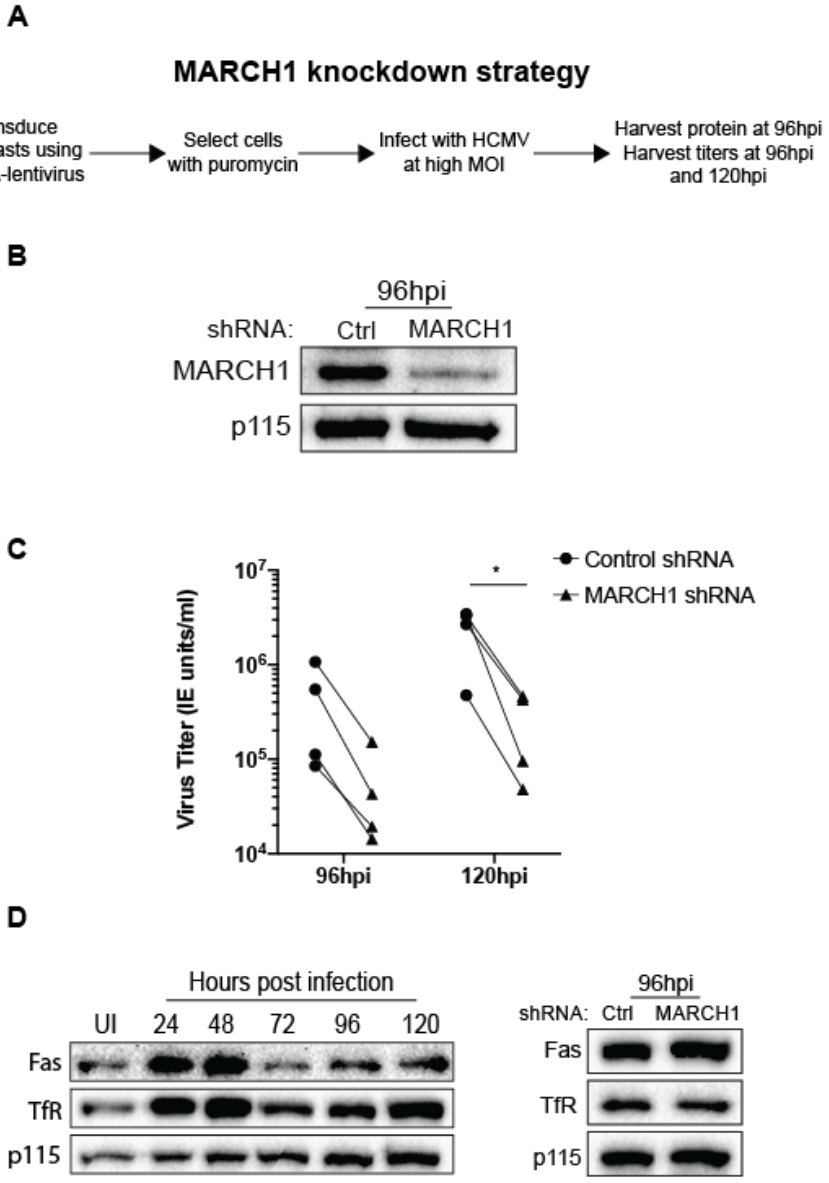

Figure 3- 3: MARCH1 knockdown reduces viral titers. (A) Outline of MARCH1 shRNA strategy. (B) Western blot analysis for MARCH1 and loading control (p115) at 96hpi in cells infected with HCMV (TB40/E strain, MOI=3) in primary fibroblasts. (C) Infectious titers at 96hpi and 120hpi for MARCH1 and control shRNA treated, infected primary fibroblasts infected as described previously in (B). (D) Western blot analysis for Fas, transferrin receptor (TfR) and loading control (p115) in uninfected cells, infected cells for a timecourse of infection (24-120hpi) and cells transduced with control or MARCH1 shRNA infected as described previously in (B) in primary fibroblasts. * indicates $p<0.05$, Student's t-test.

cellular protein, is important for the late stages of viral replication [320]. However, there was no change in STX5 expression between MARCH1 shRNA and control cells (Figure 3-4B). Thus, MARCH1 knockdown during HCMV infection decreases expression of a subset of viral proteins.

A morphological hallmark of late stage HCMV infection is the restructuring of cellular organelles and membranes adjacent to the nucleus to form the cytoplasmic viral assembly compartment (cVAC), the site of viral maturation [75]. The cVAC formation occurs at the late stages of viral replication with many viral proteins localizing to the cVAC [141]. Since there was a reduction in the expression of viral proteins, we assessed whether MARCH1 knockdown also affected the cVAC integrity. The control shRNA cells showed juxtanuclear pp28 localization at 96 hpi, similar to what has been previously reported (Figure 3-4C) [75]. Conversely, there was less pp28 staining in the MARCH1 shRNA cells and the pp28 puncta were dispersed throughout the cells within the MARCH1 shRNA sample (Figure 3-4C). We also investigated the localization of the cellular protein STX5 within the cVAC. We found that STX5 was localized adjacent to the nucleus within the Golgi ring in both the control and MARCH1 knockdown cells, similar to its reported location (Figure 3-4C) [320]. This suggests that the localization of viral proteins, but not cellular proteins, to the cVAC does not occur properly in the MARCH1 knockdown cells.

To assess the effect on viral replication and mislocalized viral proteins, we utilized electron microscopy to investigate the block in infection at a subcellular level during MARCH1 knockdown. At 96 hpi, in the control shRNA cells, there was accumulation of membrane adjacent to the nucleus in the cytoplasm, indicative of the cVAC (Figure 3-5A). The juxtanuclear region contained maturing nucleocapsids, fully enveloped virions and dense bodies (Figure 3-5A, E-F). However, the cVAC was almost devoid of maturing capsids, enveloped virions and dense bodies in the MARCH1 knockdown cells (Figures 3-5G-H). Despite the striking absence of virus activity in the cVAC, the MARCH1 knockdown cells still contained cytoplasmic membrane

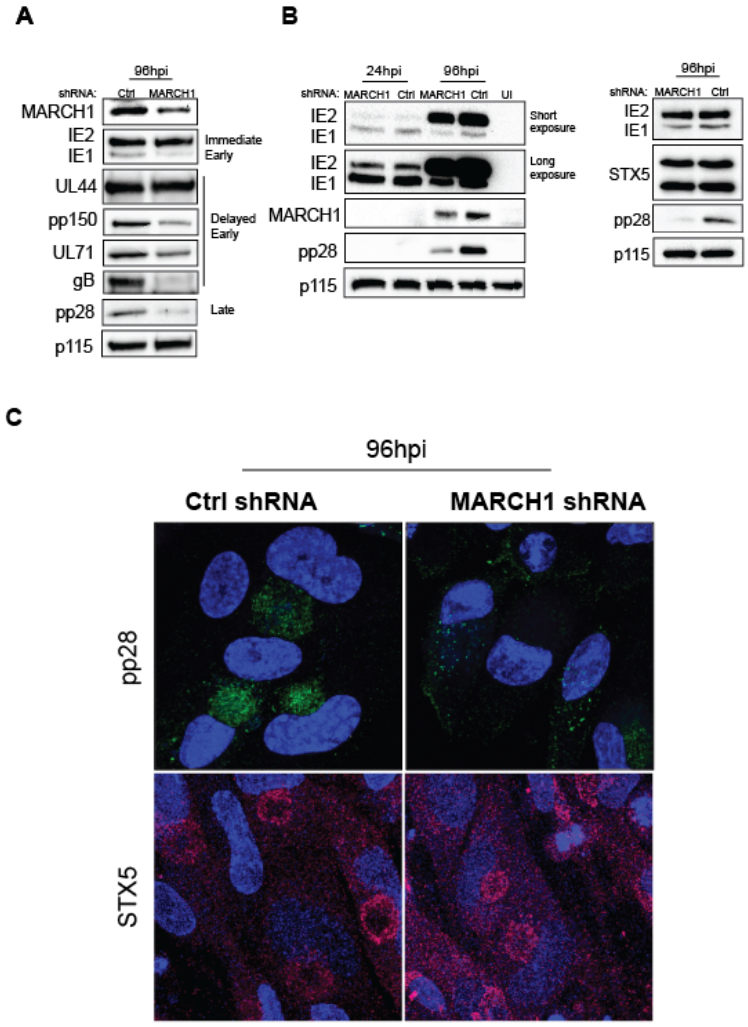

Figure 3- 4: MARCH1 knockdown decreases viral proteins. (A) Western blot analysis for MARCH1, immediate early viral proteins (IE1, IE2), delayed early viral proteins (UL44, pp150, UL71, gB), late viral protein (pp28) and loading control (p115) at 96 hpi in MARCH1 and control shRNA primary fibroblasts infected with HCMV (TB40/E strain, MOI=3). (B) Western blot analysis for MARCH1, immediate early viral proteins (IE1, IE2), late viral protein (pp28), syntaxin 5 (STX5) and loading control (p115) at 96 hpi in MARCH1 and control shRNA MRC-5 fibroblasts infected as described in (A). (C) Immunofluorescent staining for viral protein pp28 at 96 hpi in MARCH1 and control shRNA treated primary fibroblasts infected with HCMV as described previously in (A).

accumulation and nucleocapsids, similar to the control cells (Figures 3-5C-D, G-H). These results show that, while infection initiated in both the control and MARCH1 knockdown cells as seen by the presence of nucleocapsids, there was a pronounced reduction in the cytoplasmic viral activity in the MARCH1 knockdown cells.

MARCH1 localizes to the cVAC and interacts with STING

Reduction of MARCH1 protein during infection caused decreased cytoplasmic viral activity and infectious virus production. To ascertain how MARCH1 was promoting viral replication, we next wanted to determine the localization of MARCH1 during infection. Due to the lack of a MARCH1 antibody for immunofluorescent detection of the endogenous protein, we utilized lentivirus expressing EGFP (enhanced green fluorescent protein)-tagged MARCH1 to transduce fibroblasts and then infect with HCMV to visualize its localization during infection. Previous studies have reported that EGFP tagged to the N terminus of MARCH1 does not affect the function of the protein so we used the N terminal-tagged MARCH1 for the localization experiments [246, 273]. Uninfected fibroblasts transduced with EGFP-MARCH1 expressed very low amounts of EGFP-MARCH1, due to the relative instability of MARCH1 protein and constant turnover due to self-ubiquitination as was previously described (data not shown) [246]. The cVAC contains concentric layers of organelles with the Golgi and trans-Golgi network forming a ring and endosomes aggregating towards the middle within the cVAC. At 96 hpi, when the cVAC is fully formed and MARCH1 is highly expressed, EGFP-MARCH1 localized adjacent to the nucleus in a ring-like structure, surrounding the pp28 accumulation (Figure 3-6A). Importantly, EGFP-MARCH1 was not localized to early endosomes, its previously reported localization [321]. The localization of EGFP-MARCH1 similar to a ring suggested that MARCH1 was localizing to the Golgi ring within the cVAC. Hence, MARCH1 localizes to the cVAC late in infection.

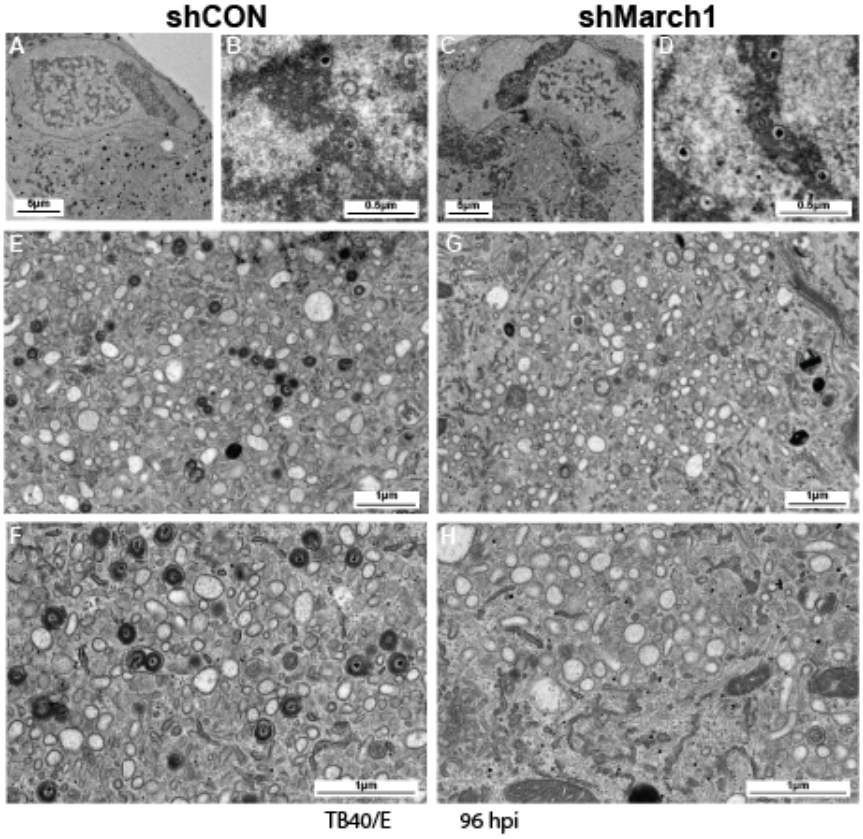

Figure 3- 5: MARCH1 knockdown reduces cytoplasmic viral activity. Electron micrographs of MARCH1 and control shRNA treated primary fibroblasts infected with HCMV (TB40/E strain, MOI=3) at 96 hpi where (A) depicts an entire control shRNA infected cell, (B) depicts the nucleus of an infected control shRNA cell, (C) depicts an entire MARCH1 shRNA infected cell, (D) depicts the nucleus of an infected MARCH1 shRNA cell. Higher magnification images of the cytoplasmic area adjacent to the nucleus where (E-F) are two independent control shRNA infected cells and (G-H) are two independent MARCH1 shRNA infected cells.

We next wondered what function MARCH1 might be playing located within the cVAC. Recently, MARCH1 has been shown to target STING and MAVS for degradation and regulate immune responses downstream of STING/MAVS signaling [238]. Interestingly, activated STING dimerizes and translocates from the ER to the Golgi complex to initiate immune signaling [322, 323]. Hence, we investigated whether STING and MARCH1 co-localized during infection. We engineered a recombinant HCMV virus with EGFP-MARCH1 expressed from the US34-TRS1 region within the HCMV genome, a region allowing robust expression of inserted genes [304]. Using this recombinant virus at a low MOI infection, we stained for STING and Golgi marker GM130 at 72 hpi. We found that STING and EGFP-MARCH1 co-localized within the Golgi during infection (Figure 3-6B). The expression of STING was reduced in the EGFP-MARCH1 expressing cells relative to the neighboring uninfected cells (Figure 3-6B). Importantly, STING was not localized to the Golgi but was expressed at relatively high levels and dispersed throughout the cell, similar to its reported localization in the ER, in the uninfected cells (Figure 3-6B). Thus, MARCH1 specifically localizes to the Golgi to target activated STING during HCMV infection.

MARCH1 decreases STING-mediated antiviral responses

Since MARCH1 localized to the Golgi during infection and its knockdown reduced cytoplasmic viral activity, we hypothesized that MARCH1 was degrading STING during HCMV infection to reduce the antiviral response. To test this, we investigated the expression of STING in the MARCH1 knockdown cells. STING protein was increased in the MARCH1 shRNA cells while MAVS levels remained unchanged, suggesting that MARCH1 was specifically acting on STING during HCMV infection, but not MAVS (Figure 3-7A). The increase in STING protein during MARCH1 knockdown could enhance antiviral responses in infected cells and could be

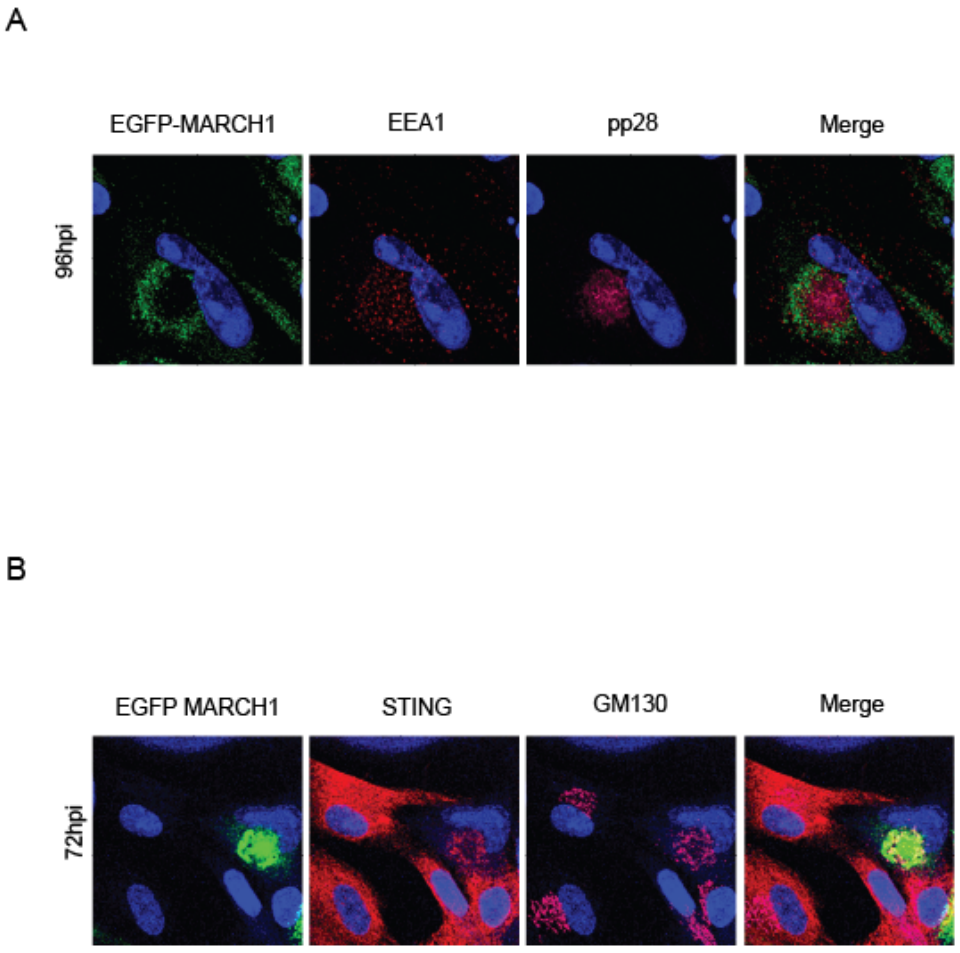

Figure 3- 6: MARCH1 is localized to the cytoplasmic viral assembly compartment (cVAC). Immunofluorescent staining for (A) early endosome marker (EEA1) and viral protein pp28 at 96 hpi in EGFP-MARCH1 transduced primary fibroblasts infected with HCMV (TB40/E strain, MOI=3) and (B) for STING, Golgi marker GM130 at 72 hpi in MRC-5 cells infected with TB40 SV40-EGFP-MARCH1 (MOI=0.3).

responsible for the decreased virus production observed in the MARCH1 shRNA cells. As MARCH1 was induced late in infection, we hypothesized that the induction of MARCH1 by HCMV is to counteract antiviral STING signaling late in infection. If the increased STING activity late in infection causes enhanced antiviral signaling, we would see a general block in infection during these later stages in the MARCH1 shRNA infected cells. We have previously observed decreased late protein synthesis in the MARCH1 knockdown cells at 96 hpi (Figure 3-4A). To see if the block in infection would extend to the transcription of late genes, we analyzed transcriptional activity of immediate early proteins and the late protein pp28 in the MARCH1 knockdown cells at 96 hpi. Similar to the protein levels, IE transcripts remained constant but pp28 transcription was reduced in the MARCH1 knockdown cells (Figure 3-7B). These findings support the hypothesis that MARCH1 is regulating STING expression levels during HCMV infection.

Upon activation, STING induces transcription of interferon-stimulated genes (ISGs) [323]. We looked at transcription of ISGs MX1, ISG15, IRF7 and IFN-β in our control and MARCH1 shRNA cells. We observed increased transcriptional activity of MX1, IRF7, IFN-β and ISG15 in the MARCH1 shRNA infected cells compared to the control shRNA infections (Figure 3-7C). Hence, HCMV induces MARCH1 to decrease STING-mediated immune responses during late stages of infection. Thus, HCMV appears to utilize MARCH1 to antagonize the antiviral signaling responses.

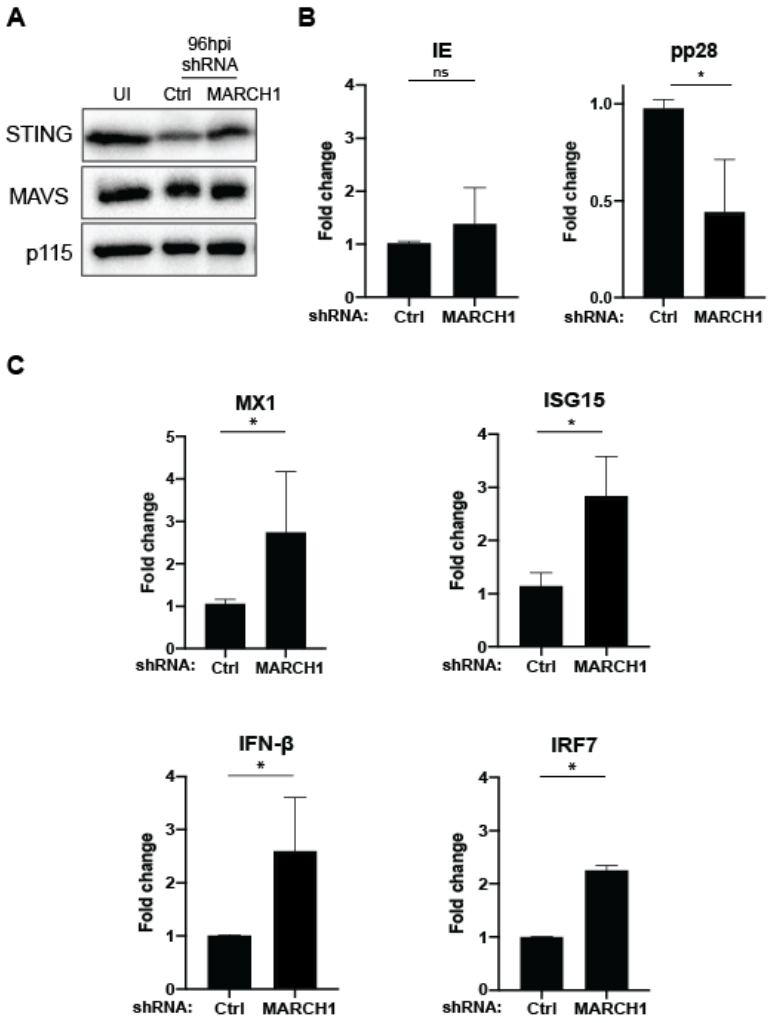

Figure 3- 7: MARCH1 knockdown increases STING and interferon stimulated genes. (A) Western blot analysis for STING, MAVS and loading control (p115) in uninfected cells and MARCH1 and control shRNA primary fibroblasts infected with HCMV (TB40/E strain, MOI=3), (B) quantitative PCR for IE and pp28, and (C) quantitative PCR for MX1, ISG15, IFN-β and IRF7 at 96 hpi in MARCH1 and control shRNA primary fibroblasts infected with HCMV (TB40/E strain, MOI=3). Values for (B) and (C)are derived from three independent experiments except IRF7 which comprises of two independent experiments. * indicates $p<0.05$, Student's t-test.

Chapter 4: Discussion

HCMV is an opportunistic pathogen that causes disease in humans under immunosuppressed conditions. The virus alters the immune signaling pathways and hijacks cellular processes for its own replication cycle. Key features of the cellular reprogramming induced by HCMV include altering the levels of immune proteins or modifying their trafficking within the cell to prevent their antiviral function. Elucidating how the virus manipulates these proteins is critical to deciphering the relationship of HCMV with the human host and finding new targets for developing better therapeutic interventions.

This dissertation describes how HCMV modulates the innate and adaptive immune responses within the host. We observed the unique induction of MARCH1, an E3 ubiquitin ligase, upon HCMV infection. We investigated the role of increased MARCH1 during HCMV infection, and found that MARCH1 is induced by HCMV to target STING, an innate immune sensor. This induction of MARCH1 is important for HCMV to alter the innate immune activation within infected cells and promote viral replication. Furthermore, our findings reveal that HCMV decreases MHC class II, an immune glycoprotein that primes CD4+ T cells. Importantly, we describe the regulation of constitutively expressed MHC class II by HCMV in myeloid cells, a cell type that is important for the viral life cycle *in vivo*. We found that HCMV decreases the synthesis of MHC class II as a measure to inhibit CD4+ T cells, important players in adaptive immunity. Hence, HCMV can differentially modulate two cellular immune proteins to attenuate both innate and adaptive immune signaling.

Regulation of endogenous MHC class II by HCMV in myeloid cells

MHC class II is expressed endogenously in APCs and induced in non-APCs [138]. The regulation of constitutive and induced MHC class II within cells is distinct in many aspects due to differences in expression of transcription factors, MIIC machinery and MHC class II trafficking proteins [157]. Thus, it is important to understand the regulation of MHC class II within both the constitutive and induced expression. Non-APCs, such as epithelial cells and fibroblasts, can be

induced to express MHC class II by overexpression of CIITA or treatment with IFN-γ to stimulate IFN-γ-driven CIITA transcription. On the other hand, APCs constitutively express CIITA to express MHC class II. The decrease of MHC class II by HCMV has been previously described in non-APCs [195, 219, 220]. These studies relied upon forced expression of MHC class II by overexpressing CIITA or induction with IFN-γ [195, 219, 220]. However, the regulation of endogenous MHC class II, devoid of stimulation by activating cytokines or forced expression, is not well-understood.

Myeloid cells are important for HCMV infections *in vivo*. CD34+ hematopoietic stem cells serve as the reservoir of HCMV-infected cells and differentiate along the myeloid lineage to monocytes and macrophages that disseminate the virus to target organs within the human host [324]. Importantly, these myeloid cells serve as APCs and a source of endogenous MHC class II expression. Within the infected myeloid cells, endogenous MHC class II could present viral antigens to prime CD4+ T cells. For example, endogenous gB is presented via MHC class II to activate CD4+ T cells [187]. In addition to their supportive role in adaptive immunity, CD4+ T cells primed to HCMV are cytolytic [291, 325]. Thus, it would be beneficial for HCMV to exert immunomodulatory control over MHC class II to prevent the CD4+ T cell response. While both induced and constitutive MHC class II regulation is important, we wanted to fill the gap in knowledge for mechanisms for endogenous MHC class II regulation during HCMV infection. To this end, we utilized Kasumi-3 cells, a myeloid cell line that expressed HLA-DR isotype of MHC class II, as an experimental model of HCMV infection. In agreement with our hypothesis, we observed decreased surface and total MHC class II due to early viral gene expression in infected cells in this relevant model system. We also utilized CD14+ monocytes to further confirm the decrease in MHC class II in primary cells and observed a similar reduction in MHC class II in infected monocytes. However, the use of primary myeloid cells is challenging due to the short life span in culture and donor variability for HCMV infectivity, limiting their use for investigating the

mechanistic regulation of MHC class II by HCMV. Thus, the Kasumi-3 cells served as an alternative model to study endogenous MHC class II regulation by HCMV. Since HCMV decreases MHC class II in both myeloid cells and non-APCs, this highlights the importance of MHC class II downregulation to HCMV infection, shared across both constitutive and induced MHC class II. However, our results demonstrate that the mechanism underlying MHC class II regulation (discussed in the following sections) in myeloid cells is distinct from these non-APCs. Thus, our findings highlight the importance of studying both endogenous and constitutive MHC class II regulation as modulated by viruses. Also, we hope that these findings encourage future studies to choose relevant model systems to fully understand MHC class II regulation during virus infections.

Role of HCMV genes in MHC class II downregulation

Consistent with the hypothesis that HCMV decreases MHC class II, different viral proteins have been implicated in modulating induced MHC class II expression in HCMV-infected non-APCs [219, 220]. We tested the role of these previously described viral genes to see if they functioned similarly in endogenous MHC class II regulation in Kasumi-3 cells. US2 and US3 decrease MHC class II in a glioblastoma cell line induced to express MHC class II. US2 degrades MHC class II α chains while US3 sequesters immature MHC class II within the cell [218, 326]. Since we used infectious virus derived from a BAC that lacks US2 and US3, the decrease in MHC class II in our system is independent of US2 and US3 [306]. Other studies have reported poor expression of US2 and the inability of US2 to decrease MHC class II in myeloid cells [327-331]. This suggests that the effects of US2 and US3 could be limited to non-APCs, and do not extend to myeloid cells.

The tegument protein pp65 was reported to decrease MHC class II in IFN-γ stimulated fibroblasts [220]. We tested the role of pp65 in our myeloid cell line by overexpression, and found no decrease in MHC class II. Since pp65 is the most abundant tegument protein, we also

tested the possibility of the incoming tegument to decrease MHC class II using UV-inactivated virus. We saw no change in MHC class II when cells were infected with UV-inactivated HCMV. These observations further supported the fact that pp65 does not decrease endogenous MHC class II in this myeloid cell line. Further, pp65 can block IFN responses by decreasing IRF1 protein levels upon infection [24] . Since IRF1 is a transcription factor required for IFN-γ-driven CIITA transcription, pp65 could function in the induced MHC class II pathway by blocking IRF1 and subsequently CIITA transcription. Thus, pp65 may function in the IFN-γ-driven MHC class II expression. However, endogenous MHC class II, expressed without activation stimuli like IFN-γ, does not appear to be regulated by pp65 during HCMV infection.

IL-10 is a potent immunosuppressive cytokine and causes a decrease in MHC class II expression. HCMV encodes a viral homolog of IL-10, namely UL111A, that mimics the viral IL-10 immunosuppressive functions [202]. Latently infected myeloid progenitor cells showed decreased MHC class II due to expression of UL111A [203, 294]. We evaluated the contribution of UL111A in our infection model, and observed that the mutant virus lacking UL111A still decreased MHC class II. HCMV infections can also induce secretion of cellular IL-10 in infected myeloid cells [297]. However, MHC class II reduction specifically occurred in infected Kasumi-3 cells, not uninfected cells within the same culture. Hence, the downregulation of MHC class II was intrinsic to the infected cells, and not due to release of soluble factors such as cellular or viral IL-10 within the culture medium to decrease MHC class II for the cell population as a whole. Thus, cellular and viral IL-10 induction does not decrease MHC class II in infected Kasumi-3 cells, suggesting a cell-intrinsic mechanism to target MHC class II.

Recently, US28 overexpression was reported to inhibit STAT1 and decrease surface MHC class II, where the US28-mediated effects on MHC class II were dependent on its G-protein function in a monocytic cell line [332]. In the same study, IFN-γ-treated cells did not have

reduced MHC class II upon US28 overexpression. These findings are contradictory since STAT1 is required to drive IFN-γ-mediated CIITA transcription and MHC class II expression [332]. In addition, the role of US28 was not verified in infected cells. Our findings show that the mutant virus with the deletion of US28 still resulted in the decrease of MHC class II. Additionally, US28 is a tegument-associated protein in the virion, but we observed no decrease in MHC class II upon infection with UV-inactivated virus. Overexpression of US28 could target other MHC class II regulatory cellular proteins to indirectly affect MHC class II but, in the context of infection, US28 has no direct role in regulating endogenous MHC class II. Further work will be required to tease out the role of US28 and its G protein signaling in MHC class II expression, but in Kasumi-3 infections, MHC class II regulation occurred in a US28-independent manner.

None of the previously reported HCMV genes that decreased MHC class II in other systems downregulated the endogenous protein. Hence, we hypothesized that the regulation of constitutively expressed MHC class II was occurring via an unknown viral gene (s), such as those encoded by the US region. The US segment of the HCMV genome plays an important function in immunomodulation. The roles of US2 and US3 in MHC class I and induced MHC class II degradation have been previously described. Further, US6 and US11 also participate in MHC class I regulation. Additionally, this region has genes that degrade NK cell-activating ligands. Moreover, many US-encoded genes are expressed with early kinetics and do not have an assigned function. Since this region is important in immune regulation, it was reasonable to assess if any of the US genes played a role in endogenous MHC class II modulation. To look at the contribution of the US region in MHC class II regulation, we attempted to delete the entire US segment but were unable to recover infectious virus. As an alternative strategy, we conducted a targeted screen of the US region by generating viral mutants deleted for four to five contiguous genes in a systematic manner to cover the entire US segment. Surprisingly, none of the viral mutants rescued the decrease in MHC class II upon HCMV infection, suggesting that the US region does

participate in MHC class II regulation in infected Kasumi-3 cells. However, it is possible that there is redundancy in the US segment with multiple genes having minor contributions to MHC class II regulation. But the major driver of the MHC class II decrease in Kasumi-3 cells is not encoded within the US region. Thus, there is a divergence in strategies utilized for MHC class I vs class II modulation by HCMV, with the MHC class II regulation being independent of the US region.

Mechanism of MHC class II decrease

MHC class II is inhibited by many different viruses as part of their immune modulation strategy. Our approach to elucidate the mechanism for MHC class II decrease was guided by observations in other cell systems by HCMV, other CMVs and herpesviruses [215, 295, 333]. Reduced MHC class II can occur due to inhibition of MHC class II synthesis, interference with the assembly process or enhanced endocytosis and degradation. Previous studies showed intracellular accumulation of MHC class II and reduced MHC class II expression on the cell surface in HCMV-infected cells [220, 294, 330]. The increased intracellular MHC class II could be the result of sequestration of endocytosed MHC class II or altered trafficking to prevent plasma membrane expression of MHC class II. We observed intracellular accumulation of MHC class II in infected Kasumi-3 cells but the total levels of MHC class II were also decreased. These observations could still be explained by enhanced degradation of MHC class II in infected cells as a potential mechanism. Since we saw MHC class II accumulating in or adjacent to LAMP1 positive compartments (Figure A-1), we hypothesized that HCMV infection was inducing lysosomal degradation of MHC class II in Kasumi-3 cells. We assessed the role of two HCMV proteins that could perform this function- UL20 and UL78. UL20 is a glycoprotein that is expressed early in infection and has partial homology to the T cell receptor γ chain [307]. Upon expression, it is trafficked to the lysosomes, and degraded [307]. Till date, UL20 has no known function, and is hypothesized to bind to and deliver an interacting protein to the lysosome for

degradation. Due to the presence of an immunoglobulin-like fold in its structure, UL20 could bind to other glycoproteins like MHC class II. The second protein we considered for MHC class II degradation is UL78, an endosomally-localized GPCR-like protein, whose homolog M78 degrades murine MHC class II in MCMV-infected myeloid cells [295, 334]. The deletion of either genes from the viral genome did not prevent the MHC class II decrease in HCMV infection, suggesting that these proteins do not function in MHC class II regulation.

To determine if increased endocytosis and degradation was the mechanism of action, we measured the internalization of cell-surface labeled MHC class II. Our results show that the rates of MHC class II endocytosis in uninfected and infected cells were similar and provide evidence that the decrease in MHC class II is not due to enhanced endocytosis and degradation. In addition, blocking lysosomal activity using concanamycin did not rescue the decrease in MHC class II in infected cells (Figure A-1). While we saw increased MHC class II in concanamycin treated cells, this increase occurred for both uninfected and infected cells (Figure A-1). Thus, the addition of lysosomal inhibitors increased MHC class II that would normally be degraded due to protein turnover, but this increase was not due to blocking active degradation of MHC class II by HCMV. These observations highlight that, in the Kasumi-3 cell model, MHC class II trafficking in infected cells is similar to uninfected cells, and increasing internalization of MHC class II for degradation is not the mechanism. Interestingly, our findings in HCMV infection contrast with the mechanisms employed by other CMVs. Both rat and murine CMVs increase endocytosis and degradation of MHC class II [295, 333]. Similar to MHC class II in other CMVs, the MHC class I regulation by HCMV also occurs through a degradative mechanism as described previously. Surprisingly, this is not the favored mechanism for the virus to inhibit MHC class II in myeloid cells. These results further depict the deviation of strategies utilized by HCMV for MHC class I and class II modulation.

Another possible mechanism for reduced MHC class II is decreased MHC class II synthesis. We found that HLA-DR α chain transcription was reduced upon HCMV infection, suggesting that the decrease in MHC class II is due to reduced transcription. CIITA, a transcriptional co-activator, is the major regulator of MHC class II transcription [157]. CIITA inhibition is a common strategy deployed by viruses to block MHC class II-mediated antigen presentation. γ-herpesviruses KSHV and EBV directly inhibit CIITA transcription while HIV interferes with the CIITA protein to prevent its binding to MHC class II transcription factors [208, 209, 213]. In HCMV-infected Kasumi-3 cells, we found that CIITA transcription was reduced. Importantly, the reduction of CIITA and MHC class II transcription was not due to a general host transcription shutoff, as the expression of other cellular genes was unaffected (Figure B-1). Targeting CIITA is an attractive strategy for the virus because CIITA is essential for both induced and constitutive expression of MHC class II. By blocking CIITA, the entire MHC class II machinery is brought to a halt as CIITA regulates transcription of the MHC class II genes and accessory factors. Moreover, in a Langerhans cell infection model, HCMV has been shown to decrease CIITA transcription in addition to sequestering MHC class II intracellularly [221, 331]. Upon activation, dendritic cells decrease CIITA transcription and MHC class II synthesis [161]. Similarly, the dampened CIITA transcription in the Langerhans cells may be the result of activation of these specialized dendritic cells upon sensing HCMV infection. Thus, in the Langerhans infection model, it is hard to distinguish whether the decreased CIITA transcription is the result of direct repression by the virus or the indirect consequence of cellular activation of these immune cells. Using Kasumi-3 cells, our data provides evidence that HCMV directly attenuates CIITA transcription via immediate early genes and the reduced CIITA transcription is accompanied by decreased surface expression of MHC class II. Hence, in infected Kasumi-3 cells, HCMV utilizes the transcriptional blockage of CIITA as a means to blunt the MHC class II pathway and suppress endogenous MHC class II expression.

CIITA promoter usage for transcription in Kasumi-3 cells

CIITA transcripts are differentially regulated during transcription by promoter usage. In humans, three active promoters (pI, pIII and pIV) mediate transcription of CIITA, where the promoter usage is dependent on cell type and cytokines. Initial characterization of these promoters established pI as the myeloid promoter due to its robust activity in DCs, pIII as the lymphoid promoter due to its usage in B cells and pIV as the IFN-γ inducible promoter [158]. However, detailed analysis in different human cells revealed that the promoter activity is promiscuous. While pI is the predominant transcript for human DCs and macrophages, monocyte-derived DCs have significant transcriptional activity of CIITA arising from pIII and pIV [161]. Additionally, each promoter can bind different transcription factors to modulate the transcriptional activity.

For Kasumi-3 cells, we found transcripts primarily generated from the pIII promoter, with no detectable transcripts from pI and very low pIV transcripts. These pIII transcripts decreased in infected Kasumi-3 cells throughout the course of infection. Since the Kasumi-3 cell line is derived from acute myeloblastic leukemia cells, the transcription from pIII promoter may occur due to its myeloid progenitor nature and ability to differentiate into monocytic lineage, where the pIII and pIV promoters are active. The lack of pI-mediated transcription in Kasumi-3 cells is intriguing since myeloid cells express pI-driven CIITA. Interestingly, plasmacytoid DCs (pDCs) are a specialized myeloid cell type that solely utilize pIII-driven CIITA transcription [335]. pDCs and conventional DCs (cDCs) are both derived from a common myeloid progenitor, but they differ in their predominant CIITA promoter usage [335, 336]. While cDCs express both pI and pIII derived transcripts, Kasumi-3 cells may represent an intermediate progenitor cell that only expresses promoter III transcripts. Kasumi-3 cells also expressed very few but detectable pIV transcripts that remained relatively unchanged during HCMV infection. Further work is needed to determine whether pIV promoter transcription is activated by IFN-γ in Kasumi-3 cells

and whether the induced transcription from this promoter is refractory to HCMV inhibition. Nonetheless, our results demonstrate that HCMV has devised a strategy to prevent CIITA expression from the constitutively active pIII promoter during infection, and has implications for understanding the impact of HCMV infections in myeloid cells *in vivo*.

Role of IE proteins in CIITA transcription and potential mechanisms

Since the MHC class II decrease in infected Kasumi-3 cells was transcriptional, we evaluated whether IE proteins, key transcriptional regulators encoded by HCMV, could be decreasing CIITA transcription. These nuclear IE proteins exert transcriptional control over many cellular and viral genes, and are essential for promoting viral infection [337]. IE protein expression was detected in infected Kasumi-3 cells and expression of the entire IE locus alone decreased surface MHC class II and CIITA transcription in Kasumi-3 cells. The IE locus overexpressed in Kasumi-3 cells is comprised of the major IE enhancer, promoter and the entire UL122/123 gene coding segment. This region expresses transcripts that undergo alternative splicing to give rise to different IE proteins [338]. We assessed the contribution of two well-known members of IE family, IE1 and IE2, that are detected in Kasumi-3 infections, and found that singular expression of either protein in Kasumi-3 cells does not decrease MHC class II. While we could detect expression of IE1 and IE2, there may be differences in the amount of IE proteins expressed singularly compared to the IE protein expression from the entire IE locus. In addition, other IE transcripts are expressed from the UL122/123 region with alternative transcription start sites generating unique ORFs in the sense frame as well as anti-sense transcripts [339, 340]. These unique transcripts generate serological responses in HCMV-infected individuals *in vivo* [340, 341]. Furthermore, ORF94, a nuclear ORF encoded by an antisense IE transcript, decreases transcription of 2'-5'-oligoadenylate synthetase-like protein (OAS-L) to aid immune dampening in infected cells [342]. Thus, the complexity of the IE region in myeloid cell

infections warrants further investigation to determine the exact products of the IE locus expressed in infected Kasumi-3 cells and their effect on MHC class II transcription.

The mechanism by which IE proteins repress CIITA transcription has not been addressed in this study. These could include direct repression of the CIITA transcription by IE proteins binding to the CIITA promoter or inhibition of cellular factors required for CIITA transcription. Interestingly, other herpesviruses also block CIITA transcription. KSHV utilizes LANA to bind to IRF4, a transcription factor required for pIII promoter activity, and decrease CIITA expression [208]. In addition, the immediate early EBV protein BZLF1 directly binds to the CIITA pIII to inhibit its transcription [208, 210]. Thus, HCMV decreases pIII promoter activity and CIITA transcription, analogous to other herpesviruses. pIII-driven CIITA transcription requires binding of transcription factor PU.1 [167]. Interestingly, IE2 binds to PU.1 to regulate IL-1β transcription [343]. While the expression of IE2 alone does not decrease MHC class II, the concerted action of multiple IE proteins or unique ORFs could affect PU.1 binding to the pIII promoter. Future work should assess the expression of the pIII transcription machinery upon IE expression and HCMV infection.

Other mechanisms to decrease MHC class II transcription include epigenetic modifications to the CIITA gene. IE proteins can bind to histone deacetylases and regulate epigenetic modification of genes through acetylation [344]. Additional experiments should determine the epigenetic status of the CIITA gene during HCMV infection and determine whether IE proteins silence the CIITA gene. Another possibility for decreased CIITA transcription is the decreased stability of the CIITA mRNA. There is no evidence of IE proteins regulating mRNA stability but this possibility still needs to be explored as a potential mechanism. Hence, the decrease in CIITA transcription is achieved by an unknown, novel function of the IE proteins during HCMV infection.

MARCH1 induction by HCMV

Many herpesviruses encode their own E3 ubiquitin ligases to target immune proteins during infection. HSV-1 encoded ICP0 is a RING E3 ubiquitin ligase that targets nuclear cellular proteins to prevent detection of viral genomes and inhibit antiviral signaling [345]. KSHV encodes RING E3 ubiquitin ligases K3 and K5, the two viral homologs of the MARCH family, to target MHC class I. Despite the large number of genes within its genome, HCMV does not encode its own E3 ubiquitin ligase. However, we found that MARCH1 expression is induced upon HCMV infection. Since HCMV lacks its own viral E3 ubiquitin ligase, the virus could commandeer a cellular E3 ubiquitin ligase like MARCH1 to target immune proteins. Thus, diverging from KSHV's strategy of pirating cellular MARCH proteins to encode K3 and K5, HCMV directly induces and utilizes MARCH1.

The endogenous expression of MARCH1 is limited to APCs such as B cells, monocytes and DCs. Even within these immune cell types, MARCH1 expression is tightly regulated with limited expression under basal conditions [230, 259, 272]. In MARCH1-expressing cells, activation stimuli inhibit MARCH1 expression while immunosuppressive cytokines such as IL-10 induce MARCH1 transcription [230, 259, 274, 276]. We saw a robust increase in transcription and protein expression for MARCH1 in HCMV-infected fibroblasts. The ability of HCMV infection to stimulate MARCH1 expression in a non-APC suggests that MARCH1 could serve a specific function during HCMV infection. In addition to IL-10 production in myeloid cells during infection, HCMV also encodes a viral homolog of IL-10 [201, 297]. Due to the known relationship of IL-10 and MARCH1 induction, we hypothesized that IL-10 contributed to the stimulation of MARCH1 expression during HCMV infection. However, there was no induction of cellular IL-10 in HCMV-infected fibroblasts, as was observed in infected monocytes. These observations are not surprising since IL-10 production is mostly limited to myeloid and lymphoid cells [346]. In addition, the deletion of the viral IL-10 homolog from the HCMV genome did not

alter MARCH1 expression. Hence, both cellular and viral IL-10 are dispensable for MARCH1 induction. While MARCH1 induction was independent of IL-10, we found that viral late gene expression was required for MARCH1 production during HCMV infection. These findings suggest that HCMV circumvents the stimulation of immunosuppressive human IL-10 and utilizes viral factors expressed late in infection to directly induce MARCH1. This is in contrast to the induction of MARCH1 by other pathogens like *Francisella* that activate cellular IL-10 to express MARCH1 [277]. While additional studies are required to determine the viral genes upregulating MARCH1, our findings reveal the unique viral induction of MARCH1 expression by HCMV, distinct from the known IL-10 pathway. Interestingly, there is an increase in MARCH1 transcription at the onset of HCMV infection but the protein is detectable only at the late stages of infection. Transcriptional regulatory elements have been described for murine MARCH1 but they only function in APCs [347]. As such, there is lack of information for the transcriptional regulation of MARCH1 expressed in non-APCs like fibroblasts. In addition, the MARCH1 protein has a short half-life and can be self-ubiquitinated and turned over [246, 272]. Whether HCMV stabilizes the MARCH1 transcript or protein during the late stages of infection is currently not known, and will require further investigation. However, our results reveal this novel induction of MARCH1 by HCMV in fibroblasts, a cell type that does not endogenously express MARCH1.

Another intriguing finding is the specific induction of MARCH1, but not its homolog MARCH8, in infected fibroblasts. MARCH8 is expressed ubiquitously in many different cell types and shares target specificity with MARCH1 [229, 233]. Fibroblasts express higher MARCH8 transcripts than MARCH1 under physiological conditions. Despite expression of MARCH8 within fibroblasts, MARCH1 transcription is specifically induced in HCMV-infected fibroblasts. Targets common to both MARCH1 and MARCH8 include Fas and transferrin receptor (TfR), proteins abundantly expressed in HCMV-infected fibroblasts. However, TfR or

Fas levels were not altered in infection or increased by MARCH1 knockdown, suggesting that MARCH1 was not acting on these proteins during viral infection. In addition, overexpression of MARCH1 was used to identify TfR and Fas as targets which is a caveat to the interpretation that these proteins are true substrates of MARCH1. Thus, this specific upregulation of MARCH1 could be the result of a viral strategy to regulate a protein that is solely a target of MARCH1, not MARCH8. Intriguingly, overexpressed MARCH1 can interact with MARCH8 to form heterodimers but the functional relevance of such interactions remains to be determined [246]. Although MARCH8 is not specifically induced, the role of endogenous MARCH8 in HCMV infections is unknown. Our findings clearly demonstrate the preference of MARCH1 induction over MARCH8 during HCMV infection, which may be due to a function exclusive to MARCH1. Thus, these findings highlight the specific regulation of cellular proteins by HCMV during infection.

Function of MARCH1 during HCMV infection

Since MARCH1 was induced by HCMV infection in non-expressing fibroblasts, we reasoned that MARCH1 was serving a specific function during HCMV infection. Previous studies have classified MARCH1 as an antiviral factor, in conjunction with MARCH2 and MARCH8, in HIV-1 infections while the homolog MARCH8 has been described as a proviral factor in HCV infections. To assess whether MARCH1 is required for HCMV infection, we utilized a shRNA-based knockdown approach to reduce endogenous MARCH1 levels and assess its impact during infection. We found reduced viral titers, less viral protein expression and a lack of cytoplasmic viral activity with MARCH1 knockdown. Thus, these findings suggest a proviral function for MARCH1 during HCMV infection and implicate MARCH1 in productive viral replication.

The predominant role of MARCH1 in APCs is to regulate antigen presentation by targeting MHC class II and CD86, essential proteins for T cell activation [233, 237, 256]. Due to

its role in MHC class II and CD86 turnover, MARCH1 modulates the development of immune cells and regulates immune responses [348-350]. However, MHC class II and CD86 are not expressed in fibroblasts physiologically. Hence, the induction of MARCH1 in non-APCs suggests it acts on other targets expressed within fibroblasts. Recently, MARCH1 has been shown to act as an inhibitor of nucleic acid sensing adaptors STING and MAVS and their downstream immune signaling pathways [238]. STING is an immune effector molecule that activates inflammatory responses and induces production of type I IFN downstream of cytosolic DNA sensors [323]. We found that MARCH1 specific knockdown increases STING expression but does not alter MAVS levels. Thus, MARCH1 specifically targets STING during HCMV infection. As mentioned previously, most of the MARCH1 targets are shared by its homolog MARCH8 and very few proteins are targeted solely by MARCH1. Although our data shows the specific increase in MARCH1 by HCMV to target STING, it would be interesting to evaluate whether MARCH8 can also target STING. Based on our findings, we hypothesize that STING interaction is exclusive to MARCH1. Hence, HCMV utilizes specific E3 ubiquitin ligases to downregulate immune proteins which is beneficial to the virus infection.

STING acts as an antiviral molecule for many DNA viruses. HCMV is targeted by STING signaling and inhibits STING via different viral proteins to prevent the induction of antiviral responses in infected cells [351]. HCMV-encoded proteins antagonize STING activity at various steps of the viral life cycle [352]. STING is blocked by the tegument protein pp71 upon viral entry and at the early stages of infections by multiple proteins as described previously [90-93]. The early inhibition of STING is important for blocking innate immune activation to allow for initiation of infection. Here, MARCH1 is induced late in infection suggesting it plays a role during the late stages of HCMV infection. Late inhibition of STING could be required because the nuclear membrane adjacent to the cVAC is altered resulting in enhanced permeability of the nucleus [353]. Consequently, a concomitant increase in cytoplasmic DNA can occur. This DNA

could then be sensed by STING, resulting in activation of type I IFN. Recently, the viral protein UL94 was described to target STING late in infection [94]. Thus, blocking STING throughout the course of infection is important for HCMV to inhibit the activation of innate immune signaling. By inducing MARCH1 to target STING late in infection, HCMV prevents STING-mediated immune signaling. Hence, MARCH1 is induced to block late stage STING-sensing of HCMV and aids in the sustained suppression of antiviral immune activity in infected cells.

Multiple mechanisms exist to inhibit STING which include degradation of STING, inhibition of STING activation and interference with STING binding to downstream effector proteins [354]. HCMV also uses various strategies to block STING as described previously. Interestingly, MARCH1 has been reported to directly bind to STING, outside of HCMV infection [238]. Since we see an increase in STING protein upon MARCH1 knockdown, our observations suggest that MARCH1 could act through a degradative mechanism to target STING. Thus, HCMV could utilize MARCH1 to bind to STING and cause its degradation. A related observation for STING is its ubiquitination at different lysine residues mediated by multiple cellular E3 ubiquitin ligases [355]. The differential ubiquitination pattern can alter STING activity and either cause its degradation or mediate its interaction with other proteins, dependent on the ubiquitination linkage [355]. It would be of interest to see if MARCH1 interacts with other viral STING-interacting proteins and ubiquitinates STING during HCMV infection. Future experiments could determine the importance of E3 ubiquitin ligase activity of MARCH1 for STING targeting and determine the lysine residues targeted by MARCH1 during HCMV infection.

MARCH1 localization and STING activation

MARCH1 localizes to early and late endosomes, and targets proteins residing within these compartments for ubiquitination. This ubiquitination event marks these proteins for lysosomal degradation. Our findings show that MARCH1 is localized to the Golgi ring within the

cVAC late in HCMV infection. This altered localization of MARCH1 in the Golgi could result in the interaction of MARCH1 with its target Golgi-associated proteins. The Golgi complex is a hub for innate immune signaling, including activated STING [356]. STING is an ER-resident membrane protein that traffics to the perinuclear ER-Golgi intermediate compartment (ERGIC) and the Golgi after activation [323]. This translocation event is important as post-translational modifications, like palmitoylation, that are required for STING activity occur at the Golgi [322, 357]. Since HCMV infection activates STING, this could result in localization of activated STING within the Golgi ring surrounding the cVAC. Here, within the Golgi ring, MARCH1 could interact with activated STING and regulate its function. In support of this, we find that MARCH1 and STING co-localize in the Golgi ring within the cVAC. Thus, MARCH1 localizes to the Golgi to inhibit activated STING during HCMV infection.

Activated STING recruits adaptor molecules that mediate downstream signaling to activate the NF-kB pathway and stimulate type I IFN production. Interestingly, the ER to Golgi translocation of STING is important for type I IFN activation, but not NF-kB activation [358]. We see elevated ISG transcription in the MARCH1 knockdown cells, consistent with the increased STING expression, but the activation of NF-κB still needs to be investigated. HCMV differentially modulates NF-κB signaling to increase expression of cytokines that aid in immunosuppression and virus spread, while inhibiting antiviral signaling. Since we observed STING interaction with MARCH1 in the Golgi post the potential NF-κB activation, it is possible that STING-mediated NF-κB signaling will not be affected by MARCH1. Hence, HCMV could be utilizing MARCH1 to specifically perturb STING-mediated type I IFN signaling, without affecting the NF-κB pathway. Activated STING binds to proteins TBK1 and IRF3 to trigger downstream signaling. Binding of STING to TBK1 kinase causes IRF3 phosphorylation and this phosphorylated IRF3 translocates to the nucleus to induce transcription of type I IFNs [359].

Based on our observations, if MARCH1 decreases the amount of STING protein, this would lower the amount of activated STING available to bind to TBK1 and IRF3. Thus, it would be important to determine the impact of MARCH1 on STING interaction with TBK1 and the subsequent IRF3 phosphorylation. In summary, HCMV specifically regulates MARCH1 to target activated STING to block type I IFN signaling.

Overexpression of MARCH1 during HCMV infection

To further study the role of MARCH1 during HCMV infection, we decided to overexpress MARCH1 and look at its impact on viral infection. Using a recombinant virus overexpressing EGFP-MARCH1, we observed decreased viral proteins and titers (Figures D-1A and B). This negative impact of MARCH1 could be due to excessive production of MARCH1 protein throughout the infection, distinct from the timely induction of MARCH1 during HCMV infection. We observed expression of EGFP-MARCH1 as early as 24hpi, with high levels expressed at 96hpi, unlike the endogenous MARCH1 expression at 72hpi during HCMV infection. The altered temporal expression and increased protein level may lead MARCH1 to ubiquitinate proteins it may not normally target during infection under MARCH1 induction conditions. In support of this, we see a decrease in TfR using this MARCH1 overexpression, while TfR remains unaffected by MARCH1 induction during HCMV infection or use of MARCH1 shRNA. Thus, HCMV finetunes the MARCH1 protein expression temporally and quantitatively, where too much or too little MARCH1 is detrimental to infection.

Implications and future directions for MARCH1

Our findings describe the unique HCMV-driven induction of MARCH1 in fibroblasts. However, MARCH1 is endogenously expressed in myeloid cells such as monocytes, macrophages and dendritic cells that serve as sites of HCMV infections in vivo. HCMV infection in macrophages and DCs initiates a robust type I IFN response largely mediated by STING [360]. Interestingly, we observe an increase in MARCH1 transcription in Kasumi-3 cells, a myeloid cell

line (Figure C-1). Thus, we hypothesize that MARCH1 may play a similar role for STING modulation during HCMV infection within myeloid cells. Furthermore, these myeloid cells are potent IL-10 producing cells, unlike fibroblasts and hence the MARCH1 induction and regulation may be more complex during HCMV infection in these cell types. Therefore, MARCH1 could function in STING modulation within HCMV-infected myeloid cells.

Our findings highlight the role of MARCH1 in modulating STING and its downstream antiviral activity during HCMV infection. However, there is lack of evidence for the role of MARCH1 in regulating immune responses in other cytomegalovirus infections. STING signaling is important for type I IFN production in MCMV infections, with MCMV antagonizing STING activation to prevent antiviral type I IFN signaling [358, 361, 362]. It would be interesting to evaluate the role of MARCH1 signaling in MCMV infections. Though MARCH1 deficient mice are a suitable model for such investigations, the critical role of MARCH1 in antigen presentation and T cell priming makes it harder to assess whether MARCH1 plays a direct role in immune responses to pathogens [95, 349, 350]. Cell-specific deletion of MARCH1 will be a useful approach to develop for future studies to carefully investigate the role of MARCH1 in immune activation.

In addition to cytomegaloviruses, it would be important to assess the impact of MARCH1 on other viruses. STING signaling inhibits replication of both DNA and RNA viruses, and thus, most viruses have developed strategies to thwart STING activation. In addition to type I IFN and NF-κB signaling, STING modulates protein translation in RNA virus infections [363]. Thus, it would be important to understand whether other viral strategies to block STING involve MARCH1. Future studies should delve into the role of MARCH1 in different DNA and RNA virus infections and its impact in the various STING antiviral functions mechanistically.

MARCH1 could serve as a potential therapeutic target for HCMV. Current remedial strategies for HCMV involve use of drugs that inhibit the viral DNA synthesis to block early

steps in the viral lifecycle [17]. However, many of these drugs have adverse effects like nephrotoxicity, limiting the administrable dose [17]. In addition, the continuous use of such therapies leads to the emergence of drug-resistant viral mutants. Hence, there is a need to identify new targets for therapeutic intervention of HCMV infections. We have identified MARCH1 to be required for late stage HCMV replication. Thus, MARCH1 presents a new druggable target to block the late stages of viral replication. Currently, there are approved inhibitors specifically targeting various E3 ubiquitin ligases. These inhibitors were developed as cancer therapeutic drugs to target E3 ubiquitin ligases that function in cellular signaling and promote cancer progression [364]. Using drug discovery strategies to develop specific inhibitors against MARCH1 is an attractive approach for inhibiting HCMV replication. Thus, MARCH1 could be a novel target for treatment of HCMV infections.

Final remarks

The work presented in this dissertation describes two distinct aspects of immune regulation by HCMV. Our findings elucidate the mechanism of endogenous MHC class II regulation by HCMV. We found that endogenous MHC class II is decreased at the transcriptional level by HCMV in infected myeloid cells. Despite an increase in MARCH1, decreased MHC class II during HCMV infection is due to a block in MHC class II synthesis and not enhanced endocytosis and degradation. These results highlight the targeting of MHC class II by HCMV to modulate the adaptive immune response. We discovered that the unique induction of MARCH1 in HCMV-infected fibroblasts is required for productive infection. They are also important for unraveling the role of MARCH1 in regulating innate antiviral responses in infected cells through its interaction with STING and pave way for future studies to uncover the role of MARCH1 in viral infections.

Chapter 5: Materials and Methods

Cell Culture

Normal human dermal fibroblasts (primary HDFs) (Cell Applications Inc. 106-05n) and normal human lung fibroblast MRC-5 cells (ATCC CCL-171) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Corning) containing 10% fetal bovine serum (Hyclone), 2 mM GlutaMAX (Gibco), 100 U/mL penicillin and 100 g/mL streptomycin (Corning). Kasumi-3 cells (ATCC CRL-2725) were maintained in RPMI-1640 medium (ATCC) supplemented as above for DMEM with the exception of 20% fetal bovine serum (FBS). Human peripheral blood monocytes (Cell Applications Inc., 6906K-50A) were maintained in the manufacturer's supplied human blood cell culture medium. Cells were maintained at 37°C with 5% CO2.

BAC Mutagenesis

Recombinant viruses were generated using BAC mutagenesis in *Escherichia coli* strain SW105 and *galK* selection as described previously [365]. Mutant viruses were generated from a TB40/E BAC expressing mCherry [304]. A virus that expresses IE2-2A-EGFP was utilized for the internalization studies [366]. The parent BAC for this IE2-2A-EGFP virus which also expressed mCherry was a gift from Eain Murphy and the mCherry sequence was replaced with the wildtype sequence as described below. Primers for recombineering for generation of all mutant strains are as follows: UL111A-galk-For 5'-TGGGACGCGCAGTTGGGTGGCGGACTGGGGCGGCATGCTGCGGCGCCTGTTGACAATTAATCATCGGCA, UL111A-galk-Rev 5'-AAAAAAGACGATCAGGACCAGAGAGGAAGAGACCATCACCGACAGTCAGCACTGTCCTGCTCCTT; UL111A-STOP-sense 5'-GACGCGCAGTTGGGTGGCGGACTGGGGCGGCATGCTGCGGCGtagCTGTCGGTGATGGTCTCTTCCTCTCTGGTCCTGATCGTCTTTTTT, UL111A-STOP-antisense 5'-AAAAAAGACGATCAGGACCAGAGAGGAAGAGACCATCACCGACAGctaCGCCGCAGC

ATGCCGCCCCAGTCCGCCACCCAACTGCGCGTC; UL78-galk-For 5'-
GGAGAGGGTATATTCGTTCGGCGAGAGCGGGCGGCGGTGGTGGGTCCTGTTGACAAT
TAATCATCGGCA, UL78-galk-Rev 5'-
GATGGACTCGGTGACTGAGGTAGTCTCCTCCACAGAAGGGGACATTCAGCACTGTCC
TGCTCCTT; US7-US12-galk-For 5'-
GGTTTATATATGACCATCCACGCTTATAACGAACCTAACAGTTTACCTGTTGACAATT
AATCATCGGCA, US7-12-galk-Rev 5'-
CCCATCGTCCCCCTTTCTCTATAAAACTTGCCGGGTACCTGAAGCTCAGCACTGTCCT
GCTCCTT; US13-US18-galk-For 5'-
GCTTCAGGTACCCGGCAAGTTTTATAGAGAAAGGGGGACGATGGGCCTGTTGACAAT
TAATCATCGGCA, US13-US18-galk-Rev 5'-
GTAACCGGGTGCTGATAAGACGGACTGTTTCATCGACGCCTACCTTCAGCACTGTCC
TGCTCCTT; US19-US24-galk-For 5'-
TCACGAGTGTGGTCAAACCGTGGCGGCACCCTGTATCCGACCCGTCCTGTTGACAAT
TAATCATCGGCA, US19-US24-galk-Rev 5'-
GGTGACGGTGTAGCGTTGCTTTCTCTGTATTTGGCTCGGCTTCTGTCAGCACTGTCCT
GCTCCTT; US26-US30-galk-For 5'-
CTCTCAGCCGGACAACCGGCGTCACTGACAGAAGCCGAGCCAAATCCTGTTGACAAT
TAATCATCGGCA, US26-US30-galk-Rev 5'-
GTCTCACCGATGAGACACCGACCGCACTCGAGAGTAAAGACAAATTCAGCACTGTCC
TGCTCCTT; US31-US34A-galk-For 5'-
TGTCTCATCGGTGAGACGAGGCCGCCGCCCGACAAGTTCGATCTCCCTGTTGACAAT
TAATCATCGGCA, US31-US34A-galk-Rev 5'-
TCGGCATCTTTGTCAATAAGACGCACGCCGCCGTGACCCATACCGTCAGCACTGTCC
TGCTCCTT; UL20-galk-For 5'-

CGGTCTTTATATATACAAACGCCGTTATGCTCAGTGTCCGGCAAGCCTGTTGACAATT
AATCATCGGCA, UL20-galk-Rev 5'-
TGCCCAGTGGTAATTCAGCATCACCAGCATAGCATGTATCCCGAGTCAGCACTGTCC
TGCTCCTT; UL20-STOPGFP-For 5'-
CGGTCTTTATATATACAAACGCCGTTATGCTCAGTGTCCGGCAAGATGGTGAGCAAG
GGCGAG, UL20-STOPGFP-Rev 5'-
TGCCCAGTGGTAATTCAGCATCACCAGCATAGCATGTATCCCGAGTTACTTGTACAG
CTCGTC; mCherry-restore-galk-For-5'-
TGTATTTGTGACTATACTATGTGCAGTCGTGTGTCGATGTTCCTATTGGGCCTGTTGA
CAATTAATCATCGGCA, mCherry-restore-galk-Rev-5'-
GATGTCTTCCTGCGTCCCACCATTCTTTATACCTCCTACATTCACACCCTTTCAGCACT
GTCCTGCTCCTT; mCherry-restore-For-5'-
TGTATTTGTGACTATACTATGTGCAGTCGTGTGTCGATGTTCCTATTGGGAAGGGTGT
GAATGTAGGAGGTATAAAGAATGGTGGGACGCAGGAAGACATC, mCherry-restore-
Rev-
5'GATGTCTTCCTGCGTCCCACCATTCTTTATACCTCCTACATTCACACCCTTCCCAAT
AGGAACATCGACACACGACTGCACATAGTATAGTCACAAATACA; SV40-EGFP-
MARCH1-galK-For-5'-
CCTAGGCTTTTGCAAAAAGCTTGCCACAACCCGGGATCCGCCACCCCTGTTGACAAT
TAATCATCGGCA, SV40-EGFP-MARCH1-galK-Rev-5'-
ATGGGTGGCAACTAAAAGGCACAGTCGAGGCTGATCAGCGAGCTCTCAGCACTGTCC
TGCTCCTT; SV40-EGFP-MARCH1-For-5'-
CCTAGGCTTTTGCAAAAAGCTTGCCACAACCCGGGATCCGCCACCATGGTGAGCAAG
GGCGAGGAGCTGTTCACCGGGGTGGTGCCCATC, SV40-EGFP-MARCH1 -Rev-5'-

ATGGGTGGCAACTAAAAGGCACAGTCGAGGCTGATCAGCGAGCTCTCAGACTGATA
CAACTTCAGGGGGGCCACCCTCTGCAGATGGCAG.

BAC sequences were PCR amplified from purified BAC DNA and verified by Sanger sequencing (GENEWIZ).

HCMV infections and virus titrations

All cytomegaloviruses stocks were generated from BACs. This includes AD169, TB40/E and the TB40/E derivatives generated as described in the previous section. Virus stocks were propagated by electroporating purified BAC DNA into MRC5 cells according to previously published protocols and subsequently freezing the infected cells as P0 stocks [367]. These stocks were added to MRC5 cells in roller bottles (Greiner) to generate P1 virus stocks. Virions produced in roller bottles were concentrated by ultracentrifugation on a 20% sorbitol cushion at 20,000 rpm, for 1 hour at 20°C in a Beckman SW32 rotor. HCMV virus stocks were titrated by serial dilutions on MRC5 cells and quantified by the immunological detection of immediate-early proteins as previously described using an antibody that detects the HCMV major immediate early proteins [368, 369]. Images of stained monolayers were acquired on a Nikon Eclipse Ti Inverted microscope and fluorescent nuclei were quantified using the NIS Elements Software.

Kasumi-3 cells were infected as previously described, with some modification [299]. Briefly, cells were cultured in X-VIVO medium (Lonza) for 48 hours prior to infection. Cells were infected with TB40/E-mCherry wild-type or mutant viruses (as described above) in X-VIVO medium with sufficient virus to result in infection of 10-20% of the cells (MOI of 10-40) and subjected to a spin of 1000 xg for 30 minutes at room temperature. Cells were incubated overnight at 37°C, washed twice with 1x PBS the following day and resuspended in fresh RPMI (with 20% FBS) following the final wash. Infected cells were cultured in RPMI (with 20% FBS) at 37°C until analysis. Where indicated, acyclovir (EMD Millipore) or DMSO (Fisher) was added at the time of infection at a concentration of 100 μg/ml and media was supplemented with

acyclovir or DMSO following washing and resuspension of cells at 24 hours. For UV inactivation, virus was placed under Mineralight UltraViolet lamp (short wave UV at 254nm) for 30 minutes at a distance of 10 centimeters. UV inactivation was confirmed by lack of mCherry-positive cells upon infection. For infection of primary monocytes, cells were resuspended in X-VIVO medium, infected with MOI of 40 and subjected to a spin of 400 xg for 30 minutes at room temperature. Cells were incubated overnight at 37°C and were washed twice with blood culture medium the following day. Infected cells were cultured in blood culture medium at 37°C until analysis.

For HCMV infections in HDFs, virus was added to HDFs and allowed to incubate for 3hrs at 37°C. Cells were washed twice with 1X PBS and replaced with fresh growth medium. Single step growth curve analysis of all BAC-generated mutant HCMV viruses were performed on HDFs infected with an MOI of 3. Virus was harvested by scraping cells in the medium, sonicating 10 times for 1 sec pulses, vortexing for 15 secs and centrifuging at 13000 rpm for 10 mins at 4°C. Supernatants were collected, aliquoted, flash frozen in liquid nitrogen and stored at -80°C until analysis. For analysis, the samples were titrated by serial dilutions and quantified as described above.

Flow Cytometry

Kasumi-3 cells were harvested, washed with cold wash buffer (2% BSA in 1X PBS with 1 mM EDTA and 0.01% sodium azide) and blocked with Human TruStain FcX for 10 minutes at room temperature, followed by incubation with antibodies against HLA-DR-APC (LN3, Thermo Fisher Scientific), HLA-DP (B7/21, Abcam) and anti HLA-DQ-FITC (Tü169, Biolegend) on ice for 30 mins. For unconjugated primary antibodies, cells were pelleted and incubated with secondary antibody (Thermo Fisher Scientific) on ice for 30 minutes. After antibody staining, cells were washed and resuspended in cold wash buffer and kept on ice until data acquisition on LSR II flow cytometer (BD Biosciences). At the indicated time point, live cells were identified

using Sytox Blue Dead Cell Stain (Thermo Fisher Scientific) or Zombie UV Fixable dye (Biolegend) and infected cells were selected by gating for mCherry. For total HLA-DR staining, cells were fixed and permeabilized using Fix/Permeabilization kit (BD Biosciences) and staining was performed as described above. Viability dyes, Sytox Blue Dead Cell Stain (Thermo Fisher Scientific) and Zombie UV Fixable dye (Biolegend), were used to discriminate between live and dead cells. Data analysis was performed for samples on FlowJo software (TreeStar) gating on live, single infected cells, with mCherry serving as a marker of infection. Geometric mean fluorescent intensity was calculated for HLA-DR on gated cell populations.

Western blotting

Kasumi-3 cells were infected with a virus lacking both US23 and US24, both of which augment virus growth when individually disrupted by transposon mutagenesis [370]. Lacking these genes allowed the virus to grow to higher titers on fibroblasts (100-1000 fold higher) to obtain a population of nearly completely infected cells (>95%). Uninfected and infected Kasumi-3 cells at 24 and 72 hours post infection were harvested in radioimmunoprecipitation assay buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 10 mM sodium phosphate [pH 7.2], 2 mM EDTA, 1mM DTT, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 0.2 mM Na3VO4, 1 µg/ml leupeptin). Cells were pelleted and resuspended in RIPA Buffer and stored at -80°C. Fibroblasts (primary and MRC-5) were infected with HCMV at MOI=3 as described previously. Uninfected or infected cell lysates were harvested at indicated time points in RIPA buffer containing protease inhibitors (as described above). Samples were analyzed by SDS-PAGE gel electrophoresis and immunoblotting on PVDF membrane blocked with 5% milk tris-buffered saline (0.1% tween). The following primary antibodies were used: αHLA-DR (TAL 1B5, Novus Biologicals), αexon2/3 against cytomegalovirus immediate early genes (gift from Jim Alwine [371]), αp115 (Proteintech), αpp71 (2H10-9, kindly provided by John Purdy [372]), αpp28 (5C3,Virusys), αpp150 (36-14,

gift from David Spector, originally generated by Bill Britt), αUL71 (1G, monoclonal antibody generated by Neil Christensen), MARCH1 (Sigma-Aldrich), GFP (gift from John Wills), αTransferrin receptor (3B8 2A1, Santa Cruz), αFas (G-9/B-10, Santa Cruz), αgB (2F12, Virusys), αGAPDH (Clone, Santa Cruz), αUL44 (CH13, Santa Cruz), αIE1/2 (D4, gift from Neil Christensen), αSTX5 (B8, SantaCruz), αSTING (ProteinTech) and αMAVS (D5A9E, Cell Signaling Technologies). The horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare. Antibody dilutions were in accordance with manufacturer's instructions. Blots were developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific).

Immunofluorescence microscopy and imaging

Kasumi-3 cells were harvested, washed twice with RPMI (20% FBS) and pelleted by centrifugation at 100 xg for 8 minutes. Cell pellets were resuspended in 100 µl of RPMI (20% FBS) and added to Poly-D-lysine coverslips (Electron Microscopy Sciences). Cells were allowed to attach for 10 minutes followed by centrifugation at 1000 xg for 10 minutes and incubation for 1 hour at room temperature. Coverslips with adhered cells were fixed by adding 100 µl of 4% paraformaldehyde directly to the cells and media for 15 minutes at room temperature. Fibroblasts (primary or MRC-5) were grown on coverslips and infected with HCMV as described previously. Uninfected or infected cells on coverslips were fixed with 4% paraformaldehyde for 15 minutes at room temperature.

Cells were blocked in PBS containing 10% human serum, 0.5% Tween-20, and 5% glycine. Triton-X 100 (0.1%) was added for permeabilization. Primary and secondary antibodies were diluted in blocking buffer. The primary antibody used were HLA-DR (L243, Biolegend), Lamp1 (D2D11, Cell Signaling Technologies), EEA1 (Thermo Scientific), STX5 (SY-SY), pp28 (Virusys), STING (ProteinTech) and GM130 (35, BD Biosciences). Alexa Fluor 488, 568 and

647 were used as the secondary antibodies (Thermo Fisher Scientific). Coverslips were mounted with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were taken on a C2+ Confocal Microscope System (Nikon). Images were minimally processed using NIS elements software. Images shown are as single slice of a Z-stack or whole volume of a Z-stack.

Reverse transcriptase and quantitative PCR

Kasumi-3 cells were infected as described for western blot analysis. Uninfected and infected cells were harvested at 24 and 72 hpi by washing with 1x PBS and resuspending in lysis buffer supplied in RNeasy Mini kit (Qiagen) for RNA extraction. Infected or uninfected fibroblasts (primary or MRC-5), at indicated timepoints, were directly lysed in wells of tissue culture plates with the RNeasy Mini kit lysis buffer (Qiagen). RNA extraction from lysates was subsequently performed using RNeasy Mini kit (Qiagen) following manufacturer's protocol. RNA concentration was quantified using NanoDrop 2000 (Thermo Fisher Scientific). cDNA was synthesized from RNA using SuperScript First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific) following manufacturer's protocol. Samples were cycled as follows on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific): 50°C 2 min, 95°C 10 min, (95°C 15 sec, 60°C 60 sec, 55°C 30 sec, 40 cycles). Primers used for quantitative PCR are as follows: HLA-DR-For 5'-CGAGTTCTATCTGAATCCTG-3', HLA-DR-Rev 5'-CTGGAGGTACATTGGTGA-3'; pan-CIITA-For 5'-AGCCTTTCAAAGCCAAGTCC 3', pan-CIITA-Rev 5'-TTGTTCTCACTCAGCGCATC-3'; CIITA-PI-For 5'-GGAGACCTGGATTTGGCCCT-3', CIITA-PIII-For 5'-GGGGAAGCTGAGGGCACG-3', CIITA-PIV-For 5'-GCGGCCCCAGAGCTGG-3', CIITA-PI-PIII-PIV-Rev 5'-GAAGCTCCAGGTAGCCACCTTCTA-3'; GAPDH-For 5'-ACCCACTCCTCCACCTTTGAC-3', GAPDH-Rev 5'-CTGTTGCTGTAGCCAAATTCGT-3'; US2-For 5'-ATGAACAATCTCTGGAAA-3'; US2-Rev 5'- GATTTGAAACCAGGGATG-3'; US3-For 5'-

GACCATCAACTGGTACCT-3', US3-Rev 5'- AATAAATCGCAGACGGGC-3'; IE1-For 5'-TTCCCAGAATTGGCCGAA-3', IE1-Rev 5'- TCTGTTTGACGAGTTCTGCCA-3'. MARCH1-For-5'- TCCCAGGAGCCAGTCAAGGTT, MARCH1-Rev-5'-CAAAGCGCAGTGTCCCAGTG; MARCH8-For-5'- ACAGGAAGCCTCCACTTCG, MARCH8-Rev-5' GACGTGGAATGTCACTGAG; human IL-10-For-5'-GGTTGCCAAGCCTTGTCTGA, human IL-10-Rev-5'- AGGGAGTTCACATGCGCCT; IE-For-5- CAAGTCCCGACACGTACC; IE-Rev-5'-TCTGTTTGACGAGTTCTGCCA, pp28-For-5'- GTGTCCCATTCCCGACTCG, pp28-Rev-5'- TTCACAACGTCCACCCACC; MX-1-For-5'- CTGTGCAGCCAGTATGAGGAG, MX-1-Rev-5'- CAGGGTGATTAGCTCATGACTG; ISG15-For-5'- GCTCCATGTCGGTGTCAGAG, ISG15-Rev-5'-CTCGAAGGTCAGCCAGAACAG; IRF7-For-5'- CCAGTTGATCCGCATAAGGT, IRF7-Rev-5'- GAGGCTCACTTCTTCCCTATTT; IFN-β-For-5'- CTCTCCTGTTGTGCTTCTCCAC, IFN-β-Rev-5'- TAGTCTCATTCCAGCCAGTGCT. Data was analyzed using delta Ct method with GAPDH as normalization control. To calculate absolute transcript numbers, standard curves for each primer set were generated by dilution series (1 nanogram to 1 femtogram). Samples from RT-PCR reaction were run on 4% agarose gel for visualization.

Plasmids

Plasmid DNA used was from plasmids pEGFPC1 (Clontech), pEGFPC1-pp65, pSVH (both gifts from Jim Alwine), pCDH (puro2AGFP), pCDH-IE1 (puro2AGFP), pCDH-IE2 (puro2AGFP), pMAX-GFP (Lonza), pCDH (EGFP-MARCH1), pLKO.1 (puro2AGFP). pCDH (puro2AGFP) was generated by stitching a T2A-EGFP sequence to the 3' end of puromycin. The EGFP sequence was amplified from pEGFPN2 with a primer that introduced an N-terminal T2A sequence downstream of 12 base pairs homologous to the 3' end of puromycin. Puromycin was amplified from pCDH-CMV-MCS-E1-Puro (Systems Biosciences) with primers that introduced C-terminal homology to 15 base pairs at the 5' end of the T2A-EGFP PCR product. The T2A-

EGFP and puromycin PCR products were stitched together in a PCR reaction that introduced a XhoI site 3' to the EGFP sequence. The resulting insert was digested with BsiWI and XhoI and ligated into pCDH-CMV-MCS-E1-Puro digested with BsiWI and SalI. pCDH-IE2 (puro2AGFP) was generated by amplifying the IE2 sequence from pIE86 with primers containing XbaI and EcoRI and ligating the resulting insert into pCDH (puro2AGFP) cut with XbaI and EcoRI. The ORF of IE1 flanked by XbaI and EcoRI sites was purchased from IDT and inserted into pCDH (puro2AGFP) cut with XbaI and EcoRI. pCDH-UL78 (puro2AGFP) was generated by amplifying the UL78 sequence from HCMV BAC (TB40/E) DNA with primers containing BamHI and EcoRI and ligated into pCDH (puro2AGFP) cut with BamHI and EcoRI. MARCH1 sequence was amplified from pCDNA3.1 MARCH1-EYFP, a kind gift from Dr. Jacques Thibodeau, using primers containing XhoI and BamHI and ligating the resulting insert into pEGFPC1 digested with XhoI and BamHI to generate the EGFP-MARCH1 construct. The resulting EGFP-MARCH1 was amplified from pEGFPC1 containing EGFP-MARCH1 with primers containing NheI and BamHI. The resulting insert was ligated into pCDH vector (gift from Jan Lammerding) digested with NheI and BamHI to generate pCDH-EGFP-MARCH1.

Amaxa transfection & cell sorting

Kasumi-3 cells (1 or 2 million cells per transfection) were centrifuged at 100 xg for 10 minutes, resuspended in 100 µl of Cell Line Nucleofector Solution R (Lonza) and mixed with 1 or 2 µg plasmid DNA. The cell suspension and reagent mixture were added to Nucleofector cuvettes and electroporation was performed using program V-001 in Nucleofector 2b device. Pre-warmed RPMI (with 20% FBS) media was added to electroporated cells and cells were incubated at 37°C until harvest for flow cytometry analysis. Electroporated cells were identified by the presence of GFP. The non-fluorescent pSVH and vector control plasmid were co-transfected with 0.2 µg pMAX-GFP to allow for detection of transfected cells. To generate RNA for cDNA synthesis and subsequent quantitative PCR analysis, Kasumi-3 cells were electroporated with

pSVH or pCDH, and co-transfected with pMax-GFP as described above. GFP-positive cells were sorted using FACSAria SORP (BD Biosciences) 48 hours post transfection and RNA was isolated immediately after sorting.

Internalization assay

For HLA-DR internalization assay, TB40/E IE2-2A-EGFP virus was used to infect Kasumi cells as described above. Infected and uninfected cells were harvested at 24 hours post infection, incubated with unconjugated anti HLA-DR (L243, Biolegend) for 30 minutes on ice, washed with cold, sterile wash buffer (2% BSA in 1X PBS) and resuspended in RPMI (20% FBS). Cells were incubated at 37°C and harvested at 0hr, 4hr, 8hr, 12hr, 24hr and 36hr post primary antibody labeling. Cells were stained with anti-mouse-AlexaFluor 647 (Thermo Fisher Scientific) and data acquisition was performed on a LSR II flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software (TreeStar) and geometric mean fluorescent intensity was calculated for HLA-DR.

Lentivirus transduction and cell selection

Lentivirus was generated using the 3rd generation packaging system with plasmid of interest and three packaging plasmids: pCMV-VSV-G (gift from Bob Weinberg, Addgene # 8454), pMDLg-RRE (gift from Didier Trono, Addgene #12251) and pRSV-Rev (gift from Didier Trono, Addgene #12253). For EGFP-MARCH1 expressing lentivirus, we used the pCDH (EGFP-MARCH1) as described above. For the shRNA plasmids, we utilized pLKO.1 plasmids with MARCH1 specific shRNA (pLKO.1 TRCN0000037019) obtained from the TRC1.0 shRNA library at Penn State College of Medicine and pLKO.1 empty vector (gift from Katherine Aird). For electron microscopy, we utilized MARCH1 specific shRNA (pLKO.1 TRCN0000037019) and scrambled shRNA (gift from David Sabatini, Addgene #1864) sequences in pLKO.1 modified to contain puro-2A-EGFP. The pLKO.1 vector with puro-2A-EGFP vector was generated by inserting a T2A-EGFP tag to the C-terminus of puromycin. The T2A-EGFP insert

was generated by PCR and fused to the end of the puromycin sequence lacking a stop codon via the gene sewing technique. Restriction sequences for BamHI and KpnI were introduced at the 5' and 3' ends of the insert, respectively, and used for cloning into the corresponding sites on the designated pLKO.1 plasmids.

Plasmid of interest (on pCDH or pLKO.1 vectors) and packaging plasmids were transfected into 293TN cells using Xtreme GENE HP DNA transfection reagent (Roche). Lentivirus containing supernatant was harvested at 72 hours post transfection and centrifuged at 2000 Xg for 10 mins at 4°C to remove cell debris. Supernatant containing lentivirus was aliquoted, flash-frozen and stored in -80°C until use. For lentivirus transduction, sub-confluent fibroblasts were seeded overnight and transduced with lentivirus containing 8μg/ml polybrene (Sigma Aldrich). Transduced cells were selected using 2μg/ml puromycin (Thermo Fisher) and passaged for two rounds of selection. Selected cells were seeded and infected with HCMV as described above.

Electron microscopy

MARCH1 shRNA and scrambled shRNA containing lentivirus was used to transduce fibroblasts as described above. Selected cells were seeded in 60 mm Permanox tissue culture dishes (Nalge Nunc International) overnight. Cells were infected with HCMV at an MOI of 3, washed two times with 1X PBS and fixed in fixation buffer (0.5% vol/vol glutaraldehyde, 0.04% vol/vol paraformaldehyde, 0.1M sodium cacodylate) for 1 hr at 4°C at 96 hpi. Cells were processed by the Microscopy Imaging Facility at Penn State College of Medicine. Briefly, the fixed samples were washed three times with 0.1 M sodium cacodylate, followed by postfixation in 1% osmium–1.5% potassium ferrocyanide overnight at 4°C. Samples were then washed 3 times in 0.1 M sodium cacodylate, dehydrated with ethanol, and embedded in Epon 812 for

staining and sectioning. Images were acquired using a JEOL JEM-1400 Digital Capture transmission electron microscope.

Statistical analyses

Statistical analysis was performed using Student's t-test and one-way ANOVA in Microsoft Excel and GraphPad Prism.

Appendix A: MHC class II in HCMV-infected Kasumi-3 cells upon lysosomal inhibition

We were interested in determining the mechanism of MHC class II decrease due to HCMV infection in Kasumi-3 cells. Since we had observed a reduction in both the surface and total MHC class II upon HCMV infection, we hypothesized that HCMV was actively degrading MHC class II. To test this, we treated uninfected and infected cells with concanamycin A, a lysosomal inhibitor, for 24hrs and stained for MHC class II. We found that the expression of intracellular MHC class II increased in both uninfected cells and infected cells at 72 hpi, but we observed no change for the120 hpi sample (Figure A-1). However, the amount of MHC class II expressed at 72 hpi and 120 hpi is much lower compared to uninfected cells (Figure A-1). This data is consistent with our observation that HCMV regulates MHC class II by blocking the transcription of MHC class II and decreasing its synthesis (Figure 2-7). Thus, the addition of concanamycin does not show a more pronounced accumulation of MHC class II in the infected cells because there is very little protein expressed in the infected cells and the synthesis of MHC class II is blocked. Furthermore, the surface MHC class II internalization assay demonstrated that the rates of MHC class II internalization and degradation are similar for infected and uninfected cells (Figure 2-6B). Thus, the lysosome inhibition experiment supports the conclusion that the mechanism of MHC class II decrease in Kasumi-3 cells is a block in the MHC class II production and not due to enhanced endocytosis and degradation.

Kasumi-3 cells were infected with TB40 virus expressing mCherry for 72 hours and 120 hours. Concanamycin (1 μm) was added to uninfected cells and infected cells 24 hours prior to fixation. Uninfected and infected cells were stained with LAMP1 and HLA DR antibodies for immunofluorescence as described previously.

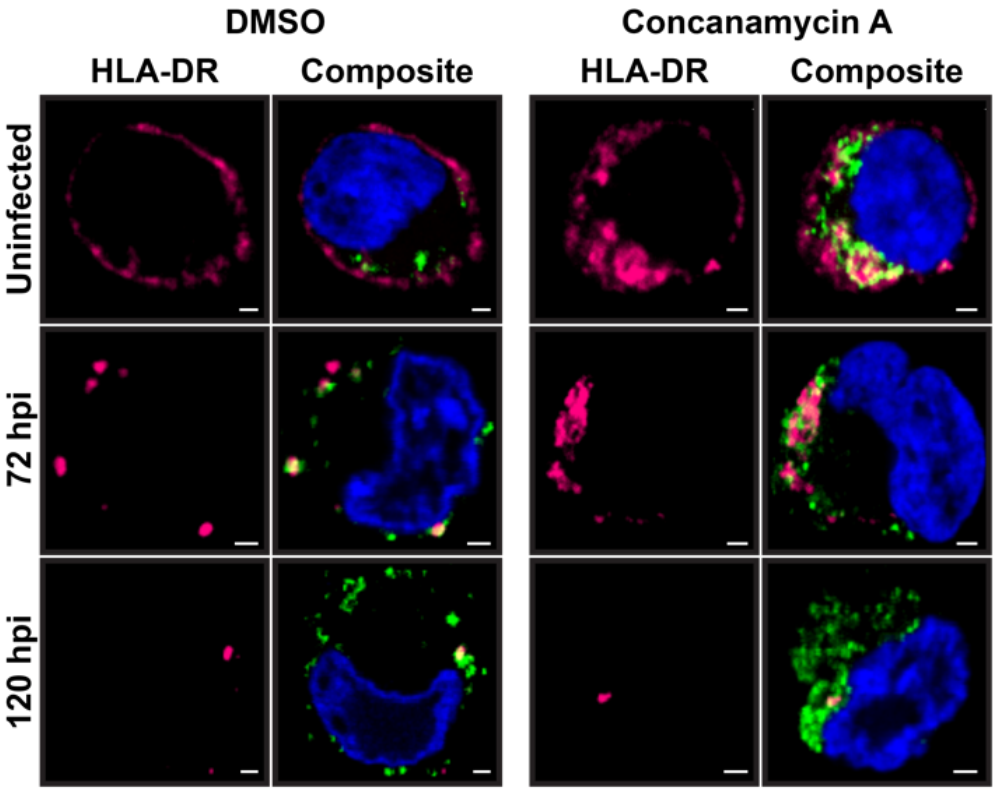

Figure A- 1: MHC class II decrease in infected Kasumi-3 cells is not due to enhanced degradation. Uninfected and HCMV-infected Kasumi-3 cells (72 & 120 hpi) were treated with DMSO or Concamycin A for 24 hours and stained for HLA-DR (pink) and LAMP1 (green). Infected cells were identified by mCherry expression (not shown). Nuclei visualized by DAPI (Blue). Scale bar: 1 μm

Appendix B: Transcription of CD63 in Kasumi-3 cells during HCMV infection

We had observed reduced transcription of HLA-DR and CIITA in HCMV-infected Kasumi-3 cells. To determine that this was not occurring due a general transcriptional block, we looked at the expression of cellular gene CD63. We found that CD63 transcription was not reduced in infected Kasumi-3 cells at 24 hpi and 72 hpi (Figure B-1). This suggests that the decrease in HLA-DR and CIITA during HCMV infection is specific and does not occur due to a global decrease in transcription of cellular genes.

Kasumi-3 cells were infected with a growth-augmented TB40 virus as described previously. Uninfected and infected cells were harvested for RNA isolation at 24 hpi and 72 hpi. Following RNA extraction, cDNA was synthesized from samples and used for quantitative PCR. Primers used for CD63 are CD63-For-5'- ACCACACTGCTTCGATCCTG and CD63-Rev-5'-TCTCCACACAGCCCTCCTTA. GAPDH was used as a control and primers used were described previously.

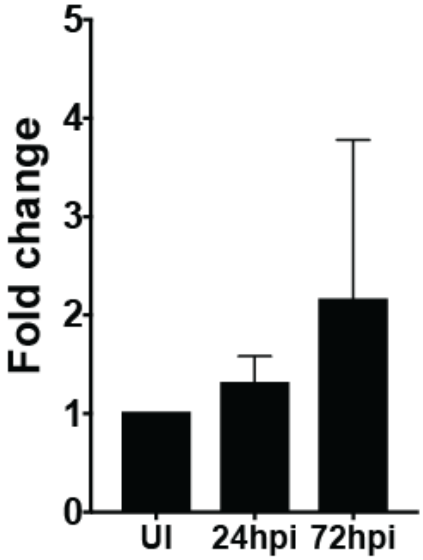

Figure B- 1: CD63 transcription does not change upon HCMV infection in Kasumi-3 cells. Quantitative PCR analysis of CD63 transcript levels in uninfected (UI) and HCMV-infected Kasumi-3 cells at 24 and 72 hpi. Values shown are relative to uninfected samples after normalization to GAPDH. Values are derived from two independent experiments.

Appendix C: MARCH1 and MARCH8 transcription in Kasumi-3 cells during HCMV infection

We had observed the presence of MHC class II in LAMP1 positive compartments in HCMV-infected Kasumi-3 cells, which could be the result of lysosomal degradation of MHC class II. Thus, we hypothesized that HCMV was increasing the rate of MHC class II endocytosis and degradation. The two cellular genes that target MHC class II for ubiquitination to promote lysosomal degradation are MARCH1 and MARCH8. Thus, we assessed the expression of MARCH1 and MARCH8 in Kasumi-3 cells upon HCMV infection. We found that MARCH1 transcription was increased upon HCMV infection while MARCH8 transcript levels remained unchanged (Figure C-1). Thus, HCMV infection increases MARCH1 transcription in infected Kasumi-3 cells.

Kasumi-3 cells were infected with TB40 virus at an MOI of 20 as described previously. Uninfected and infected cells at 48 hpi were harvested for RNA isolation and cDNA synthesis. Quantitative PCR was performed using primers specific for MARCH1, MARCH8 and GAPDH as listed previously with GAPDH as the control.

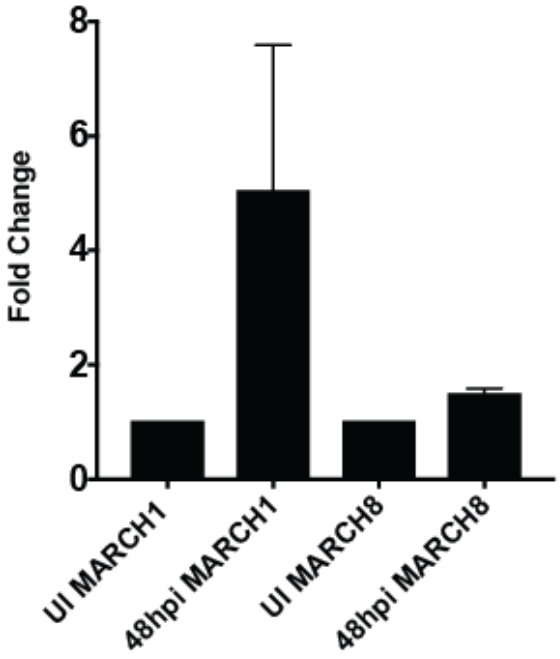

Figure C- 1: MARCH1 is increased during HCMV infection in Kasumi-3 cells. Quantitative PCR for MARCH1 and MARCH8 transcript levels in uninfected (UI) and HCMV-infected Kasumi-3 cells at 48 hpi. Values shown are relative to uninfected samples after normalization to GAPDH. Values are derived from two independent experiments.

Appendix D: Impact of MARCH1 overexpression during HCMV infection

Since MARCH1 was important for HCMV infection, we decided to overexpress MARCH1 to see whether this would lead to an increase in viral titers. For this, we utilized the EGFP-MARCH1 recombinant virus to infect fibroblasts and compare viral titers with parental strain TB40/E expressing SV40-driven mcherry (TB40 mch). Interestingly, we found that the overexpression of EGFP-MARCH1 caused a decrease in infectious virus titers compared to the parental wild-type virus both at 96 hpi and 120 hpi (Figure D-1A). The overexpressed MARCH1 appears earlier in infection and at higher levels compared to wild-type infections (Figure D-1B). We wondered if the earlier and higher expression of MARCH1 could affect other known MARCH1 targets such as TfR. Indeed, TfR protein was decreased at 96 hpi in protein lysates derived from EGFP-MARCH1 infected cells (Figure D-1B). While there is no evidence to show that TfR is essential for HCMV infection, the expression of TfR is critical for cells due to its important function of iron uptake, and could contribute to the reduction in titers due to MARCH1 overexpression. These results show that the timely induction of MARCH1 is important for HCMV, and excessive MARCH1 expression in infected cells can be detrimental.

MRC-5 fibroblasts were infected with TB40 virus expressing mcherry or EGFP-MARCH1 from its genome at an MOI of 3 as described previously. Single-step growth curve analysis for total infectious virus was performed for both viruses at 96 hpi and 120 hpi at an MOI of 3 as described previously. Uninfected and infected cells were harvested for protein lysates at 24 hpi and 96 hpi using RIPA with the protease inhibitors as listed previously.

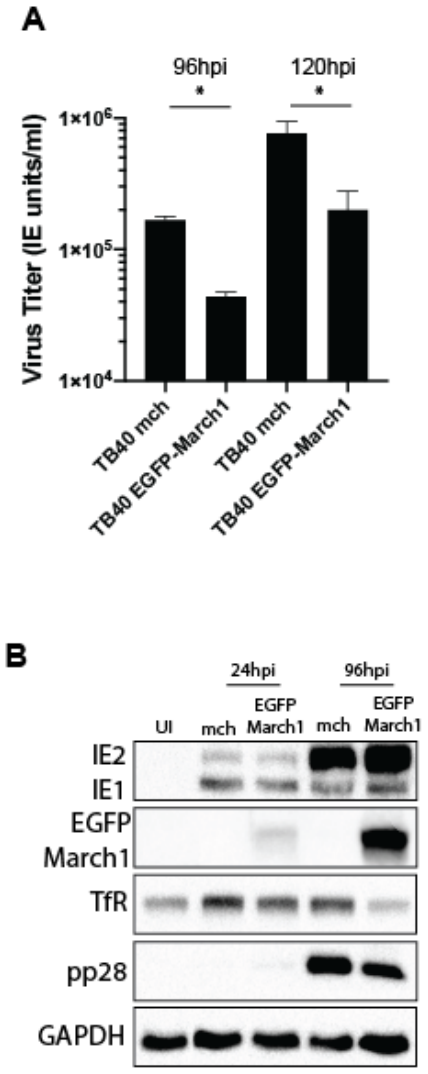

Figure D- 1: Overexpression of MARCH1 decreases viral titers. (A) Infectious titers at 96 hpi and 120 hpi for MRC-5 fibroblasts infected with HCMV TB40/E mCherry and EGFP-MARCH1 viruses (MOI=3). (B) Western blot analysis for GFP (MARCH1), transferrin receptor (TfR), immediate early viral proteins (IE1, IE2), late viral protein (pp28) and loading control (p115) in uninfected cells or cells infected at 24hpi and 96 hpi as described in (A). * indicates $p<0.05$, Student's t-test.

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Kumar R, Cruz L, **Sandhu PK**, Buchkovich NJ. UL88 Mediates the Incorporation of a Subset of Proteins into the Virion Tegument. J Virol. 2020;94(14):e00474-20.

Sandhu P, Haque M, Humphries-Bickley T, Ravi S, Song J. Hepatitis B Virus Immunopathology, Model Systems, and Current Therapies. Front Immunol. 2017;8:436.

Awards

Graduate Alumni Endowed Scholarship Award 2018
Karl H. Beyer, Jr., MD PhD Scholarship Award 2017
Richard J. Courtney Graduate Student Award 2017
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Selected presentations

Sandhu P, Buchkovich NJ. HCMV decreases endogenous CIITA and MHC class II in myeloid cell line. International Herpesvirus Workshop, July 20-24, 2019, University of Tennessee, Knoxville, TN. (Poster)
Sandhu P, Cruz L, Ferguson K, Buchkovich NJ. A cell-type specific role for UL88 during HCMV infection. International Herpesvirus Workshop, July 21-25, 2018, University of British Columbia, Vancouver, Canada. (Poster)
Sandhu P, Haque M, Fino K, Song J. Differentiation of iPSC-derived T cells specific to HBV. 19th Annual Upstate New York Immunology Conference, October 24-27, 2016, Bolton Landing, NY. (Poster)