A Study of Host Factors that Affect Herpes Simplex Virus 1 Pathogenesis: The Role of Cold Sore Susceptibility Gene 1 (CSSG1) in HSV1 Replication

Milan K. Patel
University of Massachusetts Medical School

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A STUDY OF HOST FACTORS THAT AFFECT HERPES SIMPLEX VIRUS 1 PATHOGENESIS: THE ROLE OF COLD SORE SUSCEPTIBILITY GENE 1 (CSSG1) IN HSV1 REPLICATION

A Dissertation presented

By

Milan K. Patel

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

7 December, 2017

PhD Program in Biomedical Sciences
Immunology and Virology Program
A STUDY OF HOST FACTORS THAT AFFECT HERPES SIMPLEX VIRUS 1 PATHOGENESIS: THE ROLE OF COLD SORE SUSCEPTIBILITY GENE 1 (CSSG1) IN HSV1 REPLICATION

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Milan K. Patel

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The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

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Program in Immunology and Microbiology

7 December, 2017
ABSTRACT

Numerous factors that affect herpes simplex virus 1 (HSV1)-mediated pathogenesis have been identified. Such factors directly impact the replication of HSV1 as well as modulate host immune responses following HSV1 infection. In this work, I characterize how HSV1 replication is impacted by expression of the protein encoded by C21orf91, or “Cold Sore Susceptibility Gene” (CSSG1), that has been linked to HSV1 reactivation in humans.

I investigated expression of CSSG1 mRNA expression in various tissues and found that CSSG1 mRNA was present in several tissues of importance in HSV1 disease, including brain, trigeminal ganglia (TG), cornea and spleen. Western blot analysis demonstrated that CSSG1 protein is expressed in human cells. Subcellular fractionation analysis reveals that CSSG1 is predominantly found in the cell nucleus, where it colocalizes with chromatin and with Tip60, a chromatin-binding histone modifying protein that has been shown to be essential for the replication of herpesviruses. I also discovered that CSSG1 is present in the cytosol of cells where it forms large cytosolic aggregates in presence of TRAF6, a downstream adapter that plays an important role in innate immune receptor signaling.

To determine if CSSG1 directly impacts viral replication, I generated CSSG1 knockdown human cell lines. I found that HSV1 replication was reduced in CSSG1 knockdown cells compared to control cells, whereas replication of the unrelated virus, vesicular stomatitis virus (VSV), was not affected by knockdown of CSSG1. I demonstrate that CSSG1 was necessary for efficient expression of HSV1 viral proteins during infection. Western blot analysis and measurement of expression of HSV1 proteins
expressed at various stages of viral replication illustrates that CSSG1 was required for HSV1 replication at very early stage of infection. I also noted that CSSG1 expression impacted the DNA damage response in HSV1 infected cells. Levels of H2AX phosphorylation, a marker of the DNA damage response, were increased in HSV1-infected CSSG1 knockdown cells compared to control cells. DNA damage responses are thought to promote HSV1 reactivation from latency and HSV1 gene expression, indicating a potential mechanism for role of CSSG1 in HSV1 replication through modulating the DNA damage response.

Overall, my work demonstrates that CSSG1 affects HSV1 replication and provides insight on how CSSG1 polymorphisms in humans could affect HSV1 reactivation and replication to promote cold sores. These discoveries may also lead to a better understanding of pathogenesis of other herpesviruses in humans.
ACKNOWLEDGMENTS

I want to thank my mentors Dr. Evelyn Kurt-Jones and Dr. Robert Finberg. Over the years, I have gained invaluable knowledge and wisdom from you that have helped me develop as a person and as a scientist. Your assurances and encouragement during difficult times have been crucial for providing me with confidence. I want to thank Dr. Jennifer Wang for being an external mentor and providing guidance when needed. I want to thank members of my thesis advisory committee Dr. Katherine Fitzgerald, Dr. Timothy Kowalik, Dr. Neal Silverman, and Dr. Jennifer Wang for guiding me towards completion of my dissertation research. Completion of my studies would not have been possible without your constant support and guidance.

I also want to thank all present and past members of the Finberg Lab. Thank you Mike for always being there with invaluable advice and help. Mike’s help with experiments has been crucial for completion of this scientific work. I also want to thank everyone for advice on completing experiments and getting through the long days in the lab. Lastly, I want to thank my family for their encouragement and patience. Their love and support has been invaluable for completion of my dissertation.

Thank you all once again. I will forever be grateful for your help and contributions in making this dissertation a success.
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LIST OF ABBREVIATIONS

AIM2  Absent in melanoma-2
ASC   Apoptosis-associated speck-like protein containing CARD
ATM   Ataxia–telangiectasia-mutated (ATM) protein kinase
BMDC  Bone marrow derived dendritic cell
BMDM  Bone marrow derived macrophage
C21ORF91  Chromosome 21 open reading frame 91
CARD  Caspase activation and recruitment domain
CASP1 Caspase-1
CD    Cluster of differentiation
c-di-AMP Cyclic di-adenosine monophosphate
c-di-GMP Cyclic di-guanosine monophosphate
cGAS  Cyclic GMP-AMP Synthase
cGAMP Cyclic guanosine monophosphate–adenosine monophosphate
CSSG1 Cold sore susceptibility gene 1
CTxB  Cholera toxin subunit B
Cyt. D Cytochalasin D
DAI   DNA-dependent activator of IFN-regulatory factors
DAMP  Danger-associated molecular pattern
DC    Dendritic cell
DDX   DEAD (Asp-Glu-Ala-Asp) box polypeptide
dDHX  DEAH (Asp-Glu-Ala-His) box polypeptide
dsDNA double-stranded deoxyribonucleic acid
dsRNA double-stranded ribonucleic acid
EBV   Epstein-Barr virus
ELISA Enzyme-linked immunosorbent assay
ER    Endoplasmic reticulum
FRT   Flippase recognition target
GFP   Green Fluorescent Protein
<table>
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<td>Histone H2A</td>
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<tr>
<td>pH2AX</td>
<td>Phosphorylated H2AX</td>
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<td>HCMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylases</td>
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<td>HEK293</td>
<td>Human embryonic kidney cell line</td>
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<tr>
<td>HEK293T</td>
<td>Human embryonic kidney cell line carrying SV40 T-antigen</td>
</tr>
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<td>HSE</td>
<td>Herpes simplex encephalitis</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>ICP</td>
<td>Infected cell polypeptide</td>
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<td>IFI16</td>
<td>Gamma-interferon-inducible protein 16</td>
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<tr>
<td>IFN</td>
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<tr>
<td>IFNAR</td>
<td>Interferon alpha/beta receptor</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISD</td>
<td>Interferon stimulatory DNA</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-stimulated response element</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>KD</td>
<td>Knockdown</td>
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<tr>
<td>KSHV</td>
<td>Kaposi sarcoma-associated herpesvirus</td>
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<tr>
<td>LAT</td>
<td>Latency associated transcript</td>
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<td>LAT. A</td>
<td>Latrunculin A</td>
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<td>LoxP</td>
<td>Locus of X (cross)-over in P1</td>
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<td>LRR</td>
<td>Leucin rich repeats</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>mCSSG1</td>
<td>Mouse Cold Sore Susceptibility Gene 1</td>
</tr>
<tr>
<td>NT</td>
<td>Nontargeting</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear Factor Kappa B</td>
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<tr>
<td>NLR</td>
<td>NOD-like Receptor</td>
</tr>
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<td>NLRP3</td>
<td>NOD-like receptor protein 3</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PEO</td>
<td>poly (ethylene oxide)</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PS</td>
<td>Polystyrene</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>ScEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>SeV</td>
<td>Sendai virus</td>
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<tr>
<td>SNP</td>
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<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TG</td>
<td>Trigeminal ganglia</td>
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<tr>
<td>TIP60</td>
<td>Tat Interactive Protein 60kDa</td>
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<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor homology domain</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>U2OS</td>
<td>Human bone osteosarcoma epithelial cell line</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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<td>WT</td>
<td>Wildtype</td>
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PMID:23427254. *co-first authors
CHAPTER I

INTRODUCTION
1.1 The Herpesviridae Family

Viruses from the herpesviridae family are present ubiquitously in the human population. The World Health Organization estimates that approximately 90% of the world population has been infected by members of the herpesviridae family [1]. The herpesvirus family comprises viruses carrying large double-stranded DNA (dsDNA) genomes that share structural similarity. The herpesviral genomes range in size from 120 – 230 kbp and protein-coding RNAs as well as miRNAs and non-coding RNAs (ncRNAs). The genomes of herpesviruses are encapsulated within an icosahedral capsid structure. The capsid is surrounded by tegument proteins that are responsible for activation of viral gene expression, e.g. immediate early genes. An envelope containing an array of glycoproteins surrounds the tegument protein assembly. The envelope glycoproteins are important for mediating entry of herpesviruses into the host cells. Following entry, the activation of herpesviral gene expression can lead to a productive lytic infection, resulting in the destruction of the host cells. Under circumstances related to tropism of host cells, herpesviruses can also establish a latent, asymptomatic infection characterized by relatively low expression of viral genes with persistent long term survival of latently infected cells, particularly neurons, often for the lifetime of the host (Figure 1.1).

The herpesviridae family is divided into three subfamilies, Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae, based on distinct biological properties. These properties include tropism of infection, replication cycle, and latency properties of the viruses. Alphaherpesviruses are characterized by relatively fast replication cycles, the capability of infecting a wide range of host cells, and establishment of latency in
sensory ganglia. Members of the alphaherpesvirinae include herpes simplex virus 1 and 2 (HSV1, HSV2). Beta-herpesviruses, on the other hand, have slower replication cycles and establish latency in cells of myeloid lineage. Human cytomegalovirus (HCMV) is a member of the Betaherpesvirinae subfamily. The gammaherpesviruses are more prone to latency compared to viruses from other herpesvirus subfamilies. These viruses generally infect and replicate in lymphoid cells. Epstein Barr Virus (EBV), and Kaposi sarcoma-associated herpesvirus (KSHV) are members of the gammaherpesvirinae subfamily.

In this dissertation I focus on exploring host processes involved in viral gene expression and replication of HSV1, a common human pathogen and widely studied member of the alphaherpesvirinae.

1.2 Herpes Simplex Virus 1 (HSV1)

HSV1 is a common human pathogen. A test of seropositivity for HSV1 has led to estimates of the prevalence of HSV1 being placed at 58% of individuals among the population of the United States [2]. In the developing world, HSV1 infection is considered to be almost universal, being acquired in the early childhood from close contact with a family member [3].

Primary infection of HSV1 is typically associated with infections of the oropharynx and to a lesser extent, the genitalia region. HSV1 enters through contact with the mucosal membrane and primarily infects epithelial cells, leading to ulcerations of the mucosa and skin. In some cases, primary infection of the cornea by HSV1 can lead to a
Figure 1.1: HSV1 lifecycle in humans. Acute infection of the epithelial layer by HSV1 results in a productive lytic infection. This can eventually lead to establishment of HSV1 latency in the trigeminal ganglion. In the latent stage, HSV1 genome exits in a circularized condensed chromatin stage within the nucleus. Through an incompletely understood mechanism, HSV1 can reactivate leading to a productive infection of the epithelial layer. In humans, reactivation of HSV1 can be asymptomatic or lead to conditions such as cold sores and encephalitis.
condition known as keratitis characterized by permanent vision damage. HSV1 induced keratitis is the leading cause of infectious blindness in the United States [4].

Following primary productive infection of the epithelial cells at mucosal membranes, HSV1 can also establish a latent infection in the trigeminal ganglia (TG). In the TG, HSV1 can persist as in latent state for decades. Upon exposure of the host to conditions such as heat or emotional stress, latent HSV1 can reactivate from latently infected TG and can cause pathogenesis through productive infection of mucosal surfaces enervated by TG neurons, most commonly along the lip margin. Normally, reactivation of HSV1 in humans causes herpetic lesions at oral (commonly known as cold sores) or genital mucosal surfaces. Reactivation of HSV1 in the central nervous system (CNS) can result in a morbid inflammatory complication referred to as herpes simplex encephalitis (HSE) with a fatality rate of >70% if left untreated [5].

HSV1 has a dsDNA genome of 152 kb that encodes for at least 74 open reading frames (ORFs) [6, 7]. The expression and subcellular localization of HSV1 ORFs is temporally regulated during infection, leading to distinctly characterized transcriptomes for each stage of HSV1 lifecycle. Stages of HSV1 infection include viral entry, replication of the genome, virion assembly and release, latency establishment, and reactivation.

1.3 HSV1 Replication

The following sections describe HSV1 lytic and latent lifecycles. The replication process of HSV1 is illustrated in Figure 1.2 in detail.
**Figure 1.2: Stages of HSV1 replication.** 1) Entry of HSV1 virions into the host cell mediated by binding of HSV1 proteins gB/gC to heparan sulfate resulting in endocytosis 2) Insertion of HSV1 genome into the nucleus and translocation of HSV1 transcription activator VP16 to the nucleus 3) Circularization of the HSV1 genome 4) Transcription of IE stage mRNA encoding for HSV1 proteins such as ICP4 that enter the nucleus and facilitate expression of E stage proteins 5) Transcription of mRNAs encoding for E stage HSV1 proteins such as ICP8 6) Translocation of ICP8 to the HSV1 genome located in the nucleus 7) ICP8 facilitates replication of the HSV1 genome 8) Replicated HSV1 genomes 9) Transcription of L stage HSV1 mRNAs encoding for HSV1 structural proteins 10) Capsid proteins enter the nucleus 11) Formation of HSV1 capsid 12) Insertion of the HSV1 genome into the capsid 13) Exit of HSV1 capsid structure from the nucleus and addition of the tegument proteins 14) Binding to the cell membrane 15) Release of HSV1 virions.
**HSV1 Entry**

During initial phase of infection, HSV1 infects cells of the mucosal epithelium. HSV1 entry can occur through either a pH-dependent or a pH-independent method [1, 8, 9]. During a pH-independent entry, HSV1 binds and enters directly through plasma membrane. Entry through a pH-dependent pathway requires endocytosis of HSV1 virions. The path of entry seems to be cell-type specific. The attachment of HSV1 to host cells is triggered by interaction between the HSV1 glycoproteins gB or gC and heparan sulfate expressed on host cells[10]. This results in host cell proteins called herpesvirus entry mediator (HVEM) and nectin-1 coming in contact with HSV1 gD and triggering viral entry through the HSV1 fusion machinery composed of gB and gH/gL proteins[11]. Following fusion, HSV1 capsids are released into the cytoplasm and transported to the nucleus. At the nucleus, HSV1 genomes are transported inside the nucleus through nuclear pore complexes [12].

**HSV1 Replication**

Following entry of HSV1 genome into the nucleus, HSV1 begins a complex replication cycle marked by distinct stages of coordinated HSV1 gene expression, genome replication, and frequent transport of translated HSV1 proteins between nucleus and cytosol. The HSV1 genome is reported to be free of histones and linear when it enters the nucleus [13] However prior to replication initiation, the genome is circularized and subjected to heavy heterochromatinization by an intrinsic antiviral mechanism [14-16] that results in shutting down of HSV1 gene expression. HSV1 protein ICP0 which forms part of the tegument [17] can counteract this response resulting in histone removal from HSV1 genome and reducing heterochromatization of the genomes. This allows
HSV1 transactivator protein VP16 access to the genomes to begin expression of HSV1 genes [18].

The expression of HSV1 genes occurs in three distinct stages: immediate-early (IE), early, and late. At initiation of replication, VP16 recruits HCF-1 and Oct-1 to activate the immediate-early gene promoters to induce expression of ICP0, ICP4, ICP27, ICP22, and ICP47 [19]. Following transcription, mRNAs of immediate-early genes exit the nucleus for translation. The translated immediate-early proteins ICP0, ICP4, and ICP27 return to the nucleus to activate expression of early-genes such as ICP8 and HSV-thymidine kinase. Following translation, early proteins enter nucleus to begin viral DNA replication. Following replication of HSV1 genomes, late genes (VP16, ICP5) are transcribed which make up the structure of HSV1 capsids and tegument [20].

**HSV1 Virion Assembly and Release**

Following expression of HSV1 structural proteins at the late stage, HSV1 genomes become encapsidated leading to formation of nucleocapsids containing ICP5 associated proteins. HSV1 protein UL31 helps transport the capsid containing HSV1 genomes to the nuclear membrane [21]. The capsids egress into the cytosol following a cycle of envelopment and deenvelopment of nuclear membrane. In the cytosol, the capsid becomes associated with tegument proteins and become enveloped again as they bud into the trans-golgi network. The virions are then transported to the host cell plasma membrane and secreted.
1.4 HSV1 Latency and Reactivation

As described earlier, HSV1 gene expression during the lytic phase in epithelial cells is dependent upon the VP16 transactivating complex that is formed by VP16, HCF-1 and Oct-1. HSV1 virions are transported along the axons of neurons through retrograde transport, eventually leading to HSV1 genome reaching the nucleus of sensory neurons. When HSV1 genome enters the nucleus of sensory neurons, there may be multiple explanations why lytic replication does not start. There is evidence that VP16 is not efficiently transported along axons leading to insufficient amount of protein reaching the nucleus of sensory neurons [22]. In addition, Oct-1 has been shown to be downregulated in neuronal cells [23]. Also, HCF-1 is present exclusively in the cytoplasm of neuronal cells and thus would not be available to activate HSV1 IE gene expression in the nucleus [24].

Neuronal cells also express Zhangfei protein that has been shown to have repressive effect on HSV1 gene expression[25]. Due to lack of expression of IE genes, the HSV1 genomes are unable to counteract the intrinsic chromatin remodeling response against HSV1 genomes. As a result, the viral genome in neuronal nucleus remains as an episome associated with nucleosomes.

Although HSV1 genes that promote lytic infection are suppressed in the latent stage, a latency-associated transcript (LAT) is expressed at high levels. The LAT transcript is transcribed anti-sense to the ICP0 mRNA and negatively regulates lytic gene expression [26, 27]. Through a poorly understood mechanism, HSV1 genomes can reactivate, leading to transport of HSV1 virions down the axons leading to productive infection and replication in the epithelial cells.
1.5 Role of Innate Immune Responses in HSV1 Pathogenesis

The development of herpesvirus pathogenesis involves complex interplay between HSV1 replication and the resulting immune response. Early innate immune responses characterized by production of proinflammatory cytokines and type I IFN play a crucial role in development and resolution of herpesvirus pathogenesis. The innate immune response plays a crucial role in initiation and resolution of herpesvirus pathogenesis. The two major pathways activated during an innate immune response to herpesvirus infections include a proinflammatory cytokine response and a type I IFN response. The production of proinflammatory cytokines leads to recruitment of macrophages, neutrophils, and antigen presenting cells that facilitate in recognition of HSV1 virions and clearance of infected cells. Production of type I IFN is critical for reduction of HSV1 replication and its clearance[28]. Type I IFN activates expression of ISGs that activate intrinsic cell immunity to reduce HSV1 replication[29-31]. Type I IFN also facilitates activation of the adaptive immune response by upregulating MHC-I expression and long-term T cell memory responses [32]. The importance of proinflammatory cytokines and type I IFN in HSV1 infection is evident from studies showing that IL-6, IL-1, and IFNAR deficient mice are highly susceptible to HSV1 infections [28, 33, 34].

Before an innate immune response can be initiated, the immune system needs to recognize presence of HSV1 virions. This is accomplished by recognition of HSV1 virion structural components by various intracellular and extracellular PRRs. Two major groups of PRRs are responsible for recognition of HSV1 components – 1) extracellular and endosomal Toll-like receptors (TLRs) and 2) various intracellular receptors that typically recognize viral nucleic acids. The current understanding of PRRs belonging to these two
groups is summarized below.

1.6 Toll Like Receptors (TLRs)

TLRs were first discovered in *Drosophila melanogaster* to be important for detection and clearance of bacterial and fungal infections. TLRs detect various pattern associated molecular patterns (PAMPs) to identify presence of an infection. TLRs identified so far in humans recognize various conserved DNA, RNA, and protein motifs present in pathogens. All TLRs share structural similarities[35, 36]. The ligands are detected through an extracellular leucine-rich repeats (LRR) domain that is tethered to the cytosol via a signaling cytoplasmic toll/interleukin-1 receptor (TIR) domain. The TLRs can be present on the plasma membrane (TLR 1, 2, 4, 5, 6) or in the endosome (TLR 3, 7, 8, 9) [37]. Recognition of a ligand by TLRs results in recruitment and activation of its adapter proteins (MyD88 or TRIF) and resulting production of type I IFN or proinflammatory cytokines, such as IL-6, through activation of IRF3/7 or NF-kB respectively[38].

Although all TLRs share structure similarities, they can recognize multitude of PAMPs ranging from DNA, RNA to proteins. TLRs that have been identified to recognize HSV1 components include TLRs 1, 2, 3, 6, and 9[39-41]. The role each TLR plays during HSV1 pathogenesis is summarized below.

**TLR2**

TLR2 functions as a heterodimer with either TLR1 or TLR6 to detect HSV1 glycoproteins. The resulting NF-kB activation leads to a strong proinflammatory response. In case of *in vivo* HSV1 infections, the proinflammatory response can be either protective or damaging. In the HSV1 encephalitis models, TLR2 deficient mice are
Figure 1.3: Recognition of HSV1 viral particle components by TLRs. TLR2 is expressed on the cell surface. Together with TLR1 or TLR6, TLR2 recognizes HSV1 glycoproteins and activates MyD88-dependent NF-κB pathway leading to inflammatory cytokine production. TLR3 and TLR9 are expressed on the endosomes. TLR3 recognizes dsRNA products created during HSV1 replication, while TLR9 recognizes ssDNA fragments of the HSV1 genome. Activation of TLR3 can lead to production of inflammatory cytokines and type I interferon through the NF-κB and IRFs pathways respectively.
protected and demonstrate better survival [40, 42]. Presumably, this would due to lack of a strong inflammatory response in TLR2 knockout mice in response to HSV1. In contrast, in the intranasal infection model of HSV1, TLR2 knockout mice are more susceptible to HSV1 infection [43].

In addition to HSV1, TLR2 can also detect other members of the herpesvirus family. It has been shown that during HCMV infection, TLR2 is required for NF-kB activation and IL-8 secretion in HEK293 cells [44]. TLR2 can also recognize HSV2 glycoproteins [45]. In intraperitoneal and intravaginal models of HSV2 infection, TLR2 plays a protective role in mice [42]. Thus, it seems likely that proinflammatory response to HSV1 in the brain is more detrimental than beneficial.

**TLR3**

TLR3 is an endosomal TLR sensor of nucleic acids [46, 47]. The ligand for TLR3 activation is dsRNA. TLR3 has been implicated in detection of HSV1 infections in human patients [48]. Even though HSV1 is a DNA virus, it forms dsRNA intermediary products due to the process of bidirectional transcription of its genome. Activation of TLR3 proceeds via the adapter TRIF and drives type I IFN responses [49].

**TLR9**

TLR9, like TLR3, is also an endosomal nucleic acid sensor. TLR9 recognizes unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs present in the DNA [50]. Also, activation of TLR9 results in production of type I IFN via the adapter MyD88, in contrast to TLR3-driven IFN responses, which occur via TRIF. It has been shown in multiple models that TLR9 is the sole detector for replication incompetent
herpesviruses[51]. TLR9 has been shown to be able to detect HSV1, HSV2, EBV, and MCMV[51]. TLR9 deficient mice have increased rate of mortality and viral loads in response to HSV1 infection in intranasal infection model of HSV1[43]. However, TLR9 seems to be redundant for controlling HSV1 infections in the intracranial infection model in mice[52].

**Unc93B**

The importance of endosomal TLRs in HSV1 infection is illustrated by the susceptibility of patients with mutations in Unc93B to HSE[53]. Unc93B is a protein involved in transporting TLR3 and TLR9 to the endosome. Unc93B knockout fibroblasts show decreased type I IFN production in response to HSV1 infection and increased HSV1 replication[54].

**1.7 interferon inducing intracellular nucleotide sensors of HSV1**

**IFI16**

Innate immune response initiated by IFI16 can have a major impact on replication of herpesviruses. Following detection of HSV1 genomes, IFI16 triggers production of type I IFN through activating STING and IRF3[55]. Detection of HSV1 DNA by IFI16 can occur in the cytosol or the nucleus since IFI16 is capable of shuttling between nuclear and cytosolic compartments[56, 57]. In addition to production of type I IFN, IFI16 can also activate the inflammasome pathway by forming a complex with adapter molecule ASC in response to HSV1 infection [135,136].

Another important role IFI16 plays during HSV1 infection is silencing of HSV1 genomes upon entry into the nucleus. IFI16 is part of an intrinsic host antiviral defense
Figure 1.4: Recognition of HSV1 infection by cytosolic and nuclear PRRs.

MDA5 and Rig-I recognize intermediate RNA products during HSV1 replication and activate production of type I IFN through MAVS. IFI16, cGAS, DDX41, RNA pol III, and DAI recognize HSV1 genomic DNA fragments and activate type I IFN production through STING. In addition to type I IFN production, IFI16 can also form an inflammasome complex with ASC to produce IL-1β and IL-18. Other inflammasomes involved in viral recognition include AIM2 (dsDNA) and NLRP3 (RNA).
mechanism against foreign DNA. When HSV1 genomes enter the nucleus, in absence of HSV1 protein ICP0, detection of HSV1 genome and resulting interferon production by IFI16 leads to silencing of HSV1 genome through association with heterochromatin. This prevents HSV1 from expressing its IE genes, effectively inhibiting replication[16].

RNA Pol III

RNA pol III is an indirect sensor of AT-rich cytosolic dsDNA. RNA pol III transcribes AT-rich DNA into 5’ppp RNA that is then recognized by the RIG-I pathway, leading to activation of the MAVS adapter and production of type I IFN[58]. This indirect mechanism has been shown to sense genomes of HSV1 and EBV.

Protein Kinase RNA-Activated (PKR)

PKR has been shown to detect dsRNA in the cytosol[59]. Although HSV1 is a dsDNA virus, dsRNA products are observed in HSV1-infected cells[60]. PKR phosphorylates the eIF2a initiation factor to inhibit translation of viral RNA[59]. An antiviral autophagy response is also initiated by eIF2a phosphorylation[61].

DAI

DAI (DNA-dependent activator of IFN-regulatory factors) recognizes presence of B and Z form DNA in the cytosol[62]. Upon detection of HSV1 DNA, DAI orchestrates production of type I IFN[63]. DAI-/- mice, however, respond with normal type I IFN production to presence of dsDNA in the cytosol, suggesting that role of DAI may be cell specific or redundant[64, 65].

DDX41
A member of the DEAD (Asp-Glu-Ala-Asp) box protein family, DEAD box polypeptide 41 (DDX41), has been recently shown to induce type I IFN production in response to HSV1 infection in a STING-dependent manner [110, 111]. The ligands recognized by this protein are cyclic di-AMP and cyclic di-GFMP [111].

KU70

Ku70 is a component of the DNA damage repair pathway. It is shown to form a complex with Ku80. The resulting complex is able to induce type I IFN in response to HSV1 infection [104]. Due to the significant role the DNA damage pathway plays in HSV1 replication, Ku70 may be an interesting factor to explore in context of HSV1 pathogenesis.

DDX9 and DDX36

Other members of the DExD/H box RNA helicase family, DHX9 and DHX36, have been shown to produce cytokines in response to HSV1. The PRR recognized by these helicases is CpG DNA. Upon activation by the CpG ligand, DHX36 activates NF-kB signaling pathway, leading to production of inflammatory cytokines such as IL-6 and TNFα. DHX9 activates IRF7, leading to IFNα production [109].

cGAS

Up stimulation of cells with transfected DNA, the second messenger cGAMP activates STING and downstream type I IFN signaling [66, 67]. It was discovered that it is protein cGMP-AMP synthase (cGAS) that synthesizes cGAMP from ATP and GTP after transfection of DNA [68]. Knockdown of cGAS also results in reduced production of type I IFNs following stimulation with DNA and HSV-1[68].
The cGAS deficient mice are more susceptible to HSV1 infection compared to WT mice. This susceptibility correlated with decreased type I IFN production following HSV1 infection. The HSV1 titers were also increased in these mice. BMDMs and BMDCs generated from these mice produce significantly less interferon in response to DNA ligands[69].

1.8 Inflammasomes and HSV1 disease

In addition to type I IFN, cytokines IL-1β and IL-18 have also been shown to play a significant role in HSV1 pathogenesis. Mice lacking these cytokines are more susceptible to HSV1 infections compared to WT mice [70]. This is likely because IL-1β and IL-18 are involved in crucial immune response pathways such as recruitment of neutrophils, activation of T-lymphocytes, and IFNγ production[38,39].

Release of IL-1β and IL-18 is a multistep process mediated by inflammasome complexes (Figure 1.4). First, expression of pro-IL-1β and pro-IL-18 transcripts needs to be initiated. This can be achieved by activation of Toll-like receptor 4 (TLR4) by a gram-negative bacterial cell component known as lipopolysaccharide (LPS), leading to NF-kB signaling[71]. Expression of pro-IL-1β and pro-IL-18 can also be increased by pretreatment with NK-kB activating cytokines such as TNFα[72]. The second step involves activation of the inflammasome complexes by their ligands. Following activation, inflammasome complexes such as AIM2 and NLRP3 associate with apoptosis-associated speck-like protein (ASC) via their PYD domain to form an inflammasome complex[73]. Activation of the inflammasomes results in recruitment and cleavage of pro-caspase-1 by ASC via caspase activation and recruitment domain (CARD) interactions. Cleaved caspase-1 then mediates cleavage of pro-IL-1β and pro-IL-18 to
release mature IL-1β and IL-18[74].

**AIM2**

The AIM2 inflammasome has been characterized as a cytosolic sensor of dsDNA [73, 75]. AIM2 protein possesses two major domains: HIN-200 and PYD. The HIN-200 domain of AIM2 is required for interaction with B-form dsDNA such as poly dA:dT and HSV1 genome. The PYD domain associates with ASC and facilitates cleavage of IL-1β. AIM2 activity has been shown to be essential for control and clearance of herpesvirus infections [73, 76].

**NLRP3**

The NLRP3 inflammasome, surprisingly, has been shown to be important for infection by DNA viruses. Since NLRP3 does not have DNA binding domains, it is thought its activation during infection by DNA viruses is indirect. It is likely that NLRP3 is activated due to formation of dsRNA intermediates during replication of herpesviruses[77]. It has been shown that NLRP3 inflammasome suppresses KSHV reactivation. Also, production of IL-1β is reduced in NLRP3 knockout mice following infection with adenovirus[78]. The NLRP3 inflammasome can also be activated upon stimulation with particulates such as cholesterol crystals, synthetic pariticles, and alum adjuvants[79-81].
1.9. Genome Wide Association study to determine factors that affect HSV1 pathogenesis in humans

Infection of humans by HSV1 results in a complex interplay between the immune response pathways and HSV1 replication and latency. Despite high prevalence of HSV1, its reactivation process in humans is poorly understood. A recent study aimed to identify host genes involved in reactivation of HSV1 in humans using a genome wide association study. They discovered that the genomic region of an uncharacterized open reading frame C21ORF91 contained single nucleotide polymorphisms (SNPs) that correlated with the frequency of cold sores an individual had per year [82, 83]. The open reading frame was named Cold Sore Susceptibility Gene 1 (CSSG1). The SNPs present in the CSSG1 genomic region also correlated with severity of cold sores and serum antibody levels to HSV1 in addition to annual frequency of cold sores. Recent studies also implicate CSSG1 in hepatocellular carcinoma and Down syndrome [84]. Despite association of CSSG1 with diseases that affect a significant proportion of human population, CSSG1 is poorly characterized.

In this dissertation, I explore new pathways involved in HSV1 pathogenesis. My goals are to:

1) Characterize basic properties of CSSG1
2) Determine if CSSG1 plays a role in HSV1 replication
3) Identify a potential mechanism for the role of CSSG1 in HSV1 replication
4) Develop a knockout mouse model for studying the in vivo role of CSSG1 in HSV1 pathogenesis
5) Determine potential role of CSSG1 in innate immune response pathways involved in HSV1 pathogenesis
CHAPTER II

CHARACTERIZATION OF AN OPEN READING FRAME C21orf91: C21orf91 ENCODES THE CELL-ASSOCIATED PROTEIN, COLD SORE SUSCEPTIBILITY GENE 1 (CSSG1)
Contributors

Milan Patel

Michael King

Summary of Contributions

I contributed to the design, execution, analysis and interpretation of the experiments described in this chapter. I generated the samples and performed the experiments and created the graphics shown in Figures 2.1, 2.2, 2.4 and 2.5. I was assisted by Michael King who performed the Western blot analyses shown in Figure 2.3 and 2.4.
2.1: Abstract

SNPs present in an open reading frame C21orf91 (CSSG1) are associated with HSV1 reactivation in humans. Experiments presented in this chapter aimed to characterize expression and interaction properties of this open reading frame to obtain insights into its role in HSV1 pathogenesis. It was determined that CSSG1 mRNA expression is high in tissues associated with HSV1 replication during pathogenesis such as brain and retina. Low expression of CSSG1 mRNA was observed in the trigeminal nerve, which is associated with HSV1 latency. Next, in-house antibodies were developed to show that the CSSG1 mRNA encodes for a protein detectable in human cells. Bioinformatics analysis of the CSSG1 amino acid sequence indicated presence of a coiled-coil domain important for protein-protein interactions. Using confocal microscopy, it was determined that ectopically expressed CSSG1 protein translocates to the nucleus and colocalizes with Tip60, a protein essential for herpesvirus replication. Additionally, this nuclear colocalization was unaffected by the presence of HSV1 reactivation related K115N SNP or the absence of the coiled-coil domain containing region from the CSSG1 protein.
2. 2: Introduction

One particular gene, termed “cold sore susceptibility gene -1” (CSSG1), is linked to the frequency of reactivation of HSV-1 in human subjects based on a study of more than 600 individuals that were phenotyped for HSV-1-induced cold sores [82, 83]. The SNPs present in the chromosome 21 genomic region containing CSSG1 were strongly linked with frequency and severity of HSV-1 reactivation. The presence of SNPs in individuals also correlated with serum antibody levels to HSV-1 in addition to annual frequency of cold sores. The location of the SNPs associated with HSV-1 reactivation within chromosome 21 are indicated in Figure 2.1A and described in detail below.

2.2.1 CSSG1 genomic organization

Although little is known about the function of CSSG1, the region on chromosome 21 surrounding the CSSG1 gene is well characterized. The genomic region of CSSG1 is feature-rich (Figure 2.1A). The genomic locus for CSSG1 is located on the minus strand of the long arm of chromosome 21. The exact coordinates are 17,788,967 – 17,819,386 base pairs from pter (short end) of chromosome 21. The genomic locus has a size of 30.42 kb. The longest transcript of the pre-mRNA consists of five exons and four introns. Sizes of exons in the numerical order are as follows: 84 bp (exon 1), 133 bp (exon 2), 536 bp (exon 3), 62 bp (exon 4), and 4614 bp (exon 5). Exon 1 of CSSG1 encodes the 5’ UTR. Exon 1 of CSSG1 encodes the 5’ UTR. Exon 3 is the longest, accounting for approximately 60% of predicted protein. This exon also contains all of the SNPs associated with HSV1. HSV-1 reactivation located in the protein-coding region of CSSG1. Exon 5 contains the 3’ UTR region. The sizes of the introns in the numerical
order are 977 bp (intron 1), 21,073 bp (intron 2), 1311 bp (intron 3), and 1626 bp (intron 4).

CSSG1 pre-mRNA is processed into an mRNA. Expression of CSSG1 mRNA was first identified in the undifferentiated retina of developing chick embryos[85]. In humans, the pre-mRNA of CSSG1 can be processed into three different mRNAs (Figure 2.1B). The full length mRNA (referred to as mRNA297 henceforth) is predicted to encode a protein containing 297 amino acids. The second mRNA, mRNA296, lacks three nucleotides (CTG) at the junction of exons 4 and 5 and is predicted to encode a protein of 296 amino acids. The third major mRNA, mRNA221, lacks the protein coding regions of exon 4 and 5. As a result, it is predicted to encode a protein only 221 amino acids long. All mRNAs described share common 5’ and 3’ UTR regions despite the various predicted differences at protein coding level.

In addition to a CSSG1 pre-mRNA encoding region, the C21Orf91 genomic region also consists of multiple noncoding overlapping RNA transcripts. This transcript, referred to as CSSG1-OT1, overlaps the 3’ UTR of CSSG1 mRNA and is transcribed in the sense direction (Figure 2.1A). This overlapping transcript contains the SNP rs243588, which is associated with HSV-1 reactivation in humans. There are also two additional overlapping transcripts transcribed in the anti-sense direction (Figure 2.1A). These include XR_937601.1 (8.88 kbp) and AL109761.5 (17.8 kbp). The XR_937601.1 transcript is antisense to intron 2 of CSSG1 pre-mRNA. The transcript AL109761.5 is anti-sense to majority of CSSG1 pre-mRNA (Figure 2.1A) and also contains a SNP (rs2824493) associated with HSV-1 reactivation in humans. The physiological properties and function of the overlapping transcripts are unknown.
The predicted CSSG1 protein lacks nuclear or membrane localization sequences however a study of Chromosome 21 Orfs indicates that CSSG1 could potentially bind to a nuclear protein, Tip60. CSSG1 polypeptide was shown to interact with Tip60 in a yeast-2-hybrid study [86]. Tip60 is essential for replication of herpesviruses. Knockdown of Tip60 protein expression reduces replication of EBV and HCMV while increasing their reactivation [87, 88]. Characterization of interaction between Tip60 and CSSG1 in mammalian cells was undertaken to determine the potential role of CSSG1 in HSV1 replication.

2.2.2 Goals

To determine the role of CSSG1 in HSV1 pathogenesis, it was important to characterize its basic properties. I carried out bioinformatics studies to determine the basic properties of the open reading frame C21orf91 (CSSG1). First, I conducted an analysis using various publically available RNA-seq databases (Biogps.org, proteinatlas.org) to determine expression levels of CSSG1 mRNA in various human tissues. Next, in-house antibodies that were developed against predicted CSSG1 protein were used in Western blot studies (performed by Michael King) to determine whether CSSG1 open reading frame encodes for a protein detectable in human cells and tissues. Using various bioinformatics sources, I analyzed the CSSG1 amino acid sequence to look for presence of functional domains. Lastly, I examined potential interactions of CSSG1 with Tip60, a protein essential for efficient herpesvirus replication, using confocal approaches. Overall, the experiments that I conducted in this chapter help establish basic properties of the CSSG1 protein and guide future experiments in determining its role in HSV1 infection.
Figure 2.1: Overview of the CSSG1 genomic region.

A) The genomic region of CSSG1 encodes for an mRNA predicted to encode a protein of 297 amino acids. The protein-coding region of the CSSG1 is indicated in aqua. The untranslated regions (UTR) of mRNA are indicated in yellow color. The presence of multiple overlapping transcripts is indicated by red lines. C21ORF91-OT1 is characterized as a lincRNA. Transcript XR_937601.1 is characterized as ncRNA. The relative locations of SNPs associated with HSV-1 reactivation are indicated by arrows. SNPs are present in the overlapping transcripts as well as CSSG1 mRNA.

B) Diagram illustrating three different variants of CSSG1 mRNA expressed. Full-length mRNA297 is predicted to encode a protein of 297 amino acids, while mRNA296 and mRNA221 are predicted to encode proteins of sizes 296 and 221 amino acids, respectively. Sequence data are obtained from NC_000021.9 chromosome 21 Reference GRCh38.p2 primary assembly.
2.3: Results

2.3.1: CSSG1 mRNA is expressed ubiquitously

To determine where CSSG1 mRNA is expressed in mouse tissues and whether the expression varies depending on tissue type, I isolated total mRNA from various tissues (brain, bone marrow, liver, lung, muscle, spleen, testis, thymus, and trigeminal nerve) from wild type C57BL/6 mice. Upon quantification of CSSG1 mRNA, I determined that CSSG1 is found ubiquitously in mouse tissues. Expression of CSSG1 mRNA in testis was found to be relatively high compared to other tissues. To analyze expression of CSSG1 mRNA in human tissues, I compiled RNA-seq data available from biogps.org and thehumanproteinatlas.org. I discovered that CSSG1 mRNA was also present ubiquitously in all human tissues examined (brain, retina, trigeminal ganglion, thymus, bone marrow, liver, lung, testis, ovaries, smooth and skeletal muscle) (Figure 2.2B, C), similar to results I obtained from my studies of mouse tissues.

2.3.2: CSSG1 mRNA encodes for a protein detectable in human cell lines

At the initiation of this study, it was unknown whether the predicted protein from CSSG1 mRNA was expressed endogenously in human and mouse cells. To determine whether the predicted CSSG1 protein is expressed, Michael King developed a number of monoclonal antibodies. He used a CSSG1 cDNA containing a Flag tag to ectopically express the putative protein in E. coli cells. The produced protein product was purified and used to develop anti-CSSG1 monoclonal antibodies.

Several mAb were developed that bound to human CSSG1. Two mAb cross-reacted with mouse CSSG1. Clone 1F3 bound human and mouse CSSG1 and
Figure 2.2: CSSG1 mRNA is ubiquitously expressed.

A) Total mRNA was extracted from indicated tissues isolated from wild type C57BL/6 mice. Relative expression of CSSG1 mRNA to 18S RNA was quantified using qPCR (Graphs represent mean + SEM values obtained from three mice independently measured in duplicates). Levels of CSSG1 mRNA measured as fragments per kb of total transcript per million mapped reads (FpKM) units in indicated human tissues were compiled from RNA-seq data available on B) Biogps.org and C) proteinatlas.org (Graphs represent mean + SEM values in the database collections as of 06/14/2016, minimum three samples per tissue).
recognized both the full length (297 aa) and truncated (221 aa) CSSG1 proteins. Clone 7A12 also bound both human and mouse CSSG1, but preferentially reacted with full length (297 aa) protein and only weakly bound the truncated CSSG1 (221 aa) proteins. Clones 9F3 and 3C1 both reacted with human but not mouse CSSG1.

Anti-CSSG1 mAbs were used to pull down potential endogenous CSSG1 protein from the human cell line HEK 293 (human embryonic kidney epithelial like cells). This cell line was chosen for this experiment because it was previously discovered to have detectable levels of CSSG1 mRNA, increasing the likelihood that CSSG1 protein is expressed in this cell line. The protein bound to the anti-CSSG1 antibody was run on SDS-PAGE gel and immunoblotted using anti-CSSG1 antibody. A protein band appeared close to 33 kDa (Figure 2.3A) molecular marker, the predicted size of the CSSG1 protein. Isolation of the protein band and mass spectrometry analysis confirmed the pulled down protein to be CSSG1.

2.3.3: Known and predicted properties of the CSSG1 protein

In order to determine the potential function of the CSSG1 protein and its relevance to HSV1 infection, I analyzed the amino acid sequence of CSSG1 protein by multiple bioinformatics services. The properties important for determining CSSG1 function included potential presence of functional protein domains and regulatory sites for phosphorylation and ubiquitination. The COILS server (embnet.vital-it.ch/software/COILS_form.html) reported detection of a potential coiled-coil domain located at the c-terminus of CSSG1 (Figure 2.3B). The coiled-coil domain has been shown to be involved in protein-protein interactions [89]. The CSSG1 protein product derived from the mRNA221 variant lacks the predicted coiled-coil domain. Multiple
Figure 2.3: Detection of endogenous protein encoded by a previously unknown open reading frame CSSG1*.

A) Protein product of a novel gene CSSG1 was detected by immunoblotting using in-house-developed mAbs 9F3 and 3C1. These mAbs are specific to human CSSG1 (hCSSG1) but do not bind mouse CSSG1 (mCSSG1) recombinant proteins. Endogenous CSSG1 was immunoprecipitated and visualized by western blot analysis of lysates from human HEK293 cells or mouse bone marrow derived macrophages (Mϕ). Lysis buffer with 1% BSA was included as a negative control. The predicted size of hCSSG1 is 33 kDa. Findings from one experiment representative of three independent experiments are shown.

B) Known and predicted properties of CSSG1 protein. Presence of coiled-coil domain was predicted by the COILS server database (http://www.ch.embnet.org/software/COILS_form.html, probability score of 0.96 out of 1). Known phosphorylation sites were annotated by sysPTM database (http://lifecenter.sgst.cn/SysPTM/). The 296 AA variant lacks glutamate at the junction between exon 4 and 5. The 221 AA variant lacks exon 4 and 5 along with the coiled-coil domain.
phosphorylation sites were detected by database compiled using known mass spectrometry analysis (http://lifecenter.sgst.cn/SysPTM) suggesting potential regulation of by phosphorylation and/or dephosphorylation of CSSG1 during HSV1 infection.

2.3.4: Majority of the endogenous CSSG1 protein localizes to the nucleus

HSV1 replication proceeds in distinct stages that occur in specific subcellular compartments as described previously (Figure 1.2). Determination of subcellular CSSG1 localization can provide insight into the stage of HSV1 replication that is affected by CSSG1. To determine where CSSG1 is localized in the cell and whether its localization properties change in presence of viral stimuli, U2OS (human bone osteosarcoma epithelial cell line) cells were stimulated with HSV-1 KOS strain at multiplicity of infection (MOI) of 10, poly dA:dT (1 ug/mL), or Sendai virus (80 hemagglutinin (HA) units/mL). The U2OS cells were collected following stimulation and subcellular fractions of the cells were immunoblotted with the in-house developed anti-CSSG1 mAb (Figure 2.4). CSSG1 was detected in both cytosolic and nuclear fractions. The amount of CSSG1 detected in the nuclear fraction was higher than that in the cytosol under all conditions. Upon stimulation with virus or dA:dT, the amount of CSSG1 in the cytosol increased, suggesting that the localization of CSSG1 is dynamic.

Both nuclear and cytosolic fractions contained an immunoreactive protein of ~33 kDa, consistent with the predicted size of CSSG1. The nuclear fractions contained an additional band of ~70 kDa. It is not clear if this larger band represents CSSG1 dimers or a cross-reactive nuclear protein.

The purity of cytosolic and nuclear fractions was assessed by immunoblotting for lamin A (nuclear control) and β-actin (cytosolic control). As shown in Figure 2.4, β-actin
Figure 2.4: In the U2OS cells, the majority of the endogenous CSSG1 protein is expressed in the nucleus.

Cytosolic and nuclear protein fractions were isolated from U2OS cells treated with media, poly dA:dT (1 ug/mL), HSV-1(MOI 10), and Sendai virus (80 HA units/mL) for 6 hours using a subcellular fractionation kit (Thermo Scientific). The subcellular fractions were run on an SDS-PAGE gel and immunoblotted for presence of CSSG1 using in-house developed anti-CSSG1 mAb. Presence of Lamin A and β-actin proteins were analyzed in each fraction to assess purity of cytosolic and nuclear fractions (representative data from one experiment shown, three independent experiments performed for media stimulation, single experiment for poly dA:dT, HSV1, and Sendai virus stimulations).
was detected in the cytosol while very little β-actin was detectable in the nuclear fraction. The nuclear fraction contained strong presence of lamin A while it was undetectable in cytosolic fraction. The presence of lamin A and β-actin in their respective subcellular fractions suggests high purity of cytosolic and nuclear fractions.

2.3.5: CSSG1 co-localizes with Tip60 to the nucleus, a protein essential for herpesvirus replication

Yeast two-hybrid studies suggest a potential interaction of CSSG1 with Tip60, a nuclear chromatin modifying protein[86]. To determine whether CSSG1 interacts with Tip60 in mammalian cells, HEK293T (human embryonic kidney epithelial cells expressing SV40 large T antigen) cells were transfected with CSSG1 tagged with mCherry or Tip60 tagged with GFP. This cell line was chosen for this experiment because the presence of SV40 T antigen allows for high expression of transfected cDNA containing SV40 enhancer sequences [90]. Expression of Tip60 alone led to nuclear localization of the protein (Figure 2.5A). When CSSG1 was expressed ectopically, it localized to the cytosol as reported previously (Figure 2.5A)[83]. However, when co-expressed with Tip60 protein, CSSG1 protein translocated to the nucleus and co-localized with Tip60. Co-localization of CSSG1 with Tip60 raised another interesting possibility, i.e., CSSG1 SNPs associated with HSV-1 reactivation may affect co-localization of Tip60 and CSSG1. I tested this possibility by co-expressing a CSSG1 protein variant containing a high HSV1 reactivation-associated SNP (K115) with Tip60 and visualizing their subcellular localization properties using confocal imaging. Upon co-expressing CSSG1 protein containing the high HSV1 reactivation associated K115N SNP with Tip60, translocation of CSSG1 to the nucleus was similar to that observed with
Figure 2.5: Ectopically expressed CSSG1 translocates to the nucleus with Tip60.

A) HEK 293T cells were transfected with plasmids expressing mCherry tagged CSSG1 and GFP tagged Tip60. The cells were fixed with 4% paraformaldehyde (PFA) following 24 hours and imaged with Leica SP2 confocal microscope (three independent experiments performed, representative images from one experiment are shown as an example). The arrows indicate cytosolic localization of CSSG1 in absence of Tip60 and nuclear localization in presence of Tip60. B) HEK 293T cells were transfected with CSSG1-mCherry protein containing the HSV-1 reactivation associated K115 SNP along with Tip60-GFP. The cells were processed and imaged as in A) (three independent experiments performed, representative images from one experiment are shown as an example). C) HEK 293T cells were co-transfected with plasmids expressing V5 tagged full length CSSG1 protein (297 AA) and the 221 AA protein variant lacking the predicted coiled-coil domain along with GFP-tagged Tip60 (Representative images from one experiment). The cells were fixed with 4% PFA following 24 hours and permeabilized with methanol. The cells were then stained with mouse anti-V5 (Abcam) and anti-mouse Alexa 555 (Invitrogen) antibodies and imaged using Leica SP2 confocal microscope.
the low HSV1 reactivation SNP (N115) (Figure 2.5). In addition to HSV-1 reactivation associated SNPs, CSSG1 protein also encodes for a potential coiled-coil domain involved in protein-protein interactions as discussed previously (Figure 2.2D). To determine whether the predicted coiled-coil domain of CSSG1 was needed for co-localization with Tip60, 221AA variant of CSSG1-V5 protein was co-expressed with Tip60-GFP (Figure 2.5C). Despite the lack of the predicted coiled-coil domain, 221AA CSSG1 protein also co-localized with Tip60 and translocated to the nucleus. This chapter provides information on expression and interactions of the CSSG1 protein that will be essential for studying its role in HSV1 pathogenesis. The discoveries are summarized below.

2.4: Discussion

Relative expression of CSSG1 mRNA was quantified in brain, bone marrow, liver, muscle, lung, trigeminal ganglion, thymus, testis, and spleen tissues of mice using qPCR. I found that CSSG1 is expressed ubiquitously in mouse tissues. The results are consistent with expression results available from aggregated microarray databases (Figure 2.2 B, C). High expression in retina tissue is also consistent with a report of CSSG1 mRNA expression in developing chick embryos [85]. It is important to note that relatively high expression of CSSG1 mRNA was observed in tissues where HSV1 replication occurs during pathogenesis such as retina and brain. However, the CSSG1 mRNA levels were very low but detectable in the trigeminal nerve, where HSV1 is reported to remain latent [91-93]. This observation raises the possibility of an inverse relationship between HSV1 latent infection and CSSG1 expression and a direct relationship between CSSG1 expression and HSV1 lytic infection. An in vitro model of
HSV1 latency in trigeminal nerve with ectopically expressed CSSG1 mRNA could be useful to test the possibility of CSSG1 mRNA levels affecting HSV1 reactivation [91].

At the initiation of this study, it was unknown if the CSSG1 mRNA encoded a protein. Through development of in-house developed antibodies CSSG1, my colleague Michael King and I have shown that the antibodies recognize and pull down a protein that is confirmed to be CSSG1 using mass spectrometry. Various commercial polyclonal antibodies are available, however, we have developed and tested the first monoclonal antibodies to the CSSG1 protein.

Using subcellular fractionation approach, it was also determined that endogenous CSSG1 is expressed in the cytosol and the nucleus, with majority of the protein present in the nuclear fraction. This is in contrast with results obtained from ectopic expression of CSSG1 protein indicating cytosolic localization in the absence of co-expression of Tip60[83]. Confocal imaging experiments indicate that CSSG1 colocalizes with Tip60, a protein that is activated by herpesvirus kinases following infection, in the nucleus [87, 88]. Colocalization of CSSG1 with Tip60 in the nucleus may suggest a direct role of CSSG1 in HSV1 replication, which occurs in the nucleus, and DNA damage response which is thought to regulate lytic vs. latent HSV1 infection [94]. My results also indicate that the region of CSSG1 protein containing coiled-coil domain is not required for nuclear translocation and colocalization with Tip60. A caveat of this experiment is that both CSSG1 and Tip60 are expressed with C-terminal protein tags that may interfere with the results. Since antibodies are available for detecting both proteins, it is important to determine the characteristic of interaction between endogenous, untagged CSSG1 and Tip60 proteins using confocal microscopy and
immunoprecipitation during HSV1 infection. Overall, the results obtained in this chapter have allowed me to determine basic properties of the CSSG1 protein using newly developed reagents that will guide future research into studying the role of CSSG1 in HSV1 pathogenesis. The implications of these findings for herpesvirus pathogenesis are further discussed in Chapter 4.

2.5: Materials and Methods

2.5.1: Detection of CSSG1 mRNA

To detect CSSG1 mRNA, total RNA was extracted from 1 mg of homogenized mouse tissues using TRIzol reagent (Thermo Fisher Scientific) according to manufacturer’s instructions. CSSG1 expression was quantified by quantitative PCR using primers Forward: GAAACACTCTCCTTCTGCCA and Reverse: CTTAGATCGAGGACAACCCT. Expression of 18s mRNA was quantified using a primer set available from Qiagen.

2.5.2: Development of an anti-CSSG1 mAb

A cDNA encoding the putative CSSG1 protein was Topo-cloned into a bacterial expression vector in-frame with a 6X-HIS tag (Thermo Fisher). E.coli cells were transformed with the vector and allowed to grow. The cells were lysed using sonication. A Nickel Column was attached to a MasterFlex L/S pump (Masterflex). Lysate containing CSSG1-6X His tag was run through the column twice, each time followed by binding buffer (500mM NaCl, 20mM Na₂HPO₄, 40mM Imidazole, pH 7.4). The bound protein was eluted by using elution buffer (500mM NaCl, 20mM Na₂HPO₄, 500mM Imidazole, pH 7.4). The purified protein was sent to University of Virginia antibody core for creating
anti-CSSG1 antibodies. The antibodies received were testing using by immunoprecipitating CSSG1 protein and analyzing its purity using mass spectroscopy by University of Massachusetts Medical School Protein core.

2.5.3: Immunoprecipitation and detection of CSSG1 protein

HEK293T cells were lysed in 1% Triton-X TBS with protease inhibitors. The lysate was then incubated with 20uL of protein G coated beads with 5ug of anti-CSSG1 (clone 9F3) antibody for 1h at 4°C and washed three times with PBS. The beads were added to lysates (10mg of protein lysate from Macrophages and HEK293T cells (or 1% BSA control) and incubated overnight at 4°C. Following washing beads with PBS five times, the beads were boiled in 50uL Laemmli buffer for 15mins. 40uL of the recovered immunoprecipitated proteins were run on a 4-15% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked with 1% BSA and immunoblotted with biotinylated anti-CSSG1 mAb, clone 9F3 (1ug/mL). CSSG1 protein was visualized with streptavidin HRP (0.2 ug/mL) reagent.

2.5.4: Cell culture and subcellular fractionation

U2OS cells, a HSV1 permissive human osteosarcoma cell line, were obtained from the Knipe Lab (Harvard Medical School, Boston, MA). HEK293 and HEK293T cells were obtained from ATCC. The cells were maintained in DMEM media with 10% FBS in 10% CO₂ at 37°C. Mouse macrophage cells were derived from the bone marrow of C57BL/6J mice according to a standard protocol [95]. U2OS cells were stimulated with HSV1 KOS strain (MOI 10) or Sendai virus (Cantell strain, 80 HA units/mL) or medium control for 6 hrs where indicated. To obtain cytosol and nuclear fractions, 5x10⁶ cells were collected by scraping and processed using subcellular fractionation kit available
from Thermo Scientific according to the manufacturer's protocol. Following separation of subcellular fractions, 25 ul of cytosolic and nuclear fractions were each mixed with 25 ul of 2x Laemmli sample buffer and run on SDS-PAGE. PAGE-separated cell fractions were transferred to PVDF membranes and were immunoblotted for presence of CSSG1 as described above. Antibodies specific to nuclear Lamin A (Abcam) and cytosolic β-actin (Abcam) were used at 1:5000 followed by anti-mouse HRP (1:10000, Abcam) as fractionation and loading controls.

2.5.5: Confocal experiments

HEK293T cells were plated at density of 250,000 cells/dish in a 30 mm confocal dish. The cells were transfected with 0.5 ug of plasmids encoding Flag-epitope tagged or V5-epitope tagged cDNAs for expressing indicated proteins. cDNA plasmids were transfected using GeneJuice according to manufacturer’s instructions. At 24 h post-transfection, the cells were incubated in 4 ug/mL cholera toxin B – Alexa 488 (CTxB, 4 ug/mL, Invitrogen) for 30 minutes at 37 °C where indicated to label the cell membrane. All cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. The cells were then permeabilized with 100% methanol for 5 minutes at -20 °C. The cells were then blocked with 3% BSA at room temperature for 1 hour. The anti-Flag and anti-V5 antibodies were added to the cells at 5 ug/mL in 1% BSA overnight at 4 °C. The cells were then washed with PBS and overlaid with anti-rabbit-Alexa 488 (Cell Signaling) for anti-Flag staining or anti-mouse-Alexa 488 (Invitrogen) for anti-V5 staining at 4 °C for 1 hour at concentration of 5 ug/mL with 1% BSA. The cells were washed, stained with 5 ug/mL Hoechst 34580 (Invitrogen) to visualize the nuclei, and the cells were then imaged with a Leica SP2 confocal microscope.
CHAPTER III

ROLE OF CSSG1 IN HSV1 REPLICATION
Contributors

Milan Patel

Michael King

Summary of contributions

I contributed to the design, execution, analysis and interpretation of the experiments described in this chapter. I generated the samples and performed the experiments and created the graphics shown in Figures 3.1, 3.2, 3.4, 3.5 and 3.6. I was assisted by Michael King who performed the Western blot analyses shown in Figure 3.3.
3.1: Abstract

In humans, occurrence of cold sores is a multi-stage event involving reactivation of HSV1 in the CNS to its replication in the epithelial cells. Association of CSSG1 SNPs to annual cold sore frequency and evidence for interaction of CSSG1 with Tip60 raise the possibility that CSSG1 affects cold sores frequency by affecting HSV1 replication. To investigate this possibility, CSSG1 expression was knocked down by making stable shRNA U2OS cell lines. The characteristics of HSV1 replication were analyzed in these CSSG1 knockdown (CSSG1 KD) cell lines. I show that replication of HSV1 is decreased in CSSG1 KD compared to control cells while the replication of VSV remains unaffected. It was also determined that the expression of HSV1 proteins at the IE stage (ICP4, ICP27) is reduced in CSSG1 KD cells. The DNA damage response as measured by H2AX phosphorylation in CSSG1 KD cells is also increased compared to control cells indicating defects in the DNA repair pathway and providing a potential explanation for the observed decrease in HSV1 replication.
3.2: Introduction

Reactivation of HSV1 is a complex process that involves a wide range of processes ranging from the DNA damage response to systematic immune response[96]. Multiple host proteins have been shown to play a role in these processes. For example, the protein Tip60, may affect both reactivation and replication of HSV1[88]. Given the strong evidence for interactions between CSSG1 and Tip60, I hypothesize that Tip60 and CSSG1 together directly impact HSV1 replication.

3.2.1. Determining the Role of CSSG1 in HSV-1 replication

To test whether CSSG1 affects HSV1 replication, I aimed to reduce expression of CSSG1 in human cells and then quantify HSV1 replication in these cells by measuring HSV1 protein expression and productive virion release. Choosing a human cell line that expresses CSSG1 protein for the study would allow a more direct comparison of the role of CSSG1 in HSV1 pathogenesis in humans. Furthermore, since CSSG1 was a largely uncharacterized gene, it was imperative to use an established model cell line so that the results from the study can be placed in the context of existing scientific understanding. U2OS cells (human bone osteosarcoma epithelial cells) were chosen for this purpose. Replication of multiple HSV1 strains has been characterized in this cell line [97-100]. One of these strains is the KOS strain, which is a lytic, replication-competent strain [101]. Infection of epithelial cells with this strain results in a productive infection leading to release of competent virions into the cell supernatant. I thus elected to use HSV1 KOS in my studies to determine whether CSSG1 impacts productive replication of HSV1. Another frequently used strain, HSV-1 ICP8-GFP, expresses an HSV1 E protein ICP8 that is linked to the green fluorescent protein (GFP) [102]. ICP8 protein is essential
for replication of HSV1 genome. However, due to reduced expression of ICP8 protein, the virus replicates with slower kinetics. As a result, this virus can be particularly helpful in observing early effects of CSSG1 knockdown on HSV1 replication. In addition, use of this strain provides for the opportunity to localize ICP8-GFP protein in infected cells by imaging. Finally, the HSV1 VP16-GFP strain is a replication-competent virus that expresses the late VP16 protein linked to GFP [103], allowing for simple measurement of HSV1 replication through flow cytometry or imaging modalities. The virus can also be used to examine later stages of HSV1 replication by analyzing subcellular localization of VP16-GFP.

3.2.2. Stages of HSV1 gene expression

As discussed in Chapter 1, expression of HSV1 genes proceeds in distinct stages known as IE, E, and L. By analyzing expression of HSV1 genes in the relative absence of CSSG1, I can determine the stage at which CSSG1 affects HSV1 replication. Furthermore, each replication stage is characterized by involvement of specific cellular host proteins involved in various functions such as initiation of transcription (IE), DNA replication (E), nucleotide metabolism (E), shuttling of RNA (IE) and proteins (E, L) through the nuclear membrane. Thus, involvement of CSSG1 at a specific replication stage can also provide insights into its function.

At the IE stage of replication, HSV1 genes ICP4 and ICP27 are expressed. ICP4 functions as the major transcriptional activator of HSV1 early genes. This protein has a size of 350 kDa and is required for progression of HSV1 replication beyond the IE stage. In absence of ICP4, early genes that are required for HSV1 genome replication such as ICP8 are not expressed and HSV1 replication stalls [104-106]. Expression of ICP27 is
also required for HSV1 replication. It acts as transcriptional regulator of expression of HSV1 genes and is also responsible for shutoff of host protein synthesis [107, 108]. ICP27 has been shown to directly interact with the nuclear pore complex to inhibit shuttling of host mRNAs while enabling nuclear export of viral mRNAs to the cytosol for translation.

The E stage of HSV1 replication is marked by replication of the HSV-1 genome. ICP8, an early protein product during HSV1 replication of 128 kDa, binds to single-stranded DNA (ssDNA). This protein is required for replication of HSV1 genomes by destabilizing dsDNA during initiation of replication [109]. The protein can be found associated with HSV1 genomes during early phase of infection.

During the L stage of HSV1 replication, proteins that make up the capsid and tegument are expressed. ICP5 is a major capsid protein of size 155 kDa expressed during the late stage of HSV1 replication. This protein is essential for the formation and assembly of sealed viral capsids required for egress of HSV1 DNA from the nucleus. ICP5 can organize itself into pentavalent and hexavalent capsomeres. Each formation facilitates binding and release of other structural proteins UL18, UL38, UL26.5, and UL26 during capsid maturation. HSV1 late protein VP16 (56 kDa) is important for initiation of transcription of HSV1 genes. VP16 is a major part of the tegument portion of HSV1 virion that surrounds the viral capsid [110].

3.2.3. The DNA damage response and HSV1 replication

Multiple factors have been implicated in playing a role in HSV1 replication. The DNA damage and repair mechanism affects both HSV1 replication and reactivation. Tip60, a protein that interacts with CSSG1 as discussed previously (Chapter 2.3.5), is
required for efficient replication of herpesviruses. Conserved protein kinases from HSV1, human cytomegalovirus (HCMV), Kaposi’s sarcoma-associated herpesvirus (KSHV), and Epstein-Barr virus (EBV) have been shown to interact with Tip60 leading to its phosphorylation and activation of downstream DDR [88]. Tip60 plays an important role in initiation of the DNA damage response by acetylating the ATM protein kinase leading to phosphorylation of the histone H2AX[111]. The DNA damage response induced during HSV1 infection can be quantified by measuring phosphorylation of H2AX [112]. Following phosphorylation of H2AX, proteins involved in DNA repair are recruited to the site of damage resulting in resolution of DNA damage and dephosphorylation of H2AX. U2OS cells have been used to study the mechanism for initiation of the DNA damage response following etoposide stimulation. Role of Tip60 in H2AX phosphorylation and acetylation has been extensively characterized in U2OS cells[113-115]. Thus, CSSG1 knockdown U2OS cells would be an ideal model for studying role of CSSG1 in HSV1 replication and its potential relationship to the DNA damage response.

Using the tools available above, I studied the effect of CSSG1 on HSV1 replication. I knocked down CSSG1 expression using stable shRNA U2OS cell lines and analyzing replication of HSV1 in these CSSG1 knockdown (CSSG1 KD) cell lines. Expression of HSV1 proteins expressed at the IE, E, and L stages were also measured to determine the stage at which CSSG1 affects HSV1 replication. Defects in resolution of DNA damage in CSSG1 KD U2OS cells was quantified by measuring phosphorylation of H2AX in response to stimulation with etoposide and HSV1.
3.3: Results

3.3.1. Establishment of a system to study role of CSSG1 in HSV1 replication

To examine effect of CSSG1 on HSV1 replication, a method was needed to abrogate expression of CSSG1 in U2OS cells. I thus generated CSSG1 knockdown (KD) cells. U2OS cells were stably transduced using lentivirus with shRNAs targeting the 3’ UTR region (3’ UTR KD) or the protein-coding region of CSSG1 (coding DNA sequence, or CDS KD) (Figure 3.1A). The shRNA targeting the 3’ UTR of CSSG1 gene is expected to target all known variants of CSSG1 while the shRNA targeting the coding region may only target the full length variants. Compared to the control U2OS cells expressing NT shRNA, cells expressing shRNAs targeting 3’ UTR or coding region of CSSG1 had decreased levels of CSSG1 mRNA (Figure 3.1B) and protein expression (Figure 3.1C). No adverse effects on cell growth or viability were observed in the CSSG1 KD cell lines.
Figure 3.1: Stable knockdown of CSSG1 protein expression in U2OS cells.

(A) Diagram illustrating relative target locations of shRNAs used for lentivirus mediated knockdown of CSSG1 expression. The protein-coding region of CSSG1 is targeted by shRNA CDS KD. The 3’ UTR of CSSG1 is targeted by shRNA 3’ UTR KD. U2OS cells were selected following lentivirus mediated transduction with CDS-KD, 3’ UTR-KD, and non-targeting control (NT) shRNAs and analyzed for (B) CSSG1 mRNA expression relative to the HPRT gene by qPCR (Graph shows mean + SEM of one experiment measured in duplicates) and (C) CSSG1 protein expression by immunoblotting ((three independent experiments performed, result from one experiment is shown as an example).).
3.3.2. Replication of HSV1 (ICP8-GFP) virus is reduced in CSSG1 KD cell lines

To determine whether CSSG1 affects HSV1 replication, CSSG1 KD cell lines were infected with HSV1 ICP8-GFP virus at MOI 10 or 30 for 24 hours. Upon analysis of GFP expression by flow cytometry, I discovered that expression of the E protein ICP8 is reduced in CSSG1 3’ UTR KD and CDS KD cells compared to NT control cells (Figure 3.2A). The reduction in HSV1 replication is drastic in 3’ UTR KD cells while intermediate in CDS KD cells. To determine whether the effect of CSSG1 is specific to HSV1 and independent of potential CSSG1 KD effects on cell viability, replication of the unrelated virus, vesicular stomatitis virus (VSV), was also examined in CSSG1 KD and control cells. No reduction in replication of VSV-GFP was observed in CDS KD or 3’ UTR KD cells lines compared to NT cells (Figure 3.2B) following infection at MOI 3 or 10 for 24 hours.
Figure 3.2: Expression of HSV-1 late protein ICP8 is reduced in CSSG1 KD U2OS cells.

Representative images from flow cytometry analysis of GFP expression of cells infected with HSV1 ICP8-GFP (A) or VSV-GFP (B) for 24 hours at the indicated MOI (three independent experiments performed, Result from one experiment is shown as an example). Note the reduced expression of ICP8-GFP protein in CSSG1 CDS KD and 3' UTR KD compared to NT control.
3.3.3. Expression of ICP4, ICP27, ICP8, and ICP5 proteins is reduced in HSV1 infected CSSG1 KD cells

Expression of HSV-1 ICP8 protein is regulated by the IE protein ICP4 [106]. Since a decrease in ICP8 expression was observed in CSSG1 KD cells, expression of HSV1 proteins expressed at the IE stage was determined in HSV1-KOS infected CSSG1 KD cells. Analysis of expression of ICP4 and ICP27 proteins, which are expressed during the IE stage of HSV1 replication, indicated a significant decrease in 3' UTR KD and CDS KD cell lines compared to NT control cell line (Figure 3.3). Similar decreases were observed for ICP8 and ICP5 expression, which are expressed at the E and L stage of HSV1 replication (Figure 3.3). These findings further validate the results obtained for immediate early protein.

Differences were also observed in expression of HSV1 proteins in 3' UTR compared to CDS KD cell lines. Expression of ICP4 and ICP27 in CDS KD cells elevates to expression levels observed in NT control cells following 12 hours while remaining low in 3' UTR KD. Overall, CSSG1 clearly impacts HSV1 replication before the initiation of transcription of the IE genes ICP4 and ICP27.
Figure 3.3: Expression of HSV1 genes is reduced at the IE stage of replication in CSSG1 KD cells

Expression of HSV1 immediate-early (IE) (ICP4, ICP27), early (ICP8), and late protein (ICP5) in CSSG1 KD U2OS cells infected with HSV1 KOS strain for indicated time period at MOI = 1 and analyzed by immunoblotting (three independent experiments performed, Result from one experiment is shown as an example). Note that ICP4 24hr and 48hrs were exposed for a shorter period). Notice the significant reduction in expression of ICP4 and ICP27 proteins in both 3’ UTR KD and CDS KD cells compared to NT control cells.
3.3.4. Productive replication of HSV1 is reduced in CSSG1 KD cells

To determine whether the observed defect in HSV1 protein expression in CSSG1 KD cell lines results in reduced production and release of HSV1 virions, measurement of HSV1 virions released into the supernatant was carried out by plaque assay and genome copy number following infection of CSSG1 KD cells with replication competent HSV1-KOS strain at MOI 1 and 10. Significant decrease in infectious virions released into the supernatant was observed in HSV1 infected 3’ UTR KD cells compared to NT cells (Figure 3.4A, B) after 24 and 48 hours post infection. This observation was validated by the decrease in release of total HSV1 particles from 3’ UTR KD cells at 24 and 48 hours post infection (Figure 3.4C,D). No significant difference in infectious HSV1 virions or total particles released from CDS KD cells compared to control NT cells was observed.
Figure 3.4: CSSG1 is required for efficient HSV-1 replication.

Quantification of HSV1 virions released into the supernatant from HSV1-KOS infected NT (NT) and CSSG1 KD U2OS cells for 24 hours at indicated MOIs by plaque assay (A, B) and by qPCR of genome copy number (C, D), (Graphs show mean + SEM of three independent experiments measured in duplicates) Significance values are calculated using Student’s t-test using NT versus CDS KD and NT versus 3’ UTR KD values. **: p<0.01, *: P<0.05.
3.3.5. Accumulation of DNA damage as measured by H2AX phosphorylation is increased in CSSG1 KD cells following etoposide stimulation

My data presented in Chapter 2, suggested that CSSG1 may interact with Tip60, a protein essential for herpesvirus replication [88]. One of the proposed mechanisms for involvement of Tip60 in herpesvirus replication is its role in DNA damage and repair response [87, 88]. To determine whether CSSG1 also plays a role in the DNA damage and repair pathway, I assessed the phosphorylation of H2AX in CSSG1 KD cells following induction of DNA damage using etoposide. Immunoblotting for phosphorylation of H2AX has been shown to be a reliable method for measuring DNA damage response [115]. I stimulated CSSG1 KD cells at the indicated concentration of etoposide then stained for presence of phosphorylated H2AX in the nucleus. A representative image is shown in Figure 3.5A demonstrating an increase in H2AX phosphorylation in CSSG1 3’ UTR KD cells compared to NT control cells. When I quantitated the mean nuclear intensity of phospho-H2AX in etoposide-treated CSSG1 KD cells, I found that the intensity was much higher in 3’ UTR KD cells compared to NT cells (Figure 3.5B). This observation was confirmed by immunoblotting cell lysates of etoposide-treated cells for presence phosphorylated H2AX (Figure 3.5C, D). Thus DNA damage by etoposide treatment resulted in increased phosphorylation of H2AX in 3’ UTR KD cells compared to NT control cells, suggesting that CSSG1 regulates the DNA damage response.
Figure 3.5: DNA damage accumulation following etoposide stimulation is increased in CSSG1 KD U2OS cells.

(A) CSSG1 KD and control NT U2OS cells were stimulated with 30 uM etoposide for 3 hours. The cells were then fixed with 4% paraformaldehyde, permeabilized with methanol, and stained with anti-pH2AX (Millipore) and anti-mouse Alexa 555 (Invitrogen) to visualize accumulation of DNA damage using a Nikon fluorescence microscope (Representative images from one experiment.) (B) Mean nuclear intensity of pH2AX in NT control and CSSG1 KD U2OS cells stimulated with 30 uM etoposide for 3 hours was quantified using ImageJ, (Each plotted symbol is an individual cells from one experiment, graph shows mean+SEM of pH2AX values of cell nuclei measured from one experiment). Significance values are calculated using Student’s t-test using NT versus 3’ UTR KD values. **: p<0.01, *: P<0.05 (C) Levels of phosphorylated H2AX were assessed by immunoblotting following stimulation of NT control and CSSG1 KD U2OS cells with 1, 3, 10, and 30 uM of etoposide for 4 hours. Relative phosphorylation of H2AX on immunoblot from (C) were quantified in (D) using imageJ after normalizing to β-actin control (one experiment performed).
3.3.6. Accumulation of DNA damage as measured by H2AX phosphorylation is increased in CSSG1 KD cells following HSV1 infection

HSV1 is known to induce the DNA damage response in infected cells [94]. To determine whether the increase in H2AX phosphorylation in CSSG1 KD cells also occurs during HSV1 infection, phosphorylation levels of H2AX were measured following infection with HSV1. The representative results are shown in Figure 3.6A. Following infection with VP16-GFP expressing HSV1 for 24 hours, phosphorylation of H2AX is increased in infected cells, i.e., GFP+ cells showed increased pH2AX staining. Furthermore, the induction in H2AX phosphorylation is higher in infected 3’ UTR KD cells compared to NT cells. Quantification of mean nuclear intensity in a large population of cells indicates that the observed increase in H2AX phosphorylation in 3’ UTR KD cells compared to NT KD cells following HSV1 infection is statistically significant. (Figure 3.6B).
Figure 3.6: DNA damage accumulation following HSV1 infection is increased in CSSG1 KD U2OS cells.

CSSG1 KD and control NT U2OS cells were infected with HSV1-VP16-GFP at MOI 1 for 24 hours. The cells were then fixed with 4% paraformaldehyde, permeabilized with methanol, and stained with anti-pH2AX (Millipore) and anti-mouse Alexa 555 (Invitrogen). The cells were then visualized with a Nikon fluorescence microscope. (A) Representative fluorescence images showing the DNA damage (phosphorylated H2AX) in HSV1 VP16-GFP infected cells. (B) Mean nuclear intensity of pH2AX in imaged GFP positive cells from (A) (Each plotted symbol is an individual cells from one experiment, mean + SEM are calculated from measured cells from one experiment). Significance values are calculated using Student’s t-test using NT versus 3’ UTR KD values. **: p < 0.01, *: p < 0.05.
3.4: Discussion

The appearance of cold sores is a multi-stage event initiating from HSV1 reactivation in the trigeminal nerve to HSV1 replication in the epithelial cells lining the mucosal layers. It is possible that CSSG1 affects cold sore frequency by playing a role in one or more of these pathways. Based on interaction of CSSG1 with Tip60, a protein required for herpesvirus replication, I hypothesized that CSSG1 is required for efficient HSV1 replication. In this chapter, I provide experimental evidence to support this hypothesis.

When CSSG1 KD cells were infected with ICP8-GFP HSV1, I found that expression of the ICP8 protein was decreased in 3’ UTR KD and CDS KD cells compared to NT cells. Since ICP8 protein is an E stage protein required for HSV1 genome replication, HSV1 replication should be reduced in CSSG1 KD cells [116]. Next, for further confirmation, I compared expression of HSV1 IE, and L stage proteins in the CSSG1 KD versus NT cells. I found that expression of IE (ICP4, ICP27) and L (ICP5) were decreased in CSSG1 KD cells compared to NT cells. Reduced expression of the IE ICP4 protein would have an adverse effect on HSV1 replication since it is required for transcription of downstream genes [117]. Finally, the release of HSV1 virions was measured using qPCR and plaque assay. The measurements showed that production of HSV1 virions is significantly decreased in 3’ UTR KD U2OS cells compared to NT U2OS cells as expected. Overall, the experiments not only provide evidence for the role of CSSG1 in HSV1 replication, but they also suggest that CSSG1 is affecting HSV1 replication at or prior to the IE stage of replication.

It is interesting to note the difference observed between CSSG1 3’ UTR KD and
CDS KD cells in terms of HSV1 replication. Even though both 3’ UTR KD and CDS KD cells have reduced HSV1 protein expression compared to NT cells 6hr post infection, HSV1 protein expression levels return to normal NT cells in CDS KD cells 12hr post infection. Whereas, it takes 48hrs for HSV1 protein expression levels in 3’ UTR KD cells to reach the levels seen in NT cells. Furthermore, release of HSV1 virions from 3’ UTR KD cells is significantly reduced compared to NT cells, however, there is no reduction in HSV1 virions released from CDS KD cells compared to NT cells. I believe this difference exits because both shRNAs target different CSSG1 protein variants. CSSG1 protein has a truncated natural variant in addition to full-length variants (Figure 3.1A). The shRNA targeting 3’UTR of CSSG1 targets all of the variants including the truncated 221AA variant. The CDS shRNA, however, only targets the full-length CSSG1 variants. It is possible that the 221AA variant plays a greater role in HSV1 replication. To test this, the 3’ UTR KD U2OS cells need to be rescued with full length or truncated variants of CSSG1 protein and assessed for HSV1 replication.

Since the function of CSSG1 is unknown, it is difficult to understand how CSSG1 may be affecting HSV1 replication. However, its interaction with Tip60 may provide an explanation. Tip60 plays a crucial role in initiation of the DNA damage response following etoposide and herpesvirus stimulation. Evidence suggests that host DNA damage response plays a role in supporting HSV1 replication. To determine if CSSG1 is capable of affecting the DNA damage response, 3’ UTR KD and NT U2OS cells were stimulated with etoposide or HSV1. Phosphorylation of H2AX was used as readout to measure the extent of DNA damage response. I discovered that H2AX phosphorylation is increased in HSV1 infected CSSG1 3’ UTR KD U2OS cells compared to NT U2OS cells. The effect of CSSG1 on the DNA damage response appears to be independent of
HSV1 infection since increases in H2AX phosphorylation is also observed following etoposide stimulation of CSSG1 3' UTR KD cells compared to NT cells. This suggests that HSV1 may be highjacking the intrinsic function of CSSG1 during DNA damage response to facilitate its replication. The implications of the results obtained in this section on replication and reactivation of herpesviruses in human patients are further discussed in Chapter 4.

3.5: Materials and Methods

3.5.1: Cell Culture

U2OS and Hec1B cells were provided by David Knipe (Harvard Medical School, Boston, MA). THP1 and HEK293T cells were obtained from ATCC. U2OS, Hec1B, and HEK 293T cells were grown in DMEM supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C with 10% CO₂. THP1 cells were grown in RPMI supplemented with 5% FCS, 3 μM BME, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C with 5% CO₂.

3.5.2: Establishment of CSSG1 knockdown cell line

Lentivirus vectors (PLKO.1) expressing shRNAs targeting the 3’ UTR and ORF of CSSG1 were obtained from Open Biosystems (TRCN0000127815 and TRCN0000131040, respectively). The NT shRNA construct was a gift from the Fitzgerald Lab at UMASS Medical School. The shRNA target sequences were as follows: NT - GCAAGCTGACCCTGAAGTTCA, CDS - CTCCTACAGCAATCCAGGT, 3’ UTR - GAAGAAAGTTGTGGTCCGGT. The vectors were packaged into lentivirus using a second-generation lentivirus kit and
overlaid onto U2OS cells. Following 48 hours, the cells were washed and treated with 3.5 ug/mL puromycin for 7 days. U2OS cell line expressing 3’ UTR shRNA was rescued by transducing with a lentivirus containing the CSSG1 cDNA vector obtained from Open Biosystems. This cell line was selected with 3.5 ug/mL puromycin and 10 ug/mL blasticidin for 7 days.

3.5.3: Detection of CSSG1 protein in human cell lines

To detect endogenous CSSG1 protein, U2OS, Hec1B, and HEK293T cells were lysed using 2x Laemmli buffer diluted to 1x using PBS. The lysates were run on a SDS-PAGE denaturing gel and blotted using in house developed anti-CSSG1 antibody clone 7A12 and anti-mouse HRP (Abcam). Expression of ß-actin was detected using an anti-ß actin antibody (Abcam) followed by anti-mouse HRP antibody to serve as a loading control.

3.5.4: Viral infections

HSV1-KOS, VP16-GFP, and ICP8-GFP virus strains were obtained from Dr. David Knipe (Harvard Medical School, Boston, MA). For infections with HSV1 ICP8-GFP and VSV-GFP, U2OS cells were for plated at density of 100,000 cells/plate in a 24 well plate overnight. For infection with HSV1-KOS, U2OS cells were plated at density of 400,000 cells/plate in a 6 well plate overnight. The following day, the cells were washed with DMEM and overlaid with virus at indicated MOIs in DMEM for 1 hour at 37 °C. The cells were then washed with DMEM and incubated in DMEM, 10% FBS for indicated time period.

3.5.5: Measurement of HSV1 genome copy number
To measure HSV1 genome copy number, supernatant from infected cells was removed at indicated time periods. HSV1 genomes were extracted from virions released into the supernatant using a DNeasy genomic extraction kit (Qiagen) from 50 ul of supernatant. A HSV1 genome standard was created by processing $10^9$ PFU of HSV1-KOS using the same extraction method. Purified HSV1 genomes were quantified by qPCR using the following primers: Forward, GAGGAATTCATGGCTTCGTACCCCGGCCATC; Reverse, CTCGTCGACAGGTTAGCCTCCCCCATCTCCCG. The results were fit onto a standard curve created using a HSV1 genome standard to calculate the PFU equivalent genome copy number.

### 3.5.6: Plaque assay

Plaque assays were performed using Vero cells. Briefly, 200,000 cells/well were plated overnight in a 24 well plate. Collected supernatants were serially diluted starting at 1:3 and overlaid onto Vero cells for 1 hour at 37°C. Following the incubation period, cells were washed with DMEM, 10% FBS and incubated for 72 hours at 37°C in DMEM, 10% FBS with 1.2 ug/mL human immunoglobulin. The plates were incubated and inspected following 3 days for development of plaques. To develop plaques, cells were fixed with 100% methanol for 5 minutes at room temperature and stained with 0.1x Giemsa stain for 30 mins.

### 3.5.7. Quantification of H2AX phosphorylation

U2OS cells were stimulated with either etoposide (1, 3, 10, 30 uM) for 3 hours or HSV1 (VP16-GFP, MOI 10) for 18 hours. Cells treated with HSV1 VP16-GFP were overlaid with human immunoglobulin (7 ug/mL, Thermo Fisher) 1 hour following stimulation to
neutralize extracellular virus and synchronize infection. For analysis of H2AX phosphorylation by imaging, the cells were fixed with methanol at -20 °C for 30 mins and stained with anti-pH2AX (Millipore) and anti-mouse Alexa 555 (Invitrogen) antibodies. The cells were then imaged using a fluorescent microscope. Mean nuclear intensity of pH2AX in GFP positive cells was quantified in nuclei (identified by Hoechst 34580 stained area) using ImageJ. For analysis of pH2AX by immunoblotting, lysates from etoposide-stimulated cells were run on SDS-PAGE and blotted using anti-pH2AX, anti-β actin (Abcam), and anti-mouse HRP antibodies. Amount of relative H2AX phosphorylation was quantified using ImageJ.

3.5.8: Statistical Analysis

An unpaired, two-tailed Student t test was used to determine statistical significance of independent experiments where two groups were compared. Values of p < 0.05 were considered significant with 95% confidence intervals. Statistics were performed using GraphPad (Prism version 6.0b) software.
CHAPTER IV

CSSG1 (D16ERTD472E) KNOCKOUT MOUSE FOR STUDYING THE ROLE
OF CSSG1 IN HERPESVIRUSES PATHOGENESIS
Contributors

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Summary of contributions

I contributed to the design, execution, analysis and interpretation of the experiments described in this chapter. I generated the samples and performed the experiments and created the graphics shown in Figures 4.1, 4.2, 4.3, 4.4 and 4.5. I was assisted by Anna Cerny and Emily Whicker for breeding of CSSG1 knockout mice described in section 4.3.1.
4.1 Abstract

The development of herpesvirus pathogenesis involves complex interplay between virus replication and the resulting immune response. Early innate immune responses characterized by production of proinflammatory cytokines and type I interferon play a crucial role in development and resolution of herpesvirus pathogenesis. To understand the role of CSSG1 in these pathways, I generated a mouse model deficient in CSSG1 expression using genetrap technology.

After confirmation of abrogation of CSSG1 mRNA expression in bone marrow cells extracted from the CSSG1 deficient mice, I tested the ability of the CSSG1 knockout cells to produce inflammatory cytokines in response to innate immune stimuli that mimic herpesvirus infection. I determined that stimulation of CSSG1 deficient BMDCs with Pam3CSK4, an agonist of TLR1/2 signaling pathway, led to decreased IL-6 and RANTES production compared to WT BMDC cells. To further explore potential role of CSSG1 in TLR1/2 signaling pathway, I sought evidence for interaction of CSSG1 with key proteins involved in the TLR1/2 signaling pathway. Through confocal imaging, I discovered that CSSG1 forms distinct cytoplasmic foci surrounded by TRAF6 when CSSG1 and TRAF6 were coexpressed in HEK293 cells. In addition, I observed that interferon beta promoter activation, as measured by luciferase reporter assay, in response to transfected poly dA:dT (dsDNA) was increased in presence of ectopically expressed CSSG1 compared to vector control cells.

Overall, these results warrant further investigation into analyzing the effect of CSSG1 expression on innate immune responses to herpesviruses. In combination with the in vitro models described above, the CSSG1 deficient mouse will provide a suitable model for in-depth analysis of how CSSG1 affects herpesvirus pathogenesis in vivo.
4.2: Introduction

The innate immune response plays a crucial role in initiation and resolution of herpesvirus pathogenesis. The two major pathways activated during an innate immune response to herpesvirus infections include a proinflammatory cytokine response and a type I IFN response. The production of proinflammatory cytokines leads to recruitment of macrophages, neutrophils, and antigen presenting cells that facilitate the recognition of HSV1 virions and clearance of infected cells. Production of Type I IFNs is critical for reduction of HSV1 replication and its clearance. Type I IFNs activate expression of ISGs that activate intrinsic cell immunity to reduce HSV1 replication. The importance of proinflammatory cytokines and type I IFNs in HSV1 infection is evident from studies showing that Unc93B-, IL-6-, IL-1-, and IFNAR-deficient mice are highly susceptible to HSV1 infections. This is accomplished by recognition of HSV1 virion structural components by various intracellular and extracellular PRRs (Chapter 1.6-1.9 for further details).

Since the relationship between cytokine production and resolution of herpesvirus pathogenesis is too complex to be studied effectively in vitro, a CSSG1 deficient mouse model would provide an opportunity to explore the role of CSSG1 in HSV1 pathogenesis. The CSSG1 allele in mouse is referred to as D16Ertd472e. The D16Ertd472e mRNA is predicted to encode a protein of 290 amino acids. The homology between the mouse and human CSSG1 protein is high at close to 85%. Additionally, the coils server also predicts presence of a coiled-coil domain in the D16Ertd472e protein, indicating a high probability of functional similarity between mouse and human CSSG1 proteins. Given the structural similarities between human CSSG1 and mouse D16Ertd472e proteins, development of a D16Ertd472e deficient mouse would be an
ideal model for understanding the potential role of CSSG1 in herpesvirus induced pathogenesis in humans. For simplicity, D16Ert472e is referred to as CSSG1 from here on.

In this chapter, I report the generation of CSSG1 deficient mice. CSSG1 knockout mice were visually phenotypically normal and fertile. The availability of CSSG1 deficient mice provided a well-established model for studying the role of CSSG1 in proinflammatory cytokine production. Bone marrow dendritic cells (BMDCs) have been widely used to study innate immune response to HSV1 infections and resulting production of cytokines such as il-6 and rantes [118, 119]. BMDCs derived from CSSG1 knockout mice were stimulated by ligands that activate the innate immune pathways activated during HSV1 infection to determine potential role of CSSG1 in production of proinflammatory cytokines. Confocal imaging suggested colocalization of CSSG1 with TRAF6, an adapter protein essential for activation of TLR1/2 signaling and cytokine production following HSV1 infection. Role of CSSG1 in type I interferon response was also supported by studies of IFN-β promoter activity using luciferase reporter systems.

4.3: Results

4.3.1: CSSG1 mRNA is undetectable in BMDC isolated from CSSG1 deficient mice

To generate CSSG1 knockout mice, ES cells containing a genetrap construct targeting CSSG1 gene expression were obtained from the EUCOMM consortium. The genetrap construct present in these ES cells is predicted to truncate expression of CSSG1 mRNA after exon 2. Exons 3, 4, and 5 would be replaced by mRNA encoding for β-galactosidase (lacZ). (Figure 4.1B). The genetrap is flanked by flippase recognition target (FRT) sites, allowing conditional rescue of the CSSG1 allele as desired. Following the genetrap removal, exon 3 of CSSG1 can also be removed using the X(cross)-over
Figure 4.1: Structure of the mouse CSSG1 genomic region containing the genetrap.

**A)** Mouse CSSG1 genomic region encoding for 5 exons – 299bp, 134bp, 516bp, 63bp, and 596bp in length. **B)** Genetrap containing LacZ and Neomycin resistance genes is inserted before exon 3 to truncate transcription of the mRNA following exon 2. Presence of the LoxP sites allows for conditional removal of exon 3. Presence of the FRT sites allows for conditional removal of the genetrap.
in P1 (loxP) site, allowing conditional control of CSSG1 expression.

To develop CSSG1 deficient mice, C57Bl/6 ES cells containing CSSG1 genetrap allele described above were injected into the blastocyst of albino BL6 mice by UmassMed Genomics core personnel. The resulting chimeric offspring were crossed to albino C57BL6 mice. Agouti offspring from this cross were selected for breeding to obtain CSSG1 deficient mice. To select mice for further breeding, I isolated genomic DNA from tail to be analyzed using PCR for presence of the genetrap located within CSSG1 allele. As shown in Figure 4.2A, the PCR product from a wild type (WT) mouse is approximately 300 bp. The product size of genetrap allele is detected as a band of approximately 200 bp (note both band sizes in the mice heterozygous for the presence of the genetrap (KO) allele). The sizes of the PCR amplification bands match the sizes observed in the ES cell clones by EUCOMM consortium, suggesting that the genetrap is present in the correct location within the mouse CSSG1 allele in CSSG1 KO mice.

Following confirmation of presence of the genetrap in the appropriate location within the mouse CSSG1 allele, I sought to determine whether the genetrap was functioning as intended by abrogating expression of mouse CSSG1 mRNA. To do this, mouse CSSG1 mRNA expression was quantified by qPCR in bone marrow cells derived from wildtype C57Bl/6 and CSSG1 KO mice. As expected, mouse CSSG1 mRNA expression was detected in the WT C57Bl/6 mice (Figure 4.2.B). However, the mouse CSSG1 mRNA was undetectable in the bone marrow cells derived from the CSSG1 KO mice, confirming that the insertion of the genetrap has led to abrogation of mouse CSSG1 expression.
A) Genomic DNA was isolated from tails of WT C57/Bl6 and CSSG1 heterozygous mice. Genomic region near insertion of Genetrap was amplified using PCR and run on agarose gel. The band at 400bp indicates presence of WT genomic region. Presence of 300bp indicates presence of the genetrap insertion (three independent experiments performed, result from one experiment is shown as an example.) B) Bone marrow was isolated from WT and CSSG1 KO mice. Expression of mouse CSSG1 mRNA was quantified using qPCR and normalized to expression of mGusB gene (result from one experiment performed in duplicates).

Figure 4.2: Mouse CSSG1 mRNA is undetectable in bone marrow cells isolated from CSSG1 KO mice.
4.3.2: Production of IL6 and RANTES from CSSG1 KO BMDCs is reduced in response to PAM3CSK4 stimulation

To determine if CSSG1 affects innate immune pathways involved in detection of components of HSV1 virions, BMDCs derived from WT and CSSG1 deficient mice were stimulated with agonists of PRRs that are known to be activated during HSV1 infection. The amount of IL-6 and RANTES released into the supernatant was measured by ELISA.

WT and CSSG1 KO BMDCs were stimulated with poly I:C, zymosan, LPS, cytosolic poly dA:dT, cytosolic poly I:C, or cytosolic vaccinia 70mer (ligands that activate the PRRs TLR3, TLR2/6, TLR4, Rig-I, MDA5, IFI16 respectively) [49, 55, 58, 71, 120, 121]. There was significant production of IL-6 and RANTES compared to stimulations with media and lipofectamine which served as negative controls. Stimulation of CSSG1 deficient BMDCs with the same agonists led to release of IL-6 and RANTES at similar levels compared to the wildtype BMDCs with the exception of Pam3CSK4 stimulation. The release of IL-6 and RANTES from CSSG1 deficient BMDCs was decreased following PAM3CSK4 stimulation compared to WT BMDCs (Figure 4.3.A, B), indicating CSSG1 may be selectively involved in proper activation of the TLR1/2 signaling pathway.
Figure 4.3: TLR2 activation is abrogated in BMDCs derived from CSSG1 KO mice.

BMDCs derived from WT and CSSG1 KO mice were stimulated with Pam3CSK4 (100 ng/mL), Zymosan (5 ug/mL), poly I:C (50 ug/mL), HSV1-KOS virus (MOI 100), Sendai virus (8HA/mL), Etoposide (30 uM), LPS (100 ng/ml). Following 24hrs, supernatants were collected and release of A) IL-6 and B) Rantes was measured using ELISA kits (eBioscience) according to manufacturer’s instructions (Graph shows mean + SEM of values from one experiment measured in duplicates).
4.3.3 CSSG1 forms aggregates upon activation of TRAF6, a protein involved in innate immune response to herpesvirus infection

The coiled-coil domain containing adapter protein TRAF6 plays a crucial role in the activation of the TLR2 signaling pathway leading to production of IL6[122-124]. The coils server database predicts presence of a coiled-coil domain in CSSG1 (Chapter 1.3.1). This raises a possibility of interaction between TRAF6 and CSSG1. To determine whether TRAF6 expression affects CSSG1 localization, mCherry tagged CSSG1 or mCherry alone was expressed in HEK 293T cells (Figure 4.3A) in presence or absence of TRAF6. When mCherry alone was expressed in HEK 293T cells, its expression was distributed evenly across the nuclear and cytosolic regions as observed previously. When mCherry alone was co-transfected with TRAF6, no change in localization of mCherry was observed (Figure 4.3A). Ectopic expression of CSSG1-mCherry alone also led to diffuse cytosolic localization. However, when HEK 293T cells were co-transfected with CSSG1 and TRAF6, I detected the presence of large CSSG1-mCherry aggregates in the cytosol.

To determine whether the observed reorganization of CSSG1 in presence of TRAF6 was due to co-localization of CSSG1 with TRAF6, CSSG1-mCherry and TRAF6-Flag were co-transfected and TRAF6 visualized by staining using anti-Flag-Alexa 488. In absence of transfection, anti-Flag staining is absent as illustrated by arrow pointing towards the untransfected cell, indicating high specificity of the anti-Flag antibody antibodies (Figure 4.3B). In the cells co-transfected with CSSG1-mCherry and TRAF6-Flag, the aggregates formed by the CSSG1 protein are surrounded by TRAF6 raising the possibility of presence of CSSG1 in a complex with TRAF6 during the HSV-1 induced innate immune response.
Figure 4.4: Ectopically expressed CSSG1 forms aggregates in presence of TRAF6.

A) HEK 293T cells were transfected with plasmids encoding mCherry, mCherry tagged full length CSSG1 protein, or Flag tagged TRAF6 as indicated. Following 24 hours, the cells were fixed with 4% PFA and stained with CtxB-Alexa 488 to visualize the cell membrane. Hoechst was added to stain the nuclei. The cells were imaged with Leica SP2 confocal microscope (three independent experiments performed, representative images from one experiment are shown as an example). B) HEK293T cells were co-transfected with plasmids expressing CSSG1-mCherry and TRAF6-Flag. Following 24 hours, the cells were fixed with 4% PFA, permeabilized with methanol and stained with anti-Flag Alexa 647 antibody (Invitrogen) to visualize TRAF6. Notice the presence of TRAF6 organization around CSSG1 aggregates. An untransfected cell is shown as a negative control for anti-Flag staining (arrows) (representative images from one experiment are shown as an example).
4.3.4: Expression of CSSG1 leads to increased type I interferon beta reporter activity

Since there is evidence for involvement of CSSG1 in inflammatory responses to herpesvirus infection[125-127], I sought to determine whether CSSG1 also plays a role in initiation of type I IFN response using a luciferase reporter system. To do this, U2OS cells were transfected with Firefly luciferase expressed under the control of human p125 interferon beta promoter that is activated upon IRF3 phosphorylation. The cells were also cotransfected with Renilla luciferase expressed under control of EF1α promoter. The EF1α promoter is activated ubiquitously [128], and thus, acts as a control to help account for potential variations in transfection efficiency. The U2OS cells were also cotransfected with pcDNA3.1 (empty vector) or plasmids expressing CSSG1-Flag protein variants (N115) and (K115). The cells were then stimulated with lipofectamine or media as negative controls to determine background interferon beta promoter driven activity. The cells were also stimulated with DNA (poly dA:dT) and RNA (poly I:C) cytosolic nucleotides that activate the pol III – Rig-I and MDA5 pathways, respectively. When the cells were stimulated in presence of CSSG1 (both N115 and K115) protein variants, the activation of interferon beta reporter was higher compared to the negative controls, suggesting potential role of CSSG1 in the interferon beta pathway (Figure 4.4.ABC).
Figure 4.5: Interferon beta luciferase reporter activity is increased in presence of CSSG1 in response to poly dA:dT, poly I:C, and Sendai virus.

U2OS cells were transfected with empty vector control, cDNA expressing CSSG1 protein variants N115K and K115, p125 human interferon beta promoter driven firefly luciferase, and EF1a driven renila luciferase for 24hrs. The cells were stimulated with A) transfected 1ug/mL poly dA:dT B) transfected 10ug/mL poly I:C, and C) 8HA/mL sendai virus for 18hrs. The cells were then lysed in passive lysis buffer (Promega) and subjected to luciferase assay kit (Promega) according to manufacturer’s instructions (Graph shows mean + SEM of values from one experiment measured in triplicates).
4.4: Discussion

In the interest of studying the role of CSSG1 in herpesvirus pathogenesis, we created what is currently the first known report of CSSG1 deficient mice. The CSSG1 deficient mice are fertile and devoid of visual phenotypical aberrations. The abrogation of CSSG1 mRNA expression was confirmed in bone marrow cells isolated from CSSG1 deficient mice using qPCR. BMDCs were derived from these cells and used to ascertain potential role of CSSG1 in HSV1 mediated pathogenesis.

Production of proinflammatory cytokines play a significant role in development of herpesvirus pathogenesis as discussed in Chapter 1.6. To determine whether CSSG1 plays a role in production of proinflammatory cytokines involved in HSV1 pathogenesis, IL6 and Rantes production was measured using ELISA following stimulation of WT and CSSG1 deficient BMDCs with poly I:C, zymosan, LPS, cytosolic poly dA:dT, cytosolic poly I:C, or cytosolic vaccinia 70mer. I found that production of IL6 and Rantes from CSSG1 deficient mice was reduced compared to WT BMDCs in response to PAM3CSK4, a TLR1/2 agonist. A significant role of TLR2 pathway in clearance of HSV1 infection and also, exacerbation of HSV1 induced encephalitis has been reported [40, 42, 43, 129]. A recent report has suggested that CSSG1 plays a role in basal activity of NFkB signaling pathway [84]. Since NFkB activation is required for production of IL6 following Pam3CSK4 stimulation [130], the published results agree with potential role of CSSG1 in TLR1/2 pathway activation.

Bioinfomatic studies carried out on CSSG1 amino acid sequence predict presence of a coiled-coil domain (Chapter 2.3.3). TRAF6, an adapter essential for TLR2 mediated proinflammatory cytokine production, has been shown to contain a coiled-coil domain that is required for its auto-ubiquitination and downstream signaling [124].
Coiled-coil domains play an important role in protein-protein interactions [89]. This suggested the possibility that CSSG1 may interact with TRAF6. To test this, CSSG1-mCherry and TRAF6-Flag were coexpressed in HEK293T cells and imaged using confocal microscopy. I found that CSSG1-mCherry protein forms aggregates in presence of TRAF6-Flag. Furthermore, using confocal microscopy, I show that TRAF6 encircles these CSSG1 aggregates in the cytosol. Since TRAF6 plays a crucial role in PAM3CSK4 induced IL6 production, aggregation of CSSG1 in presence of TRAF6 indicates a potential mechanism for the role of CSSG1 in TLR1/2 activation [131]. The importance of the predicted coiled-coil domain of CSSG1 also needs to be tested in this ectopic expression model. It is likely that the CSSG1 221AA variant lacking the coiled coil domain will not form cytosolic foci in presence of TRAF6. Additionally, HSV1 protein UL37 has been shown to activate TRAF6 leading to activation of NFkB and IL8 gene expression[132]. It is possible that CSSG1 is required for this activity of UL37. To test this possibility, CSSG1 3' UTR KD U2OS cells (Chapter 2.3.1) can be transfected with UL37 and subsequent expression of IL8 can be measured.

In addition to providing evidence for role of CSSG1 in the proinflammatory cytokine pathway, I also aimed to determine if CSSG1 has a role in production of type I IFN during HSV1 infection. I found that in presence of ectopically expressed CSSG1, the type I IFN production in response to cytosolic dsDNA (Rig-I agonist) and dsRNA (MDA5 agonist) is increased. It is unclear how CSSG1 can lead to an increase of type I IFN gene expression. To confirm the role of CSSG1 in the type I IFN pathways, BMDCs and MEFs derived from CSSG1 deficient mice can be stimulated with dsDNA and dsRNA stimuli and release of interferon measured using ELISA. Additionally, it is important to confirm the increase in activation of interferon beta promoter results in increased release
of interferon beta protein using bioassay.

Although more experiments are needed for confirmation of role of CSSG1 in innate immune responses, the experiments conducted in this chapter provide justification for exploring the role of CSSG1 in the innate immune responses to HSV1. Availability of CSSG1 deficient mice will be useful for this purpose.
4.5: Materials and Methods

4.5.1: Breeding of D16Ertd472e (CSSG1) genetrap mice

Embryonic stem (ES) cell clones validated for integration of a genetrap targeting the mCSSG1 gene were obtained from the EUCOMM Consortium, which also determined the location of insertion of genetrap within the mCSSG1 genomic region. ES clones of albino C57BL/6 origin were injected into C57BL/6 mice by University of Massachusetts Medical School Genomics core. These mice were then bred with C57BL/6 mice. Chimeric offspring were weaned 28 days following birth. The offspring were bred with WT C57BL/6 mice. F1 progeny were genotyped for presence of the integrated genetrap construct as described below. The F1 progeny heterozygous for the mCSSG1 genetrap allele were crossbred to obtain mice homozygous for the genetrap insertion.

4.5.2: Genotyping of the CSSG1 genetrap allele

At the weaning stage, tail-ends were collected from the offspring. Genomic DNA was extracted from the tail-ends following incubation with 75 ul of alkaline lysis buffer (0.07% NaOH, 0.7mM EDTA pH 8.0) for 30 minutes at 95 °C. The solution was then treated with 75 ul of neutralization buffer (40mM Tris-HCl, pH 5) for 5 minutes at room temperature. For detecting the genetrap, a protocol developed by the EUCOMM Consortium was used. For PCR amplification, Qiagen HotStar mix was used according to manufacturer’s instructions. The PCR cycler was set at 95 °C for 5 min, (95 °C for 10 seconds, 72 °C for 30 seconds, 58 °C for 10 seconds) x 32, 58 °C for 10 mins, followed by incubation at 4 °C. The sequences of the primers used were: D16ERTD5F –
Primers D16ERTD5F and D16ERTD3R detect the wildtype CSSG1 allele while primers D16ERTD5F and LAR3 detect the genetrap allele. Following PCR amplification, the PCR product was run on a 2% agarose gel for quantification of size.

4.5.3: Differentiation and stimulation of BMDCs and detection of cytokines

BMDC from 8 week old mice were isolated and cultured with 20 ng/mL GM-CSF in DMEM, 10% FBS at concentration of 10^6 cells/mL at 37 °C, 10% CO₂. Media was replaced every three days. Following 10 days, the cells were plated in a flat-bottom 96 well plate at density of 200,00 cells/well. The following day, the cells were stimulated with various stimuli as indicated. Nucleotides poly dA:dT (1 ug/mL) and poly I:C (5 ug/mL) were transfected using Lipofectamine 2000 at 1:1 ratio according to manufacturer’s instructions. The remaining activators of the innate immune responses were added at following concentrations: Pam3CSK4 (100 ng/mL), Zymosan (5 ug/mL), poly I:C (50 ug/mL), HSV1-KOS virus (MOI 100), Sendai virus (8HA/mL), Etoposide (30 uM), LPS (100 ng/ml). Supernatants of stimulated BMDCs were collected 24 hours following stimulation. The amount of IL-6 and RANTES released into the supernatant was measured using ELISA kits available from eBioscience according to the manufacturer’s instructions.

4.5.4: Confocal Imaging

HEK 293T cells were plated at density of 250,000 cells/dish in a 30 mm confocal dish. The cells were transfected with 0.5 ug of plasmids expressing indicated proteins.
using GeneJuice according to manufacturer’s instructions. Following 24 hours, the cells were incubated in 4 ug/mL cholera toxin B – Alexa 488 (CTxB, 4 ug/mL, Invitrogen) for 30 minutes at 37 °C where applicable. All cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. For staining with anti-Flag (Sigma) and anti-V5 (Abcam) antibodies, the cells were permeabilized with 100% methanol for 5 minutes at -20 °C. The cells were then blocked with 3% BSA at room temperature for 1 hour. The anti-Flag and anti-V5 antibodies were added to the cells at 5 ug/mL in 1% BSA overnight at 4 °C. The cells were then washed with PBS and overlaid with anti-rabbit-Alexa 488 (cell signaling) for anti-Flag staining or anti-mouse-Alexa 488 (Invitrogen) for anti-V5 staining at 4 °C for 1 hour at concentration of 5 ug/mL with 1% BSA. The cells were washed, stained with 5 ug/mL Hoechst 34580 (Invitrogen) then imaged with a Leica SP2 confocal microscope.

4.5.5: Luciferase reporter assay

U2OS cells were plated at density of 15,000 cells/well in a flat bottom 96-well plate in DMEM with 10% FBS at 37 °C and 5% CO₂. On the next day, the cells were transfected with 40 ng (Firefly luciferase expression controlled by human p125 promoter), 20 ng (Renilla luciferase expression controlled by human EF1α promoter), and 140 ng pcDNA3 or (CSSG1-V5 expressing plasmid) using GeneJuice according to manufacturer’s instructions. Following 24 hrs, the cells were stimulated with poly dA:dT (1 ug/mL), poly I:C (5 ug/mL), or Sendai virus (8 HA units/mL). Eighteen hours post-stimulation, reporter activity of the type I IFN luciferase reporter was measured using dual luciferase reporter assay kit (Promega). Briefly, the stimulated cells were lysed with 80ul of passive lysis buffer per well. Then, 50 ul of cell lysates were transferred to a fluorescence microplate.
The lysates were mixed with 50 ul of firefly renilla luciferase substrate and incubated for 10 minutes at room temperature. The fluorescence signal was measured at 485 nm. 20 ul of renilla luciferase substrate was added to the lysates and incubated for 10 mins at room temperature. The fluorescence values were measured at 485 nm. Values obtained at A485 (type I IFN firefly luciferase readout) were normalized to A465nm (EF1α renilla luciferase readout) values to obtain the relative activity of the human type I IFN promoter in stimulated cells.
CHAPTER V

DISCUSSION
Despite carrying one of the largest genomes among known viruses, HSV1 requires multiple host proteins and noncoding RNAs that facilitate its replication and reactivation. Numerous host factors involved in cell pathways such as intracellular transport, DNA repair and immune responses have already been characterized to play a role in HSV1 infection. Although significant research has been done in this area, the understanding of how HSV1 reactivation and pathogenesis occur is incomplete. A recently identified open reading frame known as C21ORF91 (CSSG1) may offer further insight into HSV1 replication and reactivation. SNPs present in the cssg1 genomic region are associated with frequency and severity of cold sores in humans caused by HSV1 reactivation[82]. The SNPs are also associated with levels of antibody titers to HSV1[83]. These associations suggest a significant role for CSSG1 in pathogenesis caused by HSV1. A major goal of this dissertation was to explore the role cssg1 plays in HSV1 infection and pathogenesis. This goal was pursued by characterizing expression and interactions of the cssg1 protein, studying its role in HSV1 replication, and exploring its role in innate immune responses involved in herpesvirus pathogenesis.

At the initiation of this study, it was unknown whether the open reading frame CSSG1 encodes for a protein or a noncoding RNA variant. Since HSV1 can utilize both host cell proteins and RNA for replication, it was important to determine whether CSSG1 encodes for a protein to assess its role in HSV1 infection. In chapter 2, my colleague Michael King and I show that the CSSG1 mRNA encodes a protein by immunoprecipitating endogenously expressed protein from human cells using in-house developed monoclonal antibodies. The identity of CSSG1 protein was confirmed using mass spectrometry analysis. There are currently no other monoclonal antibodies available for CSSG1 that can be useful for studying protein-protein interactions. The
antibodies developed in this process have been deposited for commercial use and will be an invaluable tool for exploring the role of CSSG1 in herpesvirus pathogenesis.

The ability to detect CSSG1 protein expression allowed us to determine its role in a significant event during HSV1 infection: the multiplication of its virions following entry into a host cell. In chapter 3.3, I confirm that expression of the CSSG1 protein can be knocked down in U2OS cell lines stably expressing shRNA targeting CSSG1 mRNA. Using the CSSG1 KD U2OS cells, I show that HSV1 replication as measured by release of productive virions is decreased while the replication of an unrelated virus VSV remains unaffected. The decrease in HSV1 replication was accompanied by a decrease in expression of HSV1 IE proteins ICP4 and ICP27. The IE proteins of HSV1 are the earliest proteins expressed following infection (Please see Introduction 1.4 for further details). The IE protein ICP4 is required for expression of downstream proteins required for HSV1 replication [133-135]. I propose that the reduction in HSV1 replication in CSSG1 KD U2OS cells is due to decreased expression of the IE protein ICP4. Other groups have shown that a decrease in ICP4 expression leads to a reduction in HSV1 replication since ICP4 is required for transcription of downstream HSV1 proteins [133-135]. The observed decrease in expression of HSV1 E and L stage proteins ICP8, VP16, and ICP5 in HSV1 infected CSSG1 KD cells (Chapter 3.3.) is consistent with these reports. If the decrease in HSV1 replication in CSSG1 KD U2OS cells is due to reduced ICP4 expression, it should be possible to rescue HSV1 replication in CSSG1 KD cells through ectopic expression of ICP4. Although the decrease in ICP4 expression in CSSG1 KD U2OS cells explains the HSV1 replication defect, the reason for reduction in ICP4 expression in HSV1 infected CSSG1 KD cells remains unclear. Direct involvement of CSSG1 in HSV1 replication can explain association of CSSG1 SNPs with HSV1
reactivation and cold sore severity. In absence of CSSG1, it is likely that replication of
HSV1 would be reduced leading to reduced cold sores severity and HSV1 reactivation.

Before ICP4 can be expressed from HSV1 genome following infection, multiple
events occur including HSV1 virion entry into the cell, transport of HSV1 genome and
transcription factors to the nucleus, and the recruitment of transcription factors to HSV1
genome. A defect in any of these pathways can result in reduced or delayed expression
of ICP4 in CSSG1 KD U2OS cells. Although more experiments are needed to test
potential involvement of CSSG1 in these pathways, the pathway affected by CSSG1 can
be narrowed down according to the analysis conducted on CSSG1 subcellular
localization and protein-protein interactions in Chapter 2.3. Through subcellular
fractionation, we found that majority of CSSG1 protein is expressed to the nucleus in
U2OS cells. Furthermore, based on the report of an interaction between CSSG1 and a
nuclear protein Tip60 in a yeast-2-hybrid screen [86], I ectopically co-expressed CSSG1
with tip60 in human 293T cells. I found that even though ectopically expressed CSSG1
protein is cytosolic, it translocates to the nucleus and colocalizes with ectopically
expressed Tip60. Tip60 is reported to be essential for replication and reactivation of
herpesviruses [87, 88]. The presence of CSSG1 in the nuclear subfraction and its
colocalization with tip60 in the nucleus suggested that the role of CSSG1 during HSV1
replication is in the nucleus, where transcription of HSV1 mRNAs and replication of
HSV1 DNA occur.

One proposed mechanism for how Tip60 affects HSV1 replication is its ability to
acetylate histones and proteins during DNA damage response. During initiation of DNA
damage response, Tip60 acetylates ATM [111], which then phosphorylates histone
H2AX[136]. Tip60 KD cells show decreased H2AX phosphorylation following etoposide stimulation[137]. Tip60 also acetylates H2AX[138]. This serves to recruit NuA4 chromatin remodeling complex that relaxes the chromatin and allows access of DSBs to DNA repair proteins[137, 139]. Resulting repair of DNA breaks leads to dephosphorylation of H2AX [137] (Figure 5.1A). During HSV1 infection, HSV1 kinase UL13 is thought to activate Tip60 acetylation function.

To determine if CSSG1 plays a role in this pathway, CSSG1 KD U2OS cells were stimulated by etoposide and H2AX phosphorylation was measured. I found that in CSSG1 KD cells, H2AX phosphorylation is increased following etoposide treatment. This result suggests that CSSG1 is not required for Tip60 mediated ATM acetylation which leads to H2AX phosphorylation. It is likely that CSSG1 is required for Tip60’s histone acetylation activity or recruitment of chromatin remodeling complex which is important for DNA repair and resulting dephosphorylation of H2AX (Figure 5.1A). In addition to etoposide stimulation, I also determined that H2AX phosphorylation is high in CSSG1 KD cells following HSV1 stimulation. This suggests that CSSG1 plays a similar role during HSV1 infection and etoposide stimulation.

Potential role of CSSG1 in histone acetylation or recruitment of chromatin remodeling complexes has significant implication for HSV1 infection. Following entry of HSV1 genome into the nucleus, it becomes covered with deacetylated histones through an intrinsic innate immune system[16, 140-142]. Covering of HSV1 genomes with deacetylated histones leads to chromatin condensation and suppression of HSV1 gene expression[16]. Activity of HSV1 protein ICP0 counteracts this response by increasing acetylation of histones associated with HSV1 genomes, leading to expression of HSV1
Figure 5.1: Potential mechanism for role of CSSG1 in DNA repair and HSV1 replication

A) During DNA damage response, Tip60 acetylates ATM, which results in phosphorylation of H2AX. Tip60 also acetylates H2AX and other histones present at DNA damage sites. The resulting chromatin relaxation allows DNA repair proteins to bind, leading to DNA repair and dephosphorylation of H2AX and other histones. CSSG1 likely functions during acetylation of H2AX because knocking down CSSG1 results in loss of DNA repair and accumulation of pH2AX. B) Association of HSV1 genome with deacetylated histones has been suggested to lead to suppression of HSV1 lytic gene expression, enforcing latency. Potential role of CSSG1 in histone acetylation can relieve the repression caused by deacetylated histone occupancy, leading to lytic HSV1 replication or HSV1 reactivation. During HSV1 infection, the acetyltransferase activity of Tip60 is activated by HSV1 kinase UL13.
genes and lytic infection[14, 143]. It is possible HSV1 achieves histone acetylation of its genome through Tip60 and CSSG1. Evidence suggests that HSV1 kinase UL13 binds to Tip60 and activates its acetyltransferase activity following infection [87, 88]. If CSSG1 is required for histone acetyltransferase activity of Tip60, absence of CSSG1 would cause HSV1 genomes to remain associated with condensed chromatin. This would explain decreased HSV1 gene expression and replication in CSSG1 KD cells (Figure 5.1B).

This predicted role of CSSG1 can also explain association of CSSG1 with HSV1 reactivation. It has been shown that during latency, HSV1 genome is associated with deacetylated histones in a condensed chromatin state[144, 145]. This is consistent with the observation that neuronal cells carrying latent HSV1 genome treated with HDAC inhibitors reactivate HSV1[146]. The latent genome has also been shown to reactivate through UV radiation or neuronal cell stress. Both of these events have shown to activate Tip60 mediated DNA damage response[113, 137]. It is possible that during the DNA damage response, Tip60 and CSSG1 also acetylate histones associated with latent HSV1 genome, leading to removal of the repressive chromatin and facilitating expression of immediate early HSV1 genes.

Multiple SNPs present in the CSSG1 genomic region are associated with HSV1 reactivation. The N115K SNP (rs2824495) is associated with increased HSV1 reactivation and cold sore severity. I find that presence of the N115K SNP does not affect nuclear translocation of CSSG1 protein and its colocalization with Tip60. It is possible the SNPs affect downstream function of CSSG1. Another possibility is that the SNP affects protein stability leading to decreased levels of CSSG1. By rescuing the CSSG1 KD cells with N115K SNP, the contribution of the SNPs to HSV1 replication and
DNA repair pathways can be assessed. The SNP rs10446073 is located approximately 1kb upstream of the CSSG1 coding region. It is possible that this SNP affects HSV1 reactivation by changing the transcription levels of the CSSG1 gene. Additionally, SNP rs1062202 is located in the 3’UTR region. This SNP may affect HSV1 reactivation by affecting CSSG1 mRNA levels.

In addition to directly playing a role in HSV1 replication, CSSG1 can also affect HSV1 reactivation in humans through modulating the innate immune responses involved in recognition of HSV1 infection. As discussed in chapter 1, the proinflammatory cytokines and type I interferon produced following HSV1 infection play a significant role in clearance and exacerbation of herpesviral infections. To determine if CSSG1 may play a role in PRR mediated recognition of HSV1 infections [89], I sought to determine if CSSG1 may interact with proteins involved in these pathways.

In chapter 2.3, bioinformatics analysis of CSSG1 amino acid sequence suggested presence of a coiled-coil domain. This domain is important for protein-protein interactions. I aimed to determine if CSSG1 can interact with another coiled-coil domain containing protein TRAF6 that is essential for TLR2 induced innate immune response following HSV1 infection [124, 129, 132, 147-151]. I found that although ectopic expression of CSSG1 alone leads to a diffuse cytosolic localization, coexpression with TRAF6 led to formation of large cytosolic foci (Chapter 4.3). The large foci were surrounded by presence of TRAF6 suggesting a potential interaction between TRAF6 and CSSG1. The potential interaction needs to be further tested in presence of the truncated 221AA CSSG1 protein variant that does not contain a coiled-coil domain. It is expected that the foci will not be present when 221AA CSSG1 protein is coexpressed.
Figure 5.2: Potential mechanism for the role of CSSG1 in HSV1 pathogenesis

HSV1 pathogenesis in humans is caused by HSV1 virus replication and the resulting immune response. Evidence presented in this dissertation suggests a role for CSSG1 in both of these processes. CSSG1, along with Tip60, facilitates replication of HSV1 virions following infection. Additionally, CSSG1, in combination with TRAF6, may play a role in activation of the TLR2 signaling pathway leading to inflammation of infected areas contributing to pathogenesis such as cold sores or encephalitis.
with TRAF6. Additionally, it has been shown that HSV1 tegument protein UL37 associates and activates TRAF6, leading to activation of NFkB [152]. Whether this interaction requires CSSG1 expression needs to be studied in CSSG1 KD U2OS cells to further investigate role of CSSG1 and TRAF6 during HSV1 infection.

If the formation of the foci is significant for HSV1 pathogenesis, it may have an impact on innate immune responses mediated by TLR2 since TRAF6 is required for TLR2 mediated activation of proinflammatory cytokines such as IL-6. To test this, CSSG1 knockout BMDCs were stimulated with TLR2 agonist Pam3CSK4 and release of IL6 were measured (Chapter 4.3). It was determined that release of IL6 was decreased following stimulation with Pam3CSK4 suggesting a potential role of CSSG1 in TLR1/2 pathway. This observation would explain association of CSSG1 SNPs on severity of cold sores since TLR2 signaling pathway is very important in inflammation induced HSV1 pathogenesis [40, 130, 148, 153-156]. It is likely that cold sore severity is reduced in absence of CSSG1 due to reduction in inflammation and HSV1 replication.

In addition to proinflammatory cytokines, type I interferon also plays a crucial role in limiting HSV1 reactivation and replication [29, 30, 125, 157-162]. To determine if cssg1 affects this pathway, U2OS cells were stimulated with poly dA:dT, poly I:C, and sendai virus in presence of ectopically expressed CSSG1. It was determined that activation of IFN promoter is increased in presence of CSSG1. It is interesting to note that overexpression of CSSG1 drives increased activation of IFN promoter in presence of cytosolic nucleic acids such as poly dA:dT and poly I:C. These stimuli are recognized by nucleic acid sensors such as IFI16, Rig-I, and MDA5 that are also activated during HSV1 infection. This suggests potential role of CSSG1 in detection of HSV1 dsDNA

genome and RNA intermediate products during infection. Detection of HSV1 nucleic acids and resulting type I IFN production is essential for resolution of HSV1 pathogenesis [163, 164]. Thus, role of CSSG1 in detection of HSV1 nucleic acids needs to be further studied in BMDCs and mouse embryonic fibroblast cells (MEFs) isolated from CSSG1 knockout mice. Role of CSSG1 in the interferon pathway would also explain association of CSSG1 SNPs with HSV1 reactivation.

Experimental results presented in the dissertation suggest that CSSG1 affects HSV1 pathogenesis by affecting both HSV1 replication and the resulting innate immune response (Figure 5.2). Considering the complex interplay between the immune response and HSV1 replication during pathogenesis, it may be difficult to assess the role of CSSG1 in herpesvirus pathogenesis in vitro. To address this, we have created a CSSG1 knockout mouse using genetrap technology (Chapter 4.3). This is currently the first known report of a CSSG1 knockout mouse. CSSG1 knockout mice are fertile and free of visual abnormalities. The knockout mice can be used to determine the role of CSSG1 in herpesvirus complications such as encephalitis, keratitis, or HSV1 reactivation using existing mouse infection models described here [40, 165, 166]. Based on the results obtained during this dissertation, the CSSG1 knockout mice are expected to carry decreased viral loads and have decreased resulting inflammation following infection. The overall decrease in viral replication and inflammation should lead to reduced HSV1 induced pathogenesis in CSSG1 knockout mice.

Overall, the work compiled in this dissertation makes significant contribution to understanding HSV-1 pathogenesis. My colleague Michael King and I show that our monoclonal antibodies to CSSG1 can be used for detection of CSSG1 protein
expression and for immunoprecipitation assays opening doors for analyzing CSSG1 protein-protein interactions during HSV1 infection. Furthermore, I provide evidence for interaction of CSSG1 with Tip60 and TRAF6 proteins that are known to play significant roles in HSV1 pathogenesis. I also provide evidence that CSSG1 is required for efficient HSV1 replication and DNA repair. I also present data on how polymers can be used to modulate production of inflammatory cytokines involved in herpesvirus pathogenesis. This technique may help fortify immune response to herpesviruses or to reduce inflammation during complications such as encephalitis. Finally, the in vivo role of CSSG1 in complex herpesvirus pathogenesis can to be further explored using the CSSG1 knockout mouse described in this dissertation.
APPENDIX I

MODULATION OF THE NLRP3 INFLAMMASOME ACTIVATION AND POTENTIAL FOR TREATMENT OF HERPESVIRUS COMPLICATIONS
Contributors

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Summary of Contributions

I contributed to the design, execution, analysis and interpretation of the experiments described in this chapter. I generated the samples and performed the experiments and created the graphics shown in figures A.2D, A.2E, A.7A, A.7B, A.8A, A.8B. Christine A. Vaine performed experiments in figures A.1, A.2ABC, A.3, A.4, A.5, and A.6

Sections of the following chapter are reprinted from the Journal of Immunology article:

**Tuning innate immune activation by surface texturing of polymer microparticles: the role of shape in inflammasome activation**


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A.1 Abstract

Polymeric microparticles activate the inflammasome complex and induce the processing and secretion of IL-1β, a key innate immune cytokine involved in clearance of viral infections. Recent work suggests that physical characteristics of the microparticles such as shape play an important role in the way microparticles interact with cells. We examined the role of particle surface texturing not only on phagocytosis efficiency of the microparticles but also on the subsequent immune cell activation of the inflammasome. Using a method based on emulsion processing of amphiphilic block copolymers, we prepared microparticles with similar overall sizes and surface chemistries but having either smooth or highly microtextured surfaces. In vivo, textured (budding) particles induced more rapid neutrophil recruitment to the injection site. In vitro, budding particles were more readily phagocytosed than smooth particles and induced more lipid raft recruitment to the phagosome. Remarkably, budding particles also induced stronger IL-1β secretion than smooth particles through activation of the NLRP3 inflammasome. These findings demonstrate a pronounced role of particle surface topography in immune cell activation, suggesting that shape is a major determinant of inflammasome activation.
A.2: Introduction

Inflammasomes are intracellular PRRs that are an essential component of the innate immune response to herpesviruses. Inflammasomes involved in detection of herpesviruses include NLRP3 and AIM2. Upon activation, cells release IL-1β that initiate a cascade of innate immune response resulting in recruitment of neutrophils, macrophages, and antigen presenting cells.

Despite their essential role in clearance of pathogens, inflammasomes have been frequently described as a double-edged sword (Shaw PJ et al) of the immune response. In certain cases, inflammasomes such as NLRP3 and AIM2 are essential for detection and clearance of herpesviruses (Giemenez F et al., Johnson KE et al.). Whereas, in some cases, the innate immune response can also run awry leading to inflammatory destruction of tissues as observed in viral infection induced neuroinflammation (Kaushik DK et al., Jha S et al.). In addition, inflammasome activation has also been linked to various autoimmune diseases such as rheumatoid arthritis and systematic lupus erythematosus (Shaw PJ et al). The importance of the proinflammatory cytokines produced by the inflammasome pathway are also known. IL-1β plays a significant role in activation of the innate and adaptive immune response. However, it has also been shown to play a significant role in sterile inflammation and autoimmune diseases. Treatments that block IL-1β signaling are highly effective in treating inflammatory diseases.

Due to their dual role in resolving and exacerbating pathogenesis, treatment of complications involving inflammasomes can require either induction or suppression of
the innate immune response. Thus, it is important to understand how activation of inflammasomes can be modulated in vivo to either protect against infection by herpesviruses or to reduce inflammation to protect tissue health in cases of encephalitis. In this chapter, a mechanism is described that leads to variable activation of the NLRP3 inflammasome and its implications for ability to modulate induced immune response for desired therapeutic applications involving herpesvirus pathogenesis.

In order to determine a property of inflammasomes that can be exploited for modulating their activation, the focus of the research was turned to phagocytic mechanisms involved in uptake of ligands that activate inflammasomes. The initial step to activation of the inflammasomes by a pathogen is phagocytic internalization of the pathogen. Process is a receptor-mediated, actin-dependent process carried out by a specialized subset of cells termed 'professional phagocytes', including neutrophils, monocytes, macrophages, and dendritic cells [167]. Recent pioneering work from Mitragotri's group [168] and subsequent studies [169-172], have demonstrated that the shape of a polymer microparticle has a dramatic effect on phagocytosis. Specifically, the local curvature of the particle surface that is first encountered by the phagocyte dictates whether the actin cup necessary to engulf the particle can be formed [168, 170], and thus whether the particle is internalized. This suggests that immune cells use surface curvature as a locally accessible proxy for overall particle dimension. Other work has demonstrated that for spherical microparticles, size plays a role in uptake efficiency, with maximal phagocytosis for particle diameters of 1-3 µm [172-174]. These findings were not dependent on the receptor used to initially mediate attachment or internalization, pointing to a highly universal role of particle geometry in phagocytic uptake.
Here, we take advantage of a recently developed route to prepare polymeric microparticles with complex but well-controlled surface topographies, based on emulsion processing of amphiphilic block copolymers [175, 176]. This provides a simple platform to compare the response of phagocytes to particles of similar overall size and surface chemistry, but where the particle surfaces are either smooth or densely covered with micro-scale protrusions (which we refer to as textured or ‘budding’ particles). In this way we can assess the role of shape independent of receptor interaction with particles based on their surface chemistry. Since phagocytes respond to local surface curvature [168-172, 177] we anticipated that the regions of high curvature on the budding particles should substantially alter the immune response. In this work, we examine the acute inflammatory response to polymeric microparticles via neutrophil recruitment in an in vivo mouse peritonitis model. We also analyze the mechanism of this response using mouse macrophages to compare the ability of smooth and budding particles to be phagocytosed, activate immune cells, activate the inflammasome, and induce IL-1β cytokine release.
A.3 Results

A.3.1: Preparation of budding particles

PS-PEO particles are an excellent model system as PS-PEO has been well studied in the context of generation of textured particles [176]. There have also been several studies examining the use of PS and PS-PEO particles as therapeutic agents [168, 178, 179]. Therefore, to study the role of surface texturing on the immune response, we prepared polymer microparticles consisting of PS-PEO diblock copolymer (38 and 11 kg/mol number-average molecular weights $M_n$, respectively) blended with PS homopolymer ($M_n = 12.4$ kg/mol). Initially, the polymers are dissolved in chloroform, then emulsified in water using a microfluidic flow-focusing device to provide droplets of uniform size [176]. Removal of the organic solvent by evaporation subsequently yields solid polymer microparticles of phagocytosable size (7 – 8 µm diameter). As shown in Figure. A.1A, by adjusting the mass ratio of PS-PEO:PS the morphology of the particles can be changed from ‘budding’ particles (100 : 0, Figure. A.1A), that are densely coated with vesicular protrusions of 1 – 2 mm diameter, to smooth spherical particles (20 : 80, Figure. A.1B). Due to the interfacial activity of PS-PEO, the surfaces of both types of particles are coated with a similar “brush” layer of PEO, as well as residual poly(vinyl alcohol) used to stabilize the emulsion droplets.
Figure A.1: Images of budding and spherical microparticles.

ScEM and TEM images of budding (A) and spherical (B) particles generated. Scale bars = 10 µm and 5 µm.
A.3.2: Budding particles stimulate a more robust neutrophil response at early time points *in vivo*

We first compared the *in vivo* immune response to budding particles versus spherical particles. Previous studies have shown that intraperitoneal (i.p.) injections of particulates (e.g., monosodium urate crystals or titanium particles) lead to an increase in neutrophil recruitment [180, 181]. To determine whether our polymer microparticles induced a similar increase in neutrophil recruitment, wild-type (WT) mice were first injected i.p. with budding or spherical particles at 3 different doses: approximately 6.7 x $10^5$, 2 x $10^6$, and 2.7 x $10^6$ particles (150 µg, 450 µg, and 600 µg, respectively) and lavage of the peritoneal cavity was analyzed for neutrophil influx 6 h later. We found that the 450 µg dose of budding particles induced significantly higher neutrophil recruitment (Ly6G+, 7/4+ cells) over spherical particles (Figure. A.2 A, p < 0.01, budding versus spherical). In fact, 450 µg budding particles induced neutrophil recruitment at levels similar to injection with the positive control, thioglycollate (Figure. A.2 A, p < 0.001, PBS versus Thio). Next, we compared the neutrophil recruitment to 450 µg particles at 6 or 16 h after injection. Both budding and spherical particles induced a significant neutrophil response over PBS-carrier only at 6h (p < 0.0001, both) and 16h (p < 0.01, budding; p < 0.0001, spherical) (Figure. A.2 B). Budding particles exhibited significantly higher levels of neutrophil recruitment than spherical particles at 6 h but only slightly higher levels at 16 h when compared to spherical particles (Figure. A.2 A, B).
Figure A.2: Particle-induced neutrophil recruitment depends on surface curvature and requires IL-1R and NLRP3 inflammasome-associated signaling.

Flow cytometric analysis on peritoneal neutrophil (Ly6G+, 7/4+ cells) recruitment at 6 h (A, D), 16 h (C), or at given times (B) after microparticle injections at varying doses as indicated (A) or at a fixed dose of 2 x 10^6 particles; approx. 450 µg (B, C, D) in WT (C57BL/6), IL-1RKO, NLRP3KO, or CASP1KO mice. Graphs show mean ± SEM of total number of mice indicated below, performed in 2-3 independent experiments. Y-axis scales are (x10^6) (A-D) or (x10^5) (E). Number of mice: (A) PBS (0), n=4; 150ug, n=2; 450ug, n=6 (Spher) and 8 (Bud); 600ug, n=2 (Spher) and 3 (Bud); Thio, n=3. (B) 0, n=9; 6B, n=8; 6S, n=6; 16B, n=5; 16S, n=5. (C) PBS, n=5; WT, n=5; KO, n=3. (D) n=2. Significance values are shown as Budding versus Spherical or Thio versus PBS (A), particle versus PBS injections (B), PBS versus particle injections (C), or KO versus WT (D). ****, P < 0.0001; ***, P < 0.001; **, P < 0.01; *, P < 0.05.
**A.3.3: Microparticle-induced neutrophil recruitment involves IL-1 associated signaling**

The IL-1R is required for neutrophil recruitment following exposure to stimulants/particulates in mice [180-183]. To determine whether the IL-1R was required for microparticle-induced neutrophil influx, IL-1R KO mice and WT mice were injected with budding or spherical particles for 16 h and compared to PBS alone injections. As predicted, IL-1R KO mice did not recruit neutrophils in response to particles over PBS alone, whereas WT mice exhibited a significant increase over PBS (Figure. A.2 C, p < 0.05, budding; p < 0.001, spherical).

**A.3.4: NLRP3 inflammasome is critical for microparticle-induced neutrophil recruitment**

The NLRP3 inflammasome plays a role in the response to particulate materials [79, 177, 180, 181, 183-186] through IL-1R and IL-1β secretion. To determine if components of the NLRP3 inflammasome were required for particle-induced neutrophil recruitment, we examined peritoneal neutrophil recruitment to budding particles in NLRP3 KO and CASP1 KO mice and to spherical particles in NLRP3 KO mice 6 h following particle injections. When budding particles were injected, we found that the NLRP3 KO and CASP1 KO mice exhibited significantly blunted neutrophil recruitment, compared to WT controls in response to budding particles (Figure. A.2 D, p < 0.05, WT versus all KOs). Again, spherical particles induced substantially less neutrophil recruitment than budding particles (Figure. A.2E). The neutrophil response to spherical
particles was also inhibited in NLRP3 KO compared to WT mice, and although trending did not reach statistical significance with a P value of 0.0674.

**A.3.5: Budding particles stimulate more IL-1β than spherical particles**

To determine if the observed IL-1R- and NLRP3 inflammasome-dependent neutrophil influx *in vivo* exhibited similar characteristics *in vitro*, we analyzed the IL-1β inflammasome response of murine macrophages to microparticles. Immortalized macrophages from WT mice were primed for 3 h with LPS to induce up-regulation of pro-IL-1β transcription, then incubated with budding or spherical microparticles for 6 h to induce pro-IL-1b processing to mature IL-1b. Levels of mature, secreted IL-1β were measured via ELISA using cell supernatants. As predicted by the neutrophil recruitment studies, budding particles were able to induce significantly higher levels of IL-1β secretion (Figure. A.3 A, black bars, p < 0.0001, 0.5:1 and 1:1) compared to spherical particles (Figure. A.3 A, white bars). WT macrophages responded to both types of microparticles in a dose-dependent manner with significantly higher levels of secreted IL-1β compared to priming alone at the 1:1 particle:cell ratio (4.5 x 10⁵ particles) for both types of particles as well as the 0.5:1 particle:cell ratio (2.25 x 10⁵ particles) for budding particles (Figure. A.3 A). As a positive control, macrophages were transfected with synthetic double-stranded DNA, poly-dA:dT
Figure A.3: Particle-induced IL-1β cytokine secretion is dependent on surface curvature.

Secreted IL-1β levels from WT immortalized mouse macrophages. Cells were primed with LPS for 3 h (Prime) or left unprimed (Media), then stimulated with budding or spherical particles with increasing particle-to-cell ratios (particle number:cell number; 1:1, ~ 100 µg) (A) or transfected with 400 ng poly dA:dT (B) for 6 h. Measurement of IL6 released by cells stimulated with particles [C]. Kinetics of IL-1β secretion from 100 µg budding or spherical particles (D). Cytokine levels are reported as mean + SEM and are representative of two independent experiments performed in duplicate. Significance values are shown as budding versus spherical (A, D), or Prime versus dA:dT (B). ****, P < 0.0001; **, P < 0.01; *, P < 0.05.
WT macrophages produced a significant amount of IL-1β in response to dA:dT stimulation (Figure. A.3 B, p < 0.01). As negative controls, supernatants from macrophages that received media or an LPS prime alone did not exhibit an increase in IL-1β production. Levels of NLRP3-independent cytokine IL-6 were equivalent for all samples (Figure. A.3 C). Since the neutrophil response seen with budding particles was similar to spherical particles at 16 h in vivo, we also examined the in vitro kinetics of the IL-1β response to microparticles. Unlike neutrophil recruitment, budding particles stimulated higher levels of IL-1β at all time points compared to spherical particles (Figure. 4.3 D, p < 0.01, 6 h; p < 0.05, 16 h).

**A.3.6: Microparticle-induced IL-1β production requires the NLRP3 inflammasome**

In order to further determine if the NLRP3 inflammasome signaling complex was involved in the IL-1β response to microparticles in macrophages, immortalized macrophages generated from NLRP3 KO, ASC KO, and CASP1 KO mice were analyzed for IL-1β production in response to microparticle stimulation. Supernatants from NLRP3 KO, ASC KO, and CASP1 KO macrophages each exhibited undetectable levels of secreted IL-1β in response to budding and spherical microparticles while IL-1β secretion from WT macrophages was readily detected (Figure. A.4 A, p < 0.0001, WT versus all KOs). As expected, NLRP3 KO macrophages could respond to dA:dT similar to WT cells, while ASC KO and CASP1 KO macrophages could not (Figure. A.4 B,
Figure A.4: Particle-induced IL-1β cytokine release requires the NLRP3 inflammasome.

Secreted IL-1β levels from immortalized mouse macrophages from WT, NLRP3 KO, ASC KO, and CASP1 KO mice that were primed for 3 h with LPS (Prime) then stimulated with $4.5 \times 10^5$ (1:1 particle:cell ratio) spherical or budding microparticles for 6 h (A) or transfected with poly dA:dT (B). Secreted IL6 levels from A (C). Cytokine levels are reported as mean + SEM and are representative of three independent experiments performed in duplicate. Significance values are shown as WT versus KO. ****, $P < 0.0001$; **, $P < 0.01$. 
p < 0.01, NLRP3 versus WT; p < 0.0001, ASC/CASP1 KO versus WT). WT and deficient cells also produced equivalent levels of the inflammasome-independent cytokine, IL-6 (Figure. A.4 C), following LPS stimulation.

A.3.7: Small, spherical particles do not induce significant IL-1β secretion

Studies have shown that particles within the range of 1-3 µm in diameter exhibit the highest phagocytic rates when incubated with macrophages [172-174]. The ‘buds’ on the budding particles are approximately 1-2 µm in diameter. In order to determine if the presence of these small ‘buds’ is responsible for the increased IL-1β secretion exhibited by budding particles when compared to spherical particles, we also tested the response to smaller spherical particles with diameters of 0.5 µm and 1 µm, the surfaces of which were also coated with PEO. These smaller spherical particles were incubated with WT immortalized macrophages and assayed for IL-1β secretion. Unlike the budding particles, these small (bud-sized) spherical particles did not induce a significant amount of IL-1β secretion above prime-only background values (Figure. A.5 A), even at high particle-to-cell ratios. All samples exhibited similar levels of NLRP3 independent cytokine IL6 (Figure. A.5 B) following LPS prime. Of note, uncoated 0.5 µm and 1 µm PS particles were also unable to induce significant IL-1β secretion over prime-only levels (data not shown).
Figure A.5: Small spherical particles do not induce IL-1β secretion.

Secreted IL-1β (A) and IL-6 (B) levels from WT immortalized mouse macrophages. Cells were primed with LPS for 3 h (Prime) or left unprimed (Media), then stimulated with small spherical particles (0.5 µm or 1 µm diameter) for 6 h with increasing particle-to-cell ratios (particle number:cell number; 1:1, ~ 100 µg). Cytokine levels are reported as mean ± SEM and are representative of two independent experiments performed in duplicate.
A.3.8: Budding particles are more likely to be associated with and internalized in mouse macrophages than spherical particles

Since inflammasome activation of IL-1β release generally involves phagocytosis of the stimulant, we examined the ability of macrophages to attach to and internalize spherical and budding particles. To visualize phagocytosis of microparticles, immortalized macrophages from WT mice were incubated for 6 h with budding or spherical microparticles containing a fluorescent dye, Vibrant DiI. Using confocal microscopy, we found that a significantly higher percentage of budding particles, e.g., greater than 85%, bound to or internalized in macrophages (Figure. A.6 A, D) when compared to spherical particles, where only approximately 20% were bound or internalized (Figure. A.6 B, D, p < 0.0001, budding versus spherical). We also found that budding particles were associated with more macrophages on a per particle basis, with the majority of budding particles associated with 2 – 3 macrophages each. In other words, a single budding particle was often associated with more than one macrophage at a time (Figure. A.6 C, E). In contrast, a single spherical particle only associated with a single macrophage (Figure. A.6 B, E). This data is trending towards significance with a P-value of 0.0632. As internal controls, the total number of cells per field of view and number of particles per field of view were very similar between spherical and budding particles, while the absolute number of bound budding particles was still significantly higher than bound spherical particles (Figure. A.6 H-J, p < 0.01, bound budding versus spherical).
Figure A.6: Particle phagocytosis: Budding particles associate with more macrophages than spherical particles.

Confocal microscopy images of macrophage-associated spherical (A) and budding (B, C) particles following a 3 h prime with LPS and 6 h incubation with particles. Lysosomes were visualized with LysoTracker Green. Nuclei were visualized with Hoechst 34580 (blue). Scale bars = 10 µm. Percent of and average (line) particles bound to macrophages per field of view (D). Total and average (indicated by line) number of macrophages associated with a single particle per field of view (E). Analysis was performed on seven independent fields of view per particle type. Cytokine levels are reported as mean + SEM and are representative of two independent experiments performed in duplicate. Significance values are shown as budding versus spherical particles (D). Total (circles) and average (lines) number of cells (A), particles (B), and bound particles (C) per field of view. Analysis was performed on seven independent fields of view per particle type. Scale bars = 10 µm.****, P < 0.0001; **, P< 0.01.
Optimal inflammasome activation in response to silica crystals, alum, amyloid-β, and titanium requires uptake through actin polymerization and release of cathepsin B following lysosomal destabilization [181, 183, 187]. To determine whether actin polymerization and cathepsin B are required for inflammasome activation and subsequent IL-1β production in response to microparticles, WT immortalized mouse macrophages were treated with the cathepsin B inhibitor CA-074-Me or actin inhibitors Latrunculin A (Lat. A) and Cytochalasin D (Cyt. D). Supernatants from cells pre-treated with CA-074-Me had substantially lower levels of IL-1β following a 6 h microparticle stimulation compared to untreated cells (Figure A.7A, p < 0.0001, untreated versus treated). Additionally, cells pre-treated with Lat. A or Cyt. D exhibited a complete loss of IL-1β following an 18 h microparticle stimulation (Figure A.7B, p < 0.0001, untreated versus treated). As expected, alum induced IL-1β requires both cathepsin B and actin (Figure A.7A, G, p < 0.0001, untreated versus treated, all), whereas nigericin, a potassium ionophore known to induce IL-1β through potassium efflux, lysosomal destabilization and cathepsin B release [188], requires cathepsin B but does not require actin polymerization (Figure A.7A,B, p < 0.0001, CA-074-Me treatment and p < 0.01, Lat. A and Cyt. D treatments). Furthermore, macrophages that were transfected with double-stranded DNA, poly-dA:dT (dA:dT), which induces mature IL-1β production in a NLRP3-independent manner [78], were unaffected by cathepsin B inhibition (data not shown).
Secreted IL-1β from WT immortalized macrophages stimulated for 6 h (A) or 18 h (B) with 100 μg budding or spherical particles, 130 μg/ml alum, or 5 μM nigericin in the presence (white bars) or absence (black bars) of 50 μM CA-074-Me (A), 250 nM Latrunculin A (Lat. A), or 1 μM Cytochalasin D (Cyt. D) (B). Cytokine levels are reported as mean ± SEM and are representative of two independent experiments performed in duplicate. Significance values are shown as untreated versus treated cells (A, B). ****, P < 0.0001; **, P < 0.01.
A.3.9: Budding particles localize with lipid raft components

Recruitment of lipid rafts plays an important role in phagocytosis and inflammatory cytokine secretion by anchoring scavenger receptors, innate immune receptors (such as CD14 and Dectin-1) during activation of downstream kinases [189-191]. Additionally, we have previously shown that uptake of antibody-bound membrane proteins can internalize via clathrin through the endosomal pathway [192]. In order to further elucidate the mechanisms involved in particle internalization, WT macrophages were stained with fluorescent cholera toxin subunit B (CTxB), which binds to GM1 gangliosides on the cell surface, as a marker for lipid rafts. Cells were then incubated with fluorescent particles for 6 h. We found that the surface of budding particles highly localized with CTxB (arrows), whereas spherical particles did not (Figure. A.8).
Figure A.8: Internalized budding particles localize with lipid raft components.

Confocal microscopy images of macrophage-associated spherical (A) and budding (B) particles. Cells were incubated with particles for 6 h, then fixed with 4% paraformaldehyde for 20 min prior to visualization. Lipid rafts were visualized with CTxB-Alexa 488 (green). Nuclei were visualized with Hoechst 34580 (blue). Arrows indicate localization of lipid rafts with particle. Images are representative of two independent experiments. Scale bars = 5 µm.
A.4: Discussion

This study illustrates that modulation of surface curvature of microparticles influences inflammasome mediated IL-1β production and the resulting innate immune response. The use of macrophage cell lines allowed us to interrogate the potential pathways triggered by particles and revealed roles for NLRP3, ASC, CASP1, and IL-1R and suggest that budding particles induce a significantly greater inflammasome response than spherical particles. These observations were validated in vivo using neutrophil recruitment, confirming the importance of these pathways in vivo and supporting our hypothesis that surface texture is an important determinant of inflammasome activation by particles.

It has been demonstrated that IL-1- associated signaling plays a critical role in neutrophil responses following injections of particulate stimuli (22, 23, 29, 40). Here we show that IL-1R KO mice exhibit a diminished neutrophil response following microparticle injections, further implicating IL-1 signaling in the innate immune response to budding and spherical polymer microparticles. Furthermore, we verify that this response also requires a functional NLRP3 inflammasome, as NLRP3 KO and CASP1 KO mice were unable to recruit a significant amount of neutrophils following particle injections. It is possible that sensors in addition to NLRP3 may play a role in particle induced neutrophil recruitment and this may account for the lack of a complete abolition of neutrophil recruitment in KO mice. However, regardless of whether NLRP3 is the only inflammasome receptor or one of several inflammasome receptors that are triggered by particles, these studies clearly demonstrate that the downstream ASC and CASP1
pathways and the IL-1R pathway are very important for neutrophil responses to budding and spherical particles. We determined that although there is some variation in the amount of IL-1b detected in macrophage supernatants, spherical particles consistently and reproducibly induced significantly less IL-1b than budding particles in every experiment in side-by-side comparisons. Particle IL-1b induction also occurs through activation of the NLRP3 inflammasome–signaling complex, similar to that seen with other particulate stimuli (22–29). Our kinetic studies in vitro also indicate that budding particles induced an early peak and continued high IL-1b secretion over time, whereas spherical particles induced lower IL-1b secretion levels that remained relatively constant. In vivo, it is unclear why spherical particles appear to “catch up” with budding particles for neutrophil recruitment at later time points. There is likely a complex interplay between a variety of signals in vivo, including the magnitude of inflammasome activation for IL-1b secretion, levels of IL-1b produced, IL-1b–driven neutrophil recruitment, and adherence of activated neutrophils to peritoneal tissues and/or pyroptosis/necrosis of highly activated neutrophils. Budding particles also induced a more rapid phagocytic response in vitro and were more readily taken up by macrophages than spherical particles, again suggesting that the shape of the particle affects the kinetics of the innate immune response. Our studies revealed that large (7–8 mm) polymer particles are efficiently associated with and phagocytosed by macrophages. We also noted higher concentrations of lysosomes surrounding the engulfed budding particles, suggesting that the budding particles trigger a stronger cellular response. Our findings corroborate recent studies indicating that macrophages are more likely to internalize particles if they contain regions of high positive surface curvature (15–19). Budding particles are presumably phagocytosed more efficiently because they contain higher local surface
curvature compared with spherical particles of the same overall dimensions. Although particle uptake is an important parameter for drug delivery, triggering of inflammatory responses may not require complete uptake of the particle. In fact, pathogenic crystals of uric acid, silica, b-amyloid, or cholesterol have all been shown to trigger inflammasome activation and IL-1b release by a “frustrated phagocytosis” mechanism (22, 41). The current view is that macrophages attempting to engulf large crystals form a phagolysosome around the crystal. However, in the process of engulfing very large crystals, the lysosomal membranes are ruptured, thus releasing enzymes into the cytosol that trigger cytosolic inflammasomes as a result. Our data showing multiple cells associated with a single budding particle are consistent with this proposed mechanism. However, whether budding particles are completely phagocytosed or partially phagocytosed by several cells, it is still clear that actin polymerization and cathepsin B release is necessary for IL-1b induction, because inhibitors to either completely abolish the particle induced IL-1b response. In addition to triggering a stronger cellular response, it is possible that budding particles internalize through a different mechanism of phagocytosis than spherical particles. The process of phagocytosis can occur through one of several different cell surface proteins, including complement receptors, FcRs, pathogen-specific receptors, and scavenger receptors (2, 3). Studies have indicated that scavenger receptors and caveolae/lipid rafts are involved in the internalization of a variety of therapeutic agents (48), bacteria (8, 49), and artificial particles such as latex, TiO2, silica, and polystyrene particles (9–11, 50). Using CTxB as a marker for lipid rafts, our findings indicate that particles with high surface curvature (budding particles) recruit lipid rafts during internalization, whereas particles with lower surface curvature (spherical particles) do not. These findings suggest that particles can potentially be tailored to
internalize through a specific phagocytosis pathway, based on surface curvature. Several studies have reported that small particles induce the highest amount of IL-1b from immune cells (19–21, 28). One possibility for the increased phagocytosis and increased IL-1b production seen with budding particles compared with spherical particles is that the 1- to 2-mm diameter buds mimic smaller particles. However, our findings indicate that small, spherical particles in the 0.5- to 1-mm diameter range do not induce significant IL-1b secretion (whether PEO-derivatized or not). It is clear that the immune response to particles with textured surfaces is more complicated than predicted by models based on size alone. The combination of regions of high positive and negative surface curvatures to form the more complex surfaces of the budding particles is apparently responsible for induction of high levels of IL-1b. These findings also clearly demonstrate that not all phagocytes are created equal, a concept that becomes clear when comparing studies on particle stimulation. For example, Sharp et al. (28) have suggested that particles in the range of 0.5–1 mm in diameter induce the highest amount of IL-1b in dendritic cells. Other studies have shown that particles of 1–3 mm in diameter exhibited the highest phagocytic rates when incubated with J774 mouse macrophages (19), rat alveolar macrophages (20), and peritoneal mouse macrophages (21). We have demonstrated that immortalized mouse macrophages, which have been used for inflammasome activation studies to a variety of particulate material (22, 29), appear to respond differently (and very weakly) to small, spherical particles when compared with the response from murine dendritic cells, peritoneal macrophages, and rat alveolar macrophages.

Overall, the results presented in this chapter provide insights into mechanism of the inflammasome activation pathway. The results also show that a possibility exits for
modulating the inflammasome activation and IL-1β production via controlling the surface texturing of polymeric particles.
**A.5: Materials and Methods**

**A.5.1: Microparticle preparation.** Generation of solvent-in-water emulsion droplets of well-controlled sizes via flow-focusing, and conversion to particle suspensions were conducted as previously described [176]. The resulting suspensions of polystyrene-block-poly(ethylene oxide) (PS-PEO) microparticles were dialyzed against deionized water for 2 – 3 days to remove glycerol and residual chloroform, then centrifuged and resuspended in fresh deionized water 5 – 8 times to remove excess and weakly adsorbed poly(vinyl alcohol) surfactant. Budding and spherical particles were approximately 7 – 8 µm in diameter. Stock solution concentrations of particles were approximately 1.45 x 10^7 particles/mL.

**A.5.2: PEO functionalization of small particles.** PEO-coated particles with diameters of 0.5 and 1.0 µm were prepared by modifying carboxyl-functionalized PS particles (Polysciences, Warrington, PA) with amino-PEO (α-aminomethyl, ω-methoxy PEO, 10 kDa; Nanocs, New York, NY). Briefly, PS-COOH particles (0.86 µmol COOH groups, in 500 µL) were allowed to react with 6 µmol of N-(3-dimethylaminopropyl)-N'-ethylenediimidate hydrochloride (EDC), and 9.5 µmol of N-hydroxysuccinimide (NHS) in an aqueous 100 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 6.0) at 4°C. Solutions of EDC and NHS were freshly prepared. After 1 h at room temperature, activated PS particles were washed twice with an MES solution (pH 6.0) via centrifugation and redispersion. Next, an excess of amino-PEO (1.29 µmol) in 2.58 mL of PBS (pH 7.2) was added, followed by incubation for 1 h at room temperature and then
washing twice with PBS via centrifugation and redispersion. Successful functionalization was confirmed by X-ray photoelectron spectroscopy on a sample of particles deposited on a silicon wafer. The particles were stored in aqueous suspension at 4°C until use. EDC, MES, and PBS were obtained from Sigma-Aldrich (St. Louis, MO). The stock solution concentrations of the small spherical particles were approximately $1 \times 10^{11}$ particles/mL.

**A.5.3: Electron Microscopy.** Microparticle morphologies were observed by scanning electron microscopy (ScEM) and transmission electron microscopy (TEM). For ScEM, a droplet of aqueous dispersion of particles was allowed to dry on a clean silicon wafer, followed by coating with a thin layer of gold. Samples were imaged using a JEOL 6320 FXV ScEM at an accelerating voltage of 10 kV. For TEM, a droplet of particle dispersion was allowed to dry on a copper grid coated with a carbon film (Electron Microscopy Sciences) and imaged with a JEOL 2000 FX electron microscope operated at 200 kV.

**A.5.4: Cell culture.** Immortalized mouse macrophages from WT, NLRP3-deficient, ASC-deficient, and Caspase1-deficient mice were generously provided by K. Fitzgerald and E. Latz (University of Massachusetts Medical School), and were generated as previously described [183] using a J2 recombinant retrovirus carrying v-\textit{myc} and v-\textit{raf(mil)} oncogenes. Cells were grown in DMEM supplemented with 10% FCS, 1% L-glutamine, and 1% Penicillin/streptomycin at 37°C with 5% CO$_2$. Cells were plated in GM-CSF (1 ng/mL; eBioscience)-containing media for 18 h prior to stimulations.
A.5.5: Cell Stimulations. Mouse macrophages (4 – 5 x 10⁵) were primed for 3 h with LPS (100 ng/mL – Sigma) to up-regulate pro-IL-1β expression or left unprimed (Media), then stimulated with microparticles (budding, spherical, or small particles) at given particle-to-cell ratios (particle number:cell number), 130 µg/ml alum (Thermo Scientific), 5 µM nigericin (Sigma), or transfected with 400 ng of poly(dA:dT) (Sigma) using GeneJuice (EMD Chemicals) for an additional 6 h or 18 h. Where indicated, cells were treated with 50 µM CA-074-Me (EMD Millipore), 250 nM Latrunculin A (Sigma), or 1 µM Cytochalasin D (Sigma). Secreted IL-1β was measured using ELISA (R&D Systems) according to manufacturer’s instructions.

A.5.6: Confocal Microscopy. Cells were cultured on glass-bottom 35 mm tissue-culture dishes (MatTek) in complete medium. Where indicated, cells were stained with LysoTracker Green, Hoechst 34580, and Alexa488-CTxB from Molecular Probes (Invitrogen) according to manufacturer’s instructions. Images were taken on a Leica SP2 AOBS confocal laser-scanning microscope with a 63x objective, using Leica Confocal Software. Multicolor images were acquired by sequential scanning with only one laser active per scan to avoid cross-excitation. Overall brightness and contrast of images were optimized using Adobe Photoshop CS3.

A.5.7: Mice injections. C57BL/6 (WT), IL-1R-knockout (IL-1R KO), and CASP1KO mice were obtained from Jackson Laboratories (Bar Harbor, ME). NLRP3KO mice were
provided by K. Fitzgerald (University of Massachusetts Medical School). Mice were injected i.p. with sterile PBS (400 µl), 4% thioglycollate (1 mL), or 2 x 10^6 microparticles (approx. 450 µg). Mice were sacrificed by isoflurane inhalation followed by cervical dislocation. Peritoneal exudate cells (PECs) were isolated 6 or 16 h after injections as previously described [181]. All mouse strains, age and sex-matched with appropriate controls, were bred and maintained at the University of Massachusetts Medical School animal facility. Experiments involving live animals were in accordance with guidelines set forth by the University of Massachusetts Medical School Department of Animal Medicine and the Institutional Animal Care and Use Committee.

**A.5.8: Flow cytometric analysis.** Neutrophils in PECs were enumerated as previously described [181]. Data were acquired by DIVA (BD Biosciences) and were analyzed with FlowJo 8.8.6 software (Tree Star Inc.).

**A.5.9: Statistical Analysis.** An unpaired, two-tailed Student’s t-test was used to determine statistical significance of independent experiments where two groups were compared. When more than two groups were compared, ANOVA followed by Bonferroni’s correction for post test comparisons was used. Values of P < 0.05 were considered significant with 95% confidence intervals. Statistics were performed using GraphPad (Prism v5.0d) software.
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